Pathways of the Maillard Reaction under Physiological Conditions

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CONTENTS

1 Introduction and Objectives

The non-enzymatic chemistry taking place during thermal food processing was studied since Louis-Camille Maillard discovered in 1912 that mixtures of amino acids with sugars lead to intensively brown colored structures and decarboxylation upon heating (MAILLARD 1912). Soon it became evident, that the development of color, aroma and taste are closely linked to the complex reaction pathways of the so-called Maillard reaction. The timeline of research in this field was recently summarized by Hellwig and Henle (HELLWIG and HENLE 2014). As the performance of the analytical equipment increased it was discovered that non-enzymatic browning reactions also lead to free and protein-bound Maillard products under physiological conditions. Today, these processes which lead to posttranslational modification are accepted as important pathogenic events in a number of chronic and age-related disorders such as diabetes (SINGH et al. 2001, BEISSWENGER 2014), uremia (ODANI et al. 1999a), atherosclerosis (MIYAZAWA et al. 2012) and Alzheimer's disease (SMITH et al. 1994).

On a molecular basis, the Maillard reaction results in a plethora of chemical compounds which are more generally divided into reactive intermediates and stable endproducts (advanced glycation endproducts, AGEs). *AGE formation in vivo is promoted by a general increase in oxidative stress on the one hand and the availability of reactive precursor compounds derived from both, oxidative and non-oxidative chemistry (carbonyl stress) on the other hand (BAYNES and THORPE 1999).*

α-Dicarbonyl compounds (α-DCs) have to be regarded as the most important central intermediates in the course of amino acid and protein modification. Therefore, *they represent one of the major sources of carbonyl stress and* knowledge of the *in vivo* concentrations in different matrices is required for the understanding of the mechanistic background of AGE formation and profile. However, the analytical approach to the complete α-DC spectrum is very challenging. Their high reactivity hampers direct analytical assessment and, thus, derivatization to more stable compounds is a prerequisite. Different trapping reagents like *o*-alkyl hydroxylamines, hydrazines, cysteamines, *o*-diaminobenzene derivatives and aminoguanidines are discussed in the literature (REVEL and BERTRAND 1993, SEVERIN et al. 1984, WELLS-KNECHT et al. 1995b, HAYASHI and SHIBAMOTO 1985, MCLELLAN et al. 1992, HIRSCH et al. 1992). However, derivatization of α-DCs with *o*-phenylenediamine (OPD) to yield the corresponding quinoxalines has

evolved as the most accepted procedure (GLOMB and TSCHIRNICH 2001). Furthermore, sample collection and sample preparation were found to have a major impact on the results and have to be strictly controlled (HENNING et al. 2014, MCLELLAN et al. 1992, MITTELMAIER et al. 2010, CHAPLEN et al. 1996).

Oxidative stress is quite often considered as tissue damage resulting from an imbalance between an excessive generation of highly reactive prooxidative compounds and insufficient antioxidant defense mechanisms (SIES 1997). However, generation of oxidative compounds is first and foremost physiologically relevant as an important step of inflammation. It represents one basis of the defense mechanisms against invading microorganisms and malignant cells, as well as of tissue healing and remodeling. Improper or maladaptive activation of oxidative processes, chronically present in pathological situations, such as uremia, leads to complications by contributing to cell and tissue injury (HANDELMAN 2000). Uremia is closely associated with complications like atherosclerosis and β_2-microglobulin amyloidosis which are thought to be related to oxidative tissue damage, due to the imbalance of prooxidant and antioxidant factors (DESCAMPS-LATSCHA et al. 2001). Thus, developing novel therapeutic interventions to reduce oxidative stress, i.e. by administration of antioxidants or by attenuation of chronic inflammation, is an important field of research. A necessary prerequisite in such studies is the accurate and reliable quantitation of the stress status. Here, oxidized biomolecules, e.g. proteins, are often used as targets. A term frequently used in protein oxidation research is "advanced oxidation protein products (AOPPs)" expressed as the ability of proteins to oxidize iodide to iodine (WITKO-SARSAT et al. 1996). As a major drawback of this approach the molecular nature of AOPPs is not further specified and potential mechanistic backgrounds remain unclear. A promising but analytically much more complex alternative is the detection of specific well-defined oxidation marker compounds.

The aim of the present work was to investigate the significance of the various routes for carbohydrate fragmentation in Maillard chemistry under physiological conditions. To transfer the findings of in vitro experiments to the in vivo situation, blood plasma of uremic patients vs. healthy controls was characterized with special emphasis towards carbonyl stress, oxidative stress and the content of a novel class of AGEs, the amide-AGEs.

2 Pathways of the Maillard Reaction

2.1 Initial phase

Glucose and ascorbic acid as the major carbohydrate players *in vivo* constitute the beginning of the Maillard reaction cascade. In a first step, glucose reacts to an 1,2-enaminol by nucleophilic attack of the ε-amino group of free or protein bound lysine. Rearrangement leads to 1-amino-1-deoxy-fructose (fructoselysine, Amadori product) (AMADORI 1929, HEYNS and NOACK 1962). Ascorbic acid is oxidized to dehydroascorbic acid (DHA) (SHIN and FEATHER 1990). When the Maillard reaction proceeds, both structures lead to reactive α-DCs.

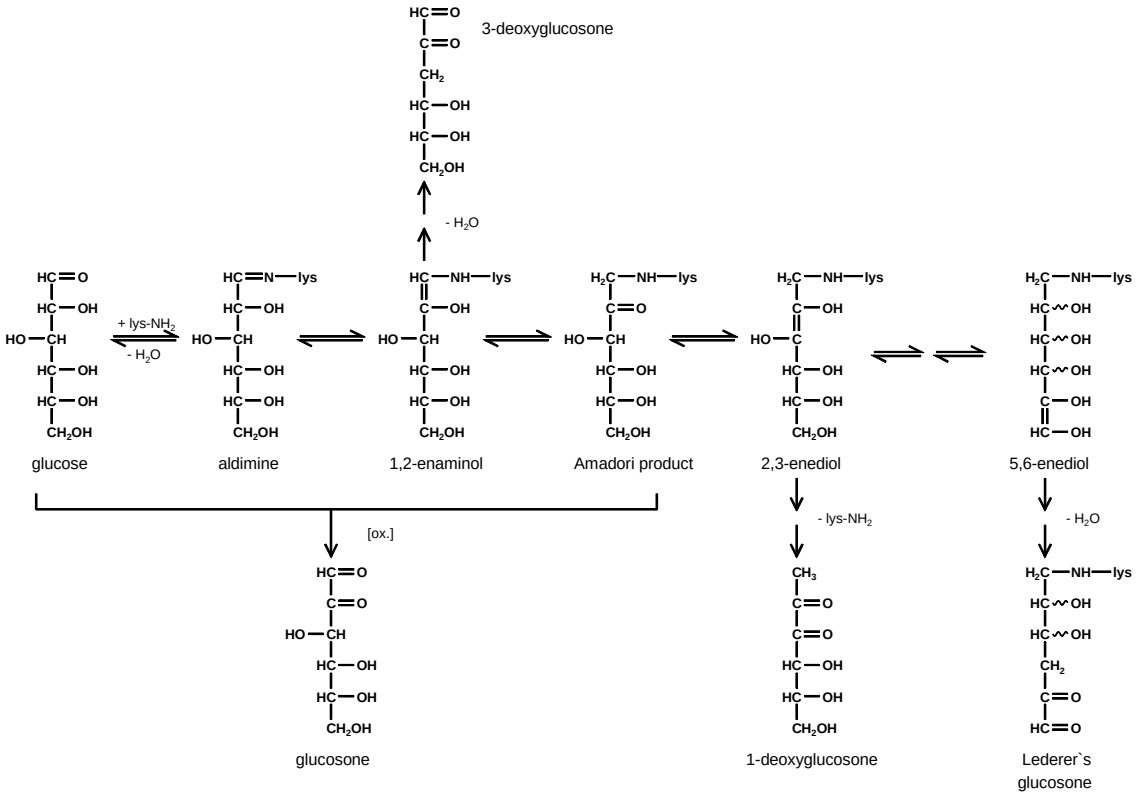

FIGURE 1. Initial state stages of amine induced glucose degradation retaining the intact C_6-carbon backbone.

The initial state preserves the C_6-carbon skeleton but includes carbonyl shifts along the entire carbohydrate backbone (Fig. 1). 3-Deoxyglucosone (3-DG) is formed non-oxidatively via 1,2-enolization and dehydration of the Amadori product (BECK et al. 1989).

2,3-Enolization leads to 1-deoxyglucosone (1-DG) (MORITA et al. 1985) and Lederer's glucosone (N^6-(3,6-dideoxyhexos-2-ulos-6-yl)-L-lysine) originates from the 5,6-enediol (BIEMEL et al. 2002b, REIHL et al. 2004a). Oxidation of the Amadori product gives rise to glucosone (GOBERT and GLOMB 2009, KAWAKISHI et al. 1991). Ascorbic acid can be recycled by enzymatic pathways from dehydroascorbic acid (WINKLER 1992, BODE et al. 1993, ARRIGONI and DE TULLIO 2002). In physiological solutions, however, part of the dehydroascorbic acid hydrolyzes irreversibly to 2,3-diketogulonic acid (2,3-DKG). These dicarbonyl compounds are direct precursors of certain AGEs, e.g. pyrraline and glucosepane, or undergo fragmentation reactions to yield further reactive α-DCs with a carbon backbone smaller than C_6. Important breakdown cascades of the carbon backbone relevant for the formation of AGEs are presented in the following sections.

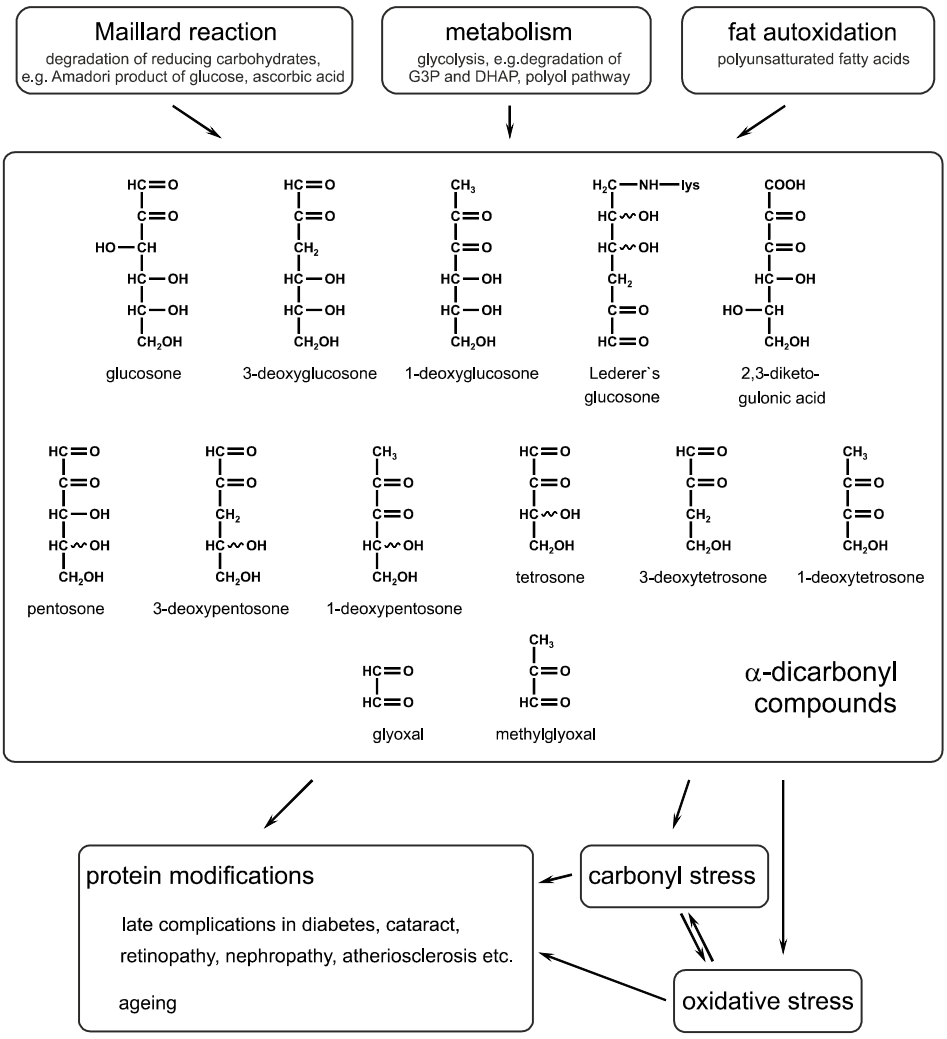

FIGURE 2. *The modern view of Maillard reaction with α-dicarbonyl compounds as central intermediates (G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate).*

The modern view of the Maillard reaction *in vivo* considers also diverse other physiological sources for α-DCs, irrespective of Maillard chemistry, like metabolism and fat autoxidation *(Fig. 2).* On the one hand, as an example, most of the methylglyoxal formed *in vivo* stems from the enzymatic and spontaneous degradation of the triose phosphates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, and the metabolism of ketone bodies (RABBANI and THORNALLEY 2012), whereas large amounts of glyoxal are formed by lipid peroxidation (FU et al. 1996). On the other hand, both compounds are detoxified by the glyoxalase system (THORNALLEY 1998).

2.2 Cyclization

Especially important in view of age-related alterations of long lived proteins are structures that crosslink two amino acid side chains. A major bivalent lysine-arginine crosslink is glucosepane which stems from Lederer's glucosone (Fig. 3).

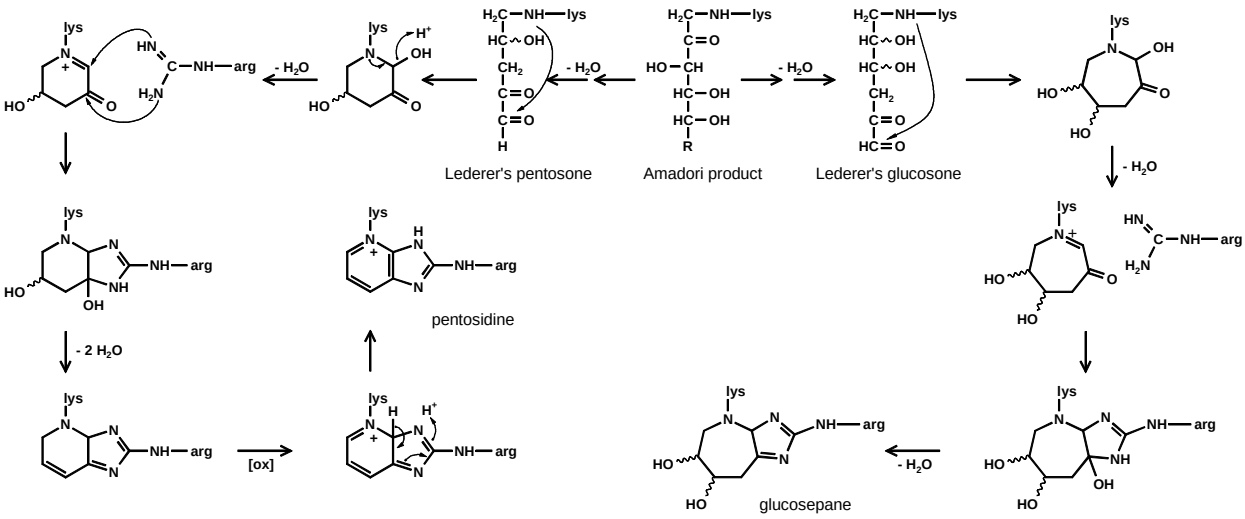

FIGURE 3. Cyclization of Lederer's glucosone (R = $–CH_2OH$) and Lederer's pentosone (R = –H) lead to bivalent lysine-arginine crosslinks glucosepane and pentosidine, respectively.

Ring closure and dehydration lead to an intramolecular aldimine which then reacts with the guanidinium group of arginine (BIEMEL et al. 2001, LEDERER and BÜHLER 1999). As a similar but quantitatively minor crosslink, pentosidine can be explained via the same mechanism with the 4,5-enediol of a pentose as the precursor (BIEMEL et al. 2001). Alternatively, fragmentation of C_6 α-DCs also yields the required C_5-precursor for

pentosidine formation. Vesperlysine (LM-1), the analogous bivalent lysine-lysine structure to pentosidine, is supposed to form via totally different pathways with ascorbic acid as the starting compound although the detailed mechanism is still unknown (NAGARAJ and MONNIER 1992, NAKAMURA et al. 1997, TESSIER et al. 1999). Pyrraline represents a monovalent lysine modification originating from 3-deoxyglucosone. The formation proceeds via nucleophilic attack of the ε-amino group of lysine at the C^2-carbonyl moiety, followed by dehydration, cyclization and aromatization (Fig. 4) (NAKAYAMA et al. 1980, MILLER and OLSSON 1985, HAYASE et al. 1989, NJOROGE et al. 1987).

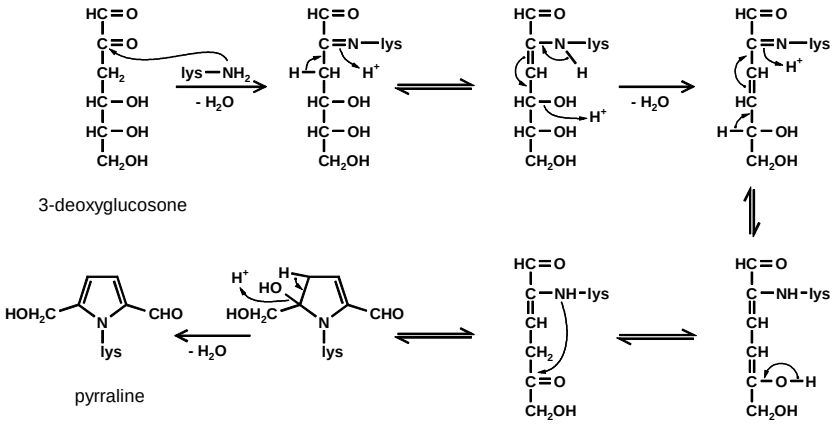

FIGURE 4. Reaction pathway for the formation of pyrraline.

2.3 Isomerization

First discovered by Baynes' group in 1986 (AHMED et al. 1986), N^6-carboxymethyl lysine (CML) is the most important monovalent lysine modification *in vivo*. They proposed the oxidative fragmentation of the Amadori product of glucose to yield CML and erythronic acid. However, a comprehensive examination of the underlying mechanism is still lacking. An alternative route proceeds with lysine and glyoxal as precursors via glyoxal-imine to the hydrate and subsequent isomerization (Fig. 5) (GLOMB and MONNIER 1995). Furthermore, glycolic acid lysine amide (GALA), glyoxal lysine amide (GOLA) and glyoxal lysine dimer (GOLD) can arise from the very same CML reaction cascade (GLOMB and PFAHLER 2001). If the nucleophilic addition of water takes place at the imine group, isomerization yields the amide-AGE GALA. A nucleophilic attack of the ε-amino group of a second lysine molecule at the carbonyl group of glyoxal-imine leads to the bivalent

lysine-lysine crosslink GOLA. Reaction of the intermediate state with a second molecule of glyoxal, ring closure and water elimination gives the imidazolium crosslink GOLD (WELLS-KNECHT et al. 1995a). Analogous imidazolium salts are methylglyoxal lysine dimer (MOLD) (BRINKMANN et al. 1995, NAGARAJ et al. 1996) and 3-deoxyglucosone lysine dimer (DOLD) (SKOVSTED et al. 1998) with the corresponding imine structures as precursors. The CML-glyoxal reaction cascade has been successfully translated to methylglyoxal leading to N^6-carboxyethyl lysine (CEL) and N^6-lactoyl lysine. However, here in contrast to CMLs additional Amadori product route, the formation via isomerization is exclusive.

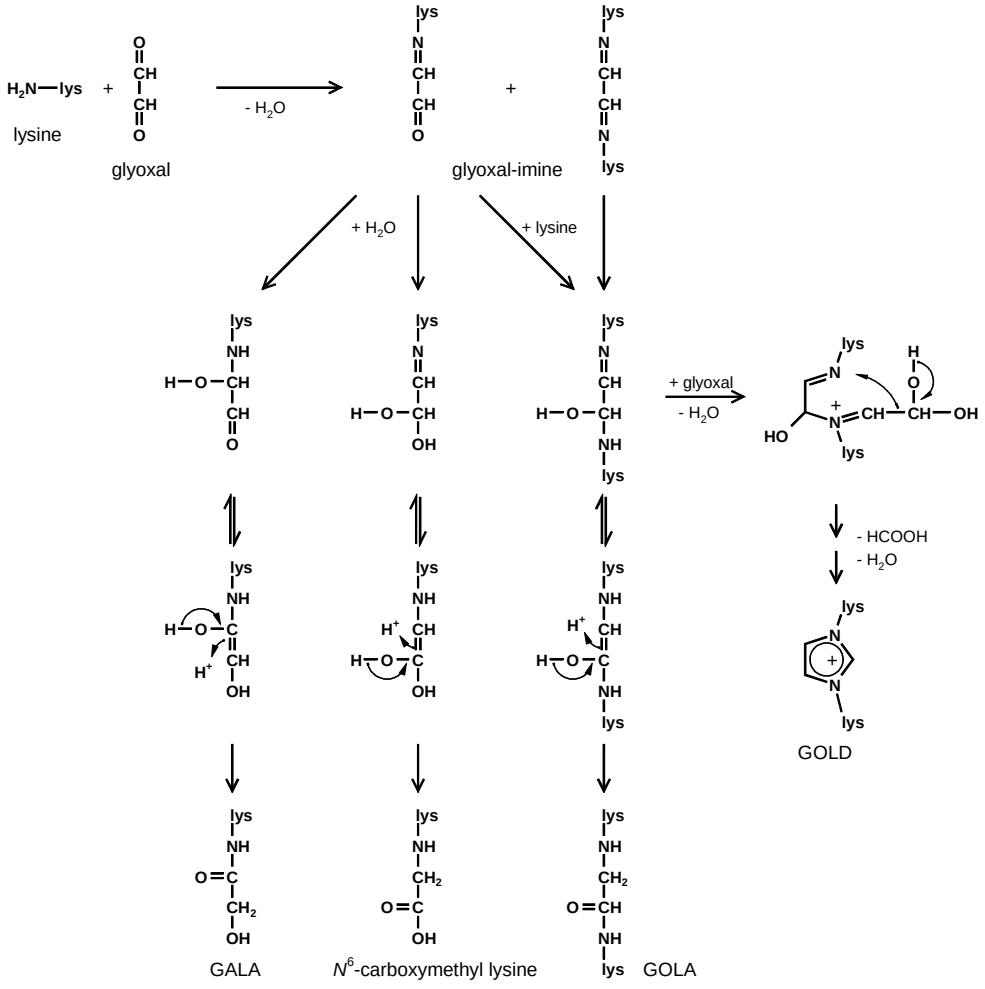

FIGURE 5. The N^6-carboxymethyl lysine (CML) reaction cascade explains the formation of glycolic acid lysine amide (GALA), glyoxal lysine amide (GOLA) and glyoxal lysine dimer (GOLD) via isomerization of the hydrated glyoxal-imine.

In addition to the ε-amino group of lysine the guanidinium group of arginine is prone to yield various Maillard modifications. Here, only α-DCs are known precursors for monovalent modifications. Our workgroup investigated the mechanistic pathway of the reaction with glyoxal (GLOMB and LANG 2001). The intermediate 2-imino-4,5-dihydroxy-imidazolidine is slowly converted to N^7-carboxymethyl arginine (CMA) through ring opening of the imidazolidine to the open-chained hemiaminal and intramolecular disproportionation, i.e. isomerization. An alternative route, only present under strong acidic conditions, leads to glyoxal imidazolinone 3 (G-H3). Thus, formation of G-H3 is not possible under physiological conditions but occurs as an artifact of sample cleanup. CMA has to be regarded as the only stable arginine modification with glyoxal. Methylglyoxal as a precursor gives a different picture. Here, as illustrated in Fig. 6, the homologous structure N^7-carboxyethyl arginine (CEA) (ALT and SCHIEBERLE 2005b, ALT and SCHIEBERLE 2005a) is a minor intermediate of the equilibrium between a kinetically controlled (MG-H3) and a thermodynamically controlled imidazolinone (MG-H1) (KLÖPFER et al. 2010). In the methylglyoxal reactions the various cyclic imidazolinone and pyrimidine derivatives are of much higher quantitative importance.

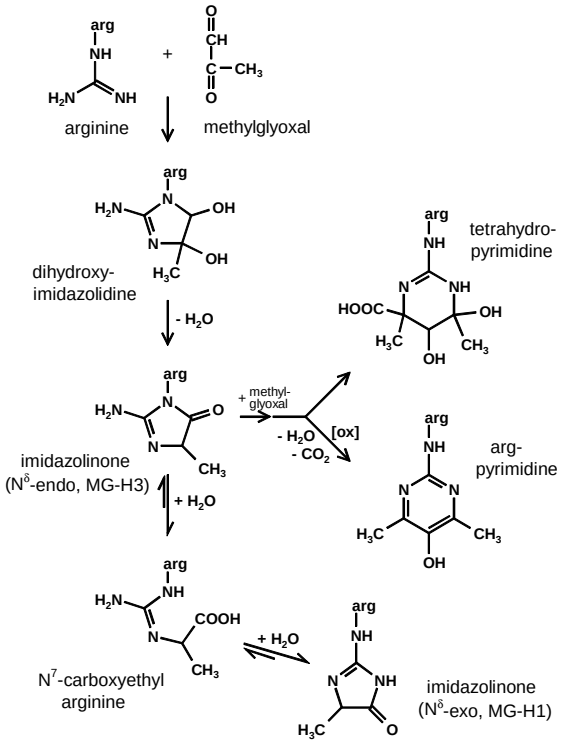

FIGURE 6. Formation pathways of methylglyoxal-arginine modifications.

In general, three constitutional isomers of the imidazolinones originating from glyoxal, methylglyoxal and 3-DG can be formulated. The isomer type 1 refers to the imidazolinone with an exocyclic δ-amino group whereas isomer type 2 and type 3 possess an endocyclic δ-nitrogen atom (AHMED et al. 2002b, THORNALLEY et al. 2003). In addition, tautomerization of the imino group of MG-H1 or racemization of the chiral center of the imidazolinone ring system of MG-H2 and MG-H3 lead to a multitude of further isomeric structures (KLÖPFER et al. 2010). A stable and fluorescent endproduct of the reaction of arginine with methylglyoxal, argpyrimidine (AP), was identified by Shipanova et al. (SHIPANOVA et al. 1997). The formation proceeds via the same precursor compound as the reaction to CEA with incorporation of a second molecule of methylglyoxal. An alternative route yields a tetrahydropyrimidine (THP) (OYA et al. 1999).

While the described isomerization reactions maintain the carbon backbone, the following sections discuss possible fragmentation pathways (Fig. 7).

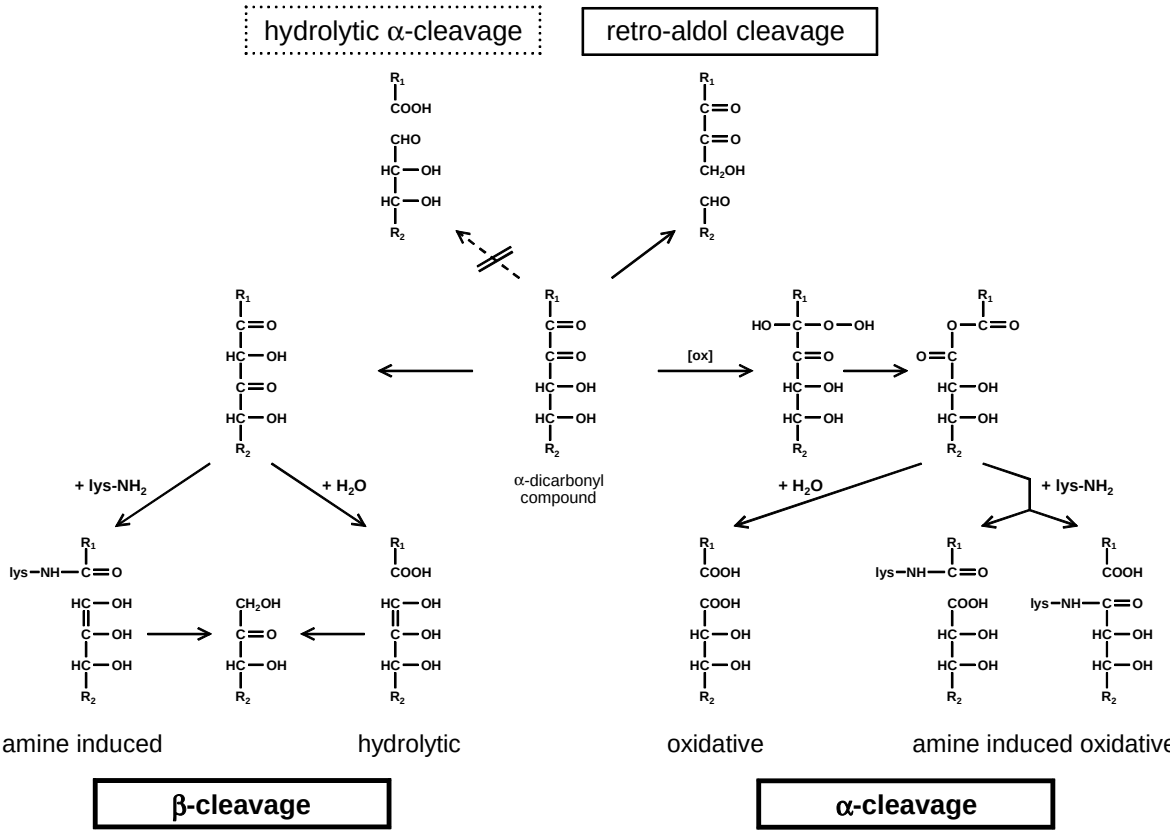

FIGURE 7. Fragmentation pathways of α-dicarbonyl compounds. The relevant mechanisms *in vivo* are set in bold fonds.

Basically, three different mechanisms are described in the literature (WEENEN 1998, TRESSL and REWICKI 1999): retro-aldol cleavage, α-dicarbonyl cleavage and β-dicarbonyl cleavage. This concept has to be re-evaluated in the light of new findings. First, differentiation into a hydrolytic (KIM and BALTES 1996) and an oxidative α-dicarbonyl cleavage (DAVIDEK et al. 2006) has to be made. Second, both the hydrolytic β-dicarbonyl and the oxidative α-dicarbonyl cleavage have to be complemented by an amine-catalyzed mechanism (SMUDA et al. 2010).

2.4 Retro-aldol cleavage

β-Hydroxy carbonyl compounds (aldols) can undergo retro-aldolization by cleavage of the carbon-carbon bond between C^{α} and C^{β} next to the carbonyl group. This mechanism is still the most accepted fragmentation pathway in Maillard reactions for sugars, Amadori and Heyns rearrangement products and α-DCs used to explain formation of shorter-chain hydroxyl ketones, hydroxyl aldehydes and dicarbonyl compounds (HODGE 1953, WEENEN 1998, THORNALLEY et al. 1999, YAYLAYAN and KEYHANI 2000). However, as stated in a recent review, there is no convincing experimental proof for retro-aldolization in α-DC fragmentation (SMUDA and GLOMB 2013a). The relevance of retro-aldol fragmentation under physiological conditions is more than questionable.

2.5 Hydrolytic α-cleavage

A second, frequently reported concept of α-DC fragmentation is the hydrolytic α-cleavage (KIM and BALTES 1996, GINZ et al. 2000, BRANDS and VAN BOEKEL 2001, MARTINS et al. 2003). Based on an intramolecular disproportionation, the carbon-carbon bond fission reaction takes place between both carbonyl moieties yielding a carboxylic acid and an aldehyde. However, experiments of Davídek et al. and others clearly disproved this mechanism and proposed an oxidative α-dicarbonyl cleavage mechanism instead (DAVÍDEK et al. 2006).

2.6 Oxidative α-cleavage

The oxidative α-cleavage proposed by Davídek et al. (DAVÍDEK et al. 2006) was further verified by *in vitro* experiments with 1-deoxyglucosone (DAVIDEK et al. 2006). As expected, acetic acid and the counterpart erythronic acid were detected. More recently, we performed a mechanistic in-depth investigation of the degradation pathways of ascorbic acid (SMUDA and GLOMB 2013b). Based on the incorporation of activated molecular oxygen generated by e.g. photooxidation processes or hydroperoxide species the mechanism starts with an attack at the C^2 or C^3 carbonyl moiety of 2,3-DKG (Fig. 8).

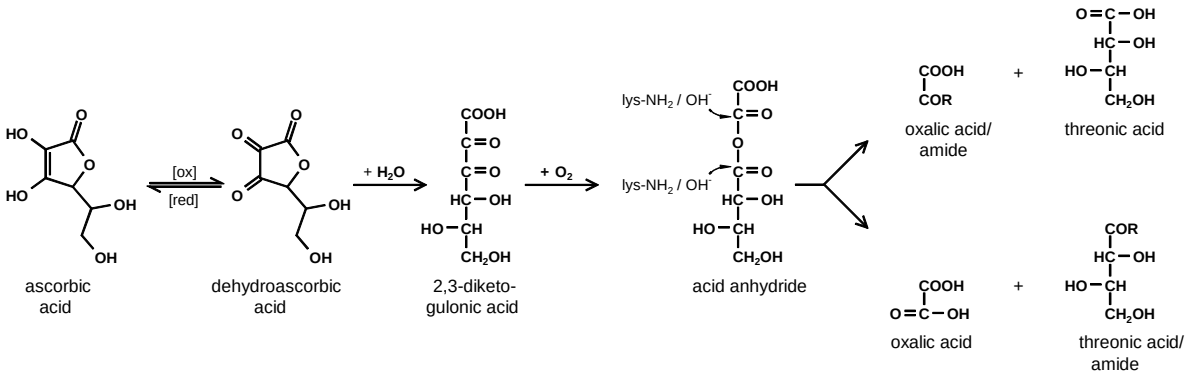

FIGURE 8. Oxidative α-dicarbonyl cleavage reaction of 2,3-diketogulonic acid which is the product of ascorbic acid oxidation and hydrolytic ring opening. Hydrolytic attack leads to oxalic acid and threonic acid (R = –OH) whereas lysine induced fragmentation (R = –NH-lys) yields the amide structure and the corresponding acid.

After rearrangement hydrolysis of the intermediate acid anhydride yields a mixture of oxalic acid and threonic acid. Acylation is an amine driven alternative route where the ε-amino group of lysine initiates a nucleophilic attack on the anhydride to directly yield the amide-AGEs N^6-oxalyl lysine and N^6-threonyl lysine.

2.7 Hydrolytic β-cleavage

Hydrolytic β-dicarbonyl cleavage of sugars was already mentioned in 1961 as an alternative fragmentation pathway to retro-aldol reactions (HAYAMI 1961). Again, Davídek et al. were the first to perform detailed mechanistic studies (MILLS et al. 1970, DAVIDEK et al. 2005). The results were confirmed by investigations of our workgroup (VOIGT and

GLOMB 2009, VOIGT et al. 2010). We studied the degradation of 1-DG which is isomerized into its 2,4-tautomer. Addition of water leads to a hydrate which induces scission to acetic acid and a C_4-enediol intermediate. Isomerization eventually yields tetruloses and tetrosones (Fig. 9).

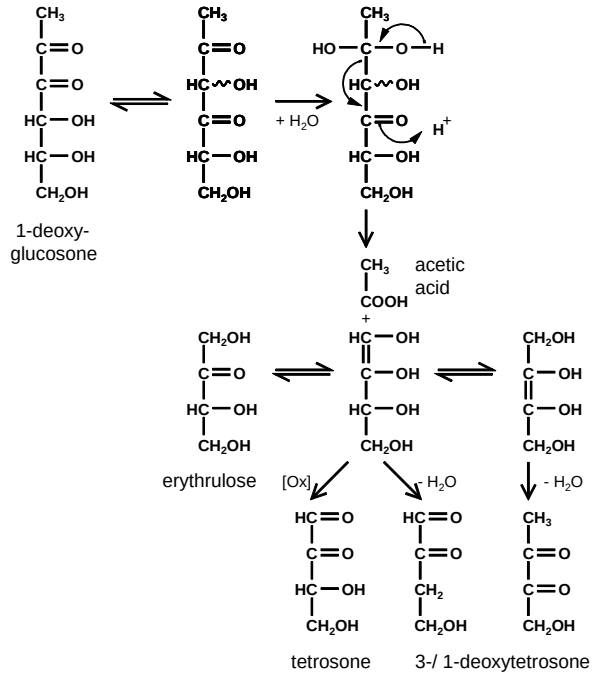

FIGURE 9. Hydrolytic β-dicarbonyl cleavage reaction of 1-deoxyglucosone.

Similarly, 2,3-DKG can fragment to oxalic acid and the corresponding C_4-enediol based on the same pathway. Hydration at the C^2 carbonyl moiety without prior tautomerization results in decarboxylation as an alternative reaction pathway, which indeed follows the same mechanistic principle. The C_5-enediol intermediate can dehydrate to form xylosone or isomerize to yield xylonic acid and lyxonic acid, respectively (Fig. 10) (SMUDA and GLOMB 2013b).

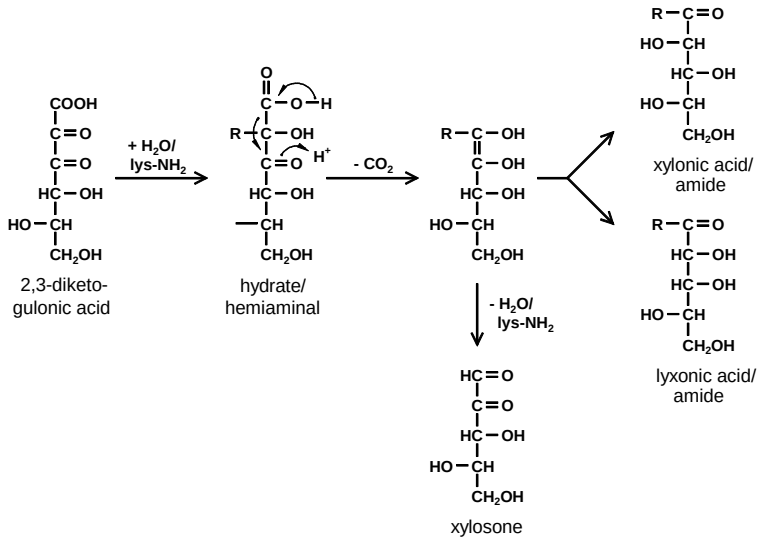

FIGURE 10. Hydration (R = –OH) or amination (R = –NH-lys) and subsequent decarboxylation of 2,3-diketogulonic acid leads to the respective C_5-enediol or enaminol.

2.8 Amine induced β-cleavage

Recently, a novel class of AGEs (amide-AGEs) was discovered during the degradation of 1-DG in aqueous solutions under physiological conditions (SMUDA et al. 2010). *The quantitative relevance in vivo was established for N^6-formyl, N^6-lactoyl and N^6-glycerinyl lysine for the first time in publication A in human blood plasma and later also in human lens (HENNING et al. 2011, SMUDA et al. 2015).* The formation of amide-AGEs was explained via an amine induced β-cleavage mechanism which parallels the above hydrolytic pathways (Fig. 11).

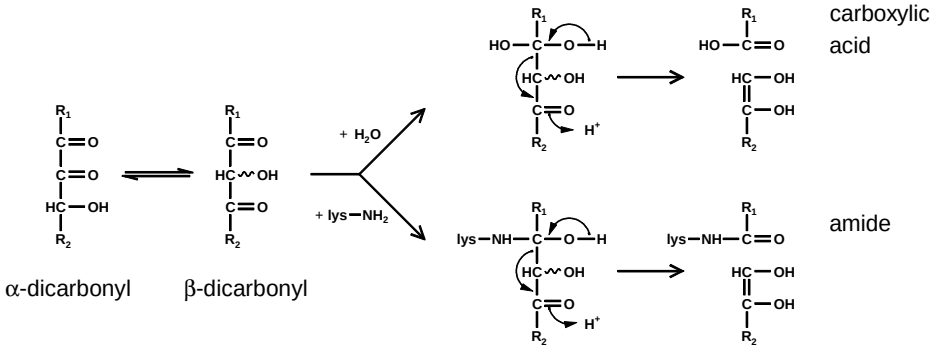

FIGURE 11. Hydrolytic β-dicarbonyl cleavage leads to an enediol structure and a carboxylic acid as the counterpart. The analogous amine induced β-dicarbonyl cleavage yields the amide structure as the counterpart of the enediol.

Indeed, incubations of α-DCs in the presence of lysine yielded the enediol in form of the respective ketose, the matching carboxylic acid and the corresponding amide structure (e.g. N^6-acetyl lysine with 2,4-deoxyglucosone as the precursor or N^6-oxalyl lysine with 2,4-diketogulonic acid as the precursor) (SMUDA et al. 2010, SMUDA and GLOMB 2012).

3 Relevance *in vivo*

3.1 α-Dicarbonyl Compounds as reactive intermediates

As already discussed above, the basis for the understanding of the complex Maillard processes *in vivo* is the knowledge of the complete spectrum of α-DCs. However, analytical investigations were often limited to few selected compounds like glyoxal, methylglyoxal and 3-DG (MIRZA et al. 2007, ODANI et al. 1999a, LAPOLLA et al. 2005b, LAPOLLA et al. 2005a, KNECHT et al. 1992, MCLELLAN et al. 1992, NAKAYAMA et al. 2008, LOPEZ-ANAYA and MAYERSOHN 1987, HAIK et al. 1994). A more comprehensive approach including important intermediates from a mechanistic point of view is needed, e.g. 1-DG and 2,3-DKG. Initial investigations for a complete set of α-DCs in human plasma *(publication B)* and lens were recently published and should be continuously extended (HENNING et al. 2014, NEMET and MONNIER 2011).

TABLE 1. *Levels of all relevant α-dicarbonyl compounds in human blood plasma (mean ± SD of 15 healthy subjects and 24 patients undergoing hemodialysis (HD patients), replicate analyses n = 3, the Student's t-test was used for statistical evaluation of significant differences between both groups). (HENNING et al. 2014)*

	healthy subjects						HD patients					
quinoxaline	mean	±	SD	range			mean	±	SD	range		
	pmol/ml						*pmol/ml*					
glucosone	46	±	11	28	–	67	96	±	49 [**]	57	–	276
1-DG	22	±	3	16	–	28	30	±	16	12	–	82
3-DG	43	±	5	35	–	56	65	±	20 [**]	36	–	125
Lederer's glucosone	< *LOD*						7.0	±	2.5 [a]	< *LOD*	–	13
pentosone	15	±	10 [a]	< *LOD*	–	32	11	±	5 [a]	< *LOD*	–	23
1-DP	3.6	±	1.2 [a]	2.6	–	7.8	6.2	±	2.6 [a **]	3.2	–	15
3-DP	11	±	2	9	–	17	33	±	9 [**]	20	–	53
threosone	5.4	±	0.7 [a]	4.2	–	6.6	9.0	±	3.5 [a **]	4.2	–	19
1-DT	3.1	±	0.3	2.6	–	3.8	3.8	±	1.0 [*]	2.0	–	6.6
3-DT	10	±	2	8	–	14	45	±	11 [**]	28	–	75
methylglyoxal	61	±	7	51	–	76	219	±	129 [**]	42	–	617
glyoxal	491	±	47	405	–	564	1273	±	980 [**]	400	–	4914
pyruvic acid	7250	±	2549	2818	–	12038	35874	±	19080 [**]	8399	–	97935
glyoxylic acid	1264	±	353	783	–	1942	2031	±	608 [**]	1026	–	3514
oxalic acid	40	±	21	18	–	82	36	±	12	16	–	70
3,4-DDP	6.0	±	1.0	4.4	–	7.7	19	±	8 [**]	6	–	42
DHA precursor	687	±	351 [b]	337	–	1303	1925	±	1106 [b **]	308	–	4829
DHA	15400	±	4019 [b]	6809	–	23967	12538	±	7183 [b]	2737	–	26680
2,3-DKG	1741	±	674 [c]	567	–	2669	411	±	238 [c **]	145	–	937

[a] *LOD* < *x* < *LOQ*. [b] The sum of DHA and DHA precursor account for approximately 38 % of the true DHA content. [c] Values adjusted as described in the discussion of publication B. [*] $p < 0.01$ *versus* healthy subjects. [**] $p < 0.001$ *versus* healthy subjects.

The LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) method presented in publication B provides for the first time the opportunity to identify and quantitate the complete spectrum of relevant α-DCs in human plasma in a single chromatographic run. For 14 compounds, the plasma levels were determined. Three compounds were below the LOQ but were unequivocally identified. Ten substances have not been reported for plasma samples before. To evaluate the clinical relevance of the assay described herein an initial set of 24 uremic patients undergoing hemodialysis was analyzed. Uremia is related to an increase in oxidative and carbonyl stress and thus should lead to a clear shift in the dicarbonyl spectrum. Indeed, most α-DCs were considerably higher in HD patients. Glucose derived glucosone exhibited a two-fold increase which is expected under conditions of elevated oxidative stress. In contrast, 1-DG does not require an oxidation step for its formation and remained nearly at the level of healthy subjects. Interestingly, plasma levels of 2,3-DKG were considerably decreased in HD patients. This must be explained by a significantly accelerated degradation via oxidative pathways. Glyoxal and methylglyoxal are further compounds of published interest in regard to certain chronic diseases like uremia. A 3-fold elevation of glyoxal and a 4-fold increase of methylglyoxal were observed in uremic plasma. This is in line with the literature (ODANI et al. 1999a, LAPOLLA et al. 2005b, NAKAYAMA et al. 2008), although the absolute values differ significantly, depending on the respective study. The assessment of α-DC plasma levels depends strongly on the analytical approach, specifically on work-up conditions, derivatization procedure and chromatographic method. Most importantly, the derivatization was conducted in the presence of protein to assess both free and reversibly bound α-DCs. Therefore, a direct comparison of the plasma levels of the present study and those reported previously is not possible.

The C_6-dicarbonyl compound glucosone and its analogues 1-DG and 3-DG can not arise from ascorbate degradation and therefore are markers for glucose derived α-DCs in the context of Maillard chemistry (Fig. 12). 3-DG is formed non-oxidatively from the Amadori product of glucose via 1,2-enolization and dehydration, whereas 2,3-enolization yields 1-DG (NIWA 1999).

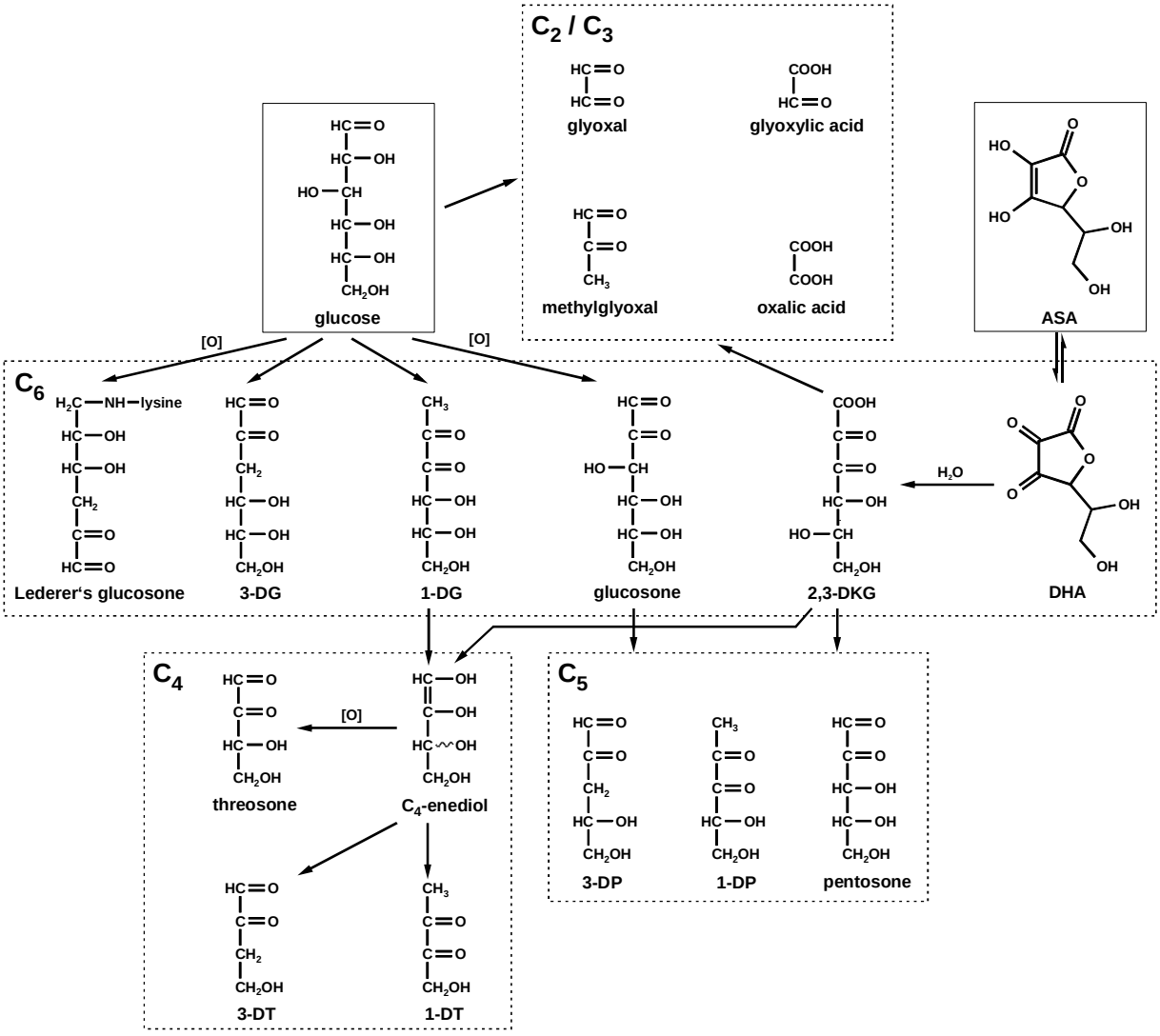

FIGURE 12. *Formation pathways of α-DCs from glucose and ascorbic acid. (HENNING et al. 2014)*

Oxidation of the Amadori compound leads to glucosone (GOBERT and GLOMB 2009). Besides from Maillard reaction, an important endogenous route leading to 3-DG formation from glucose is the enzymatic polyol pathway (TSUKUSHI et al. 1999). 3-DG is reviewed in literature as the most abundant C_6-dicarbonyl in vivo (VISTOLI et al. 2013) but poses only a very limited glycating reactivity (GOBERT and GLOMB 2009). Thus, the chemistry of 3-DG has to be considered as of minor relevance regarding Maillard processes under physiological conditions. 1-DG and glucosone were identified as the central intermediates leading to C_4- and C_5-fragments, respectively (GLOMB et al. 2010). Their reductone structure with an α-oxo-enediol moiety boasts significant higher reactivity. More generally, this applies to all analogue C_4- and C_5-dicarbonyls but 1-DG is

by far the most reactive and thus important α-DC intermediate regarding glucose-derived AGEs. In particular, as already mentioned above, amine-induced β-cleavage in the presence of lysine leads directly to carboxylic acid amides (amide-AGEs).

Lederer's glucosone was not detected in plasma of healthy subjects even though it is a relative stable non reductone structure like 3-DG. However, unlike 3-DG the required enolization along the entire carbon backbone makes this α-DC susceptible to multiple degradation processes. The existence of Lederer's glucosone in vivo is evident from the detection of its AGE follow-up structure glucosepane at low levels in human blood and extracellular matrix (BIEMEL et al. 2002a, SELL et al. 2005).

DHA is a C_6-dicarbonyl structure exclusively assigned to the degradation of ascorbic acid (ASA), formed by oxidation. DHA in aqueous solution hydrolyzes irreversibly to 2,3-DKG, which is the direct precursor of fragmentation products with a carbon backbone smaller than C_6.

α-DCs with a carbon skeleton smaller than C_6 arise from the degradation of glucose as well of ASA. As established in previous papers (SMUDA and GLOMB 2013b, VOIGT and GLOMB 2009), the C_4-dicarbonyls threosone, 1-deoxythreosone (1-DT) and 3-deoxythreosone (3-DT) are formed from both 1-DG and 2,3-DKG via β-dicarbonyl cleavage with an C_4-enediol as the reactive intermediate. Oxidation of the latter leads to threosone whereas dehydration at C^3 results in 3-DT. The enediol may also undergo isomerization to give 1-DT in an equivalent reaction. As a consequence under deaeration, which represents the situation in vivo, 3-DT was the prominent structure as the reductone 1-DT has to be considered a much more reactive and, thus, short-lived intermediate.

The plasma levels of pentosone were comparatively high and did not fit into the picture. Decarboxylation is a well established mechanism of ASA degradation and leads to C_5-compounds including pentosone (SHIN and FEATHER 1990, REIHL et al. 2004b) but under physiological conditions the formation of C_4-dicarbonyls from 2,3-DKG is favored (NEMET and MONNIER 2011, SMUDA and GLOMB 2013b). Glucose derived pentosone stems from glucosone by the same mechanism of hydrolytic β-cleavage as threosone from 1-DG as the precursor. After isomerization and hydration formic acid is cleaved off and gives an C_5-1,2-enediol as the reactive intermediate. Oxidation leads to pentosone, whereas water elimination yields 3-deoxypentosone (3-DP) or after 2,3-enolization 1-deoxypentosone (1-DP) (GLOMB et al. 2010). Considering the need of an oxidation step in order to obtain pentosone from both 2,3-DKG or glucosone, formation of 1-DP and 3-DP should be

favored. The results therefore strongly suggest an additional alternative source for pentosone formation in vivo.

In Maillard chemistry, glyoxylic acid is assigned to disaccharide chemistry (SMUDA and GLOMB 2011) but can also arise from oxidation of glyoxal (ROSSNER et al. 2001) and degradation of DHA (SHIN and FEATHER 1990, TAKAGI and MORITA 1987). However, this can not account solely for the plasma levels which were 2.5-fold higher than glyoxal and in the same range as 2,3-DKG. An alternative source is the degradation of hydroxyproline with the subsequent glyoxylate metabolism in human organism which is rather complex involving several enzymatic and non-enzymatic reactions and is subject to recent investigation (DUARTE et al. 2007).

Oxalic acid can originate via β-dicarbonyl fragmentation as well as via oxidative α-DC cleavage from 2,3-DKG and is the main degradation product of the latter (SMUDA and GLOMB 2013b). In addition, oxalate is also part of the glyoxylate metabolism mentioned above. However, under assay conditions the dicarboxylic acid oxalic acid is not converted to its corresponding quinoxaline. This is expected, because at the chosen workup pH the carboxylic acid groups do not show sufficient carbonyl activity to react with OPD. Consequently, there must be alternative precursors for the quinoxaline of oxalic acid other than oxalic acid itself. 3,4-Dideoxypentosone (3,4-DDP) is a known intermediate of maltose degradation but was neither found in glucose nor ASA reaction systems (SMUDA and GLOMB 2011). Hence, the origin of the detected quinoxaline of 3,4-DDP remains unknown.

3.2 Advanced Glycation Endproducts

The analytical assessment of AGE free adducts (AGE-modified amino acids) in different biological matrices is a difficult task. Even more challenging is the quantitation of protein bound AGEs because hydrolysis of the peptide bounds is a necessity. Total hydrolysis is achieved by strong acidic treatment at high temperatures. However, under these harsh conditions, many of the above described AGEs are not stable. To circumvent this problem to achieve a complete AGE spectrum different protocols of enzymatic protein hydrolyses were developed (GLOMB and PFAHLER 2001, HENLE et al. 1991, SMUDA et al. 2015, AHMED and THORNALLEY 2002). It is important in this regard to determine the efficiency

of the enzymatic digestion and to reassure the stability of e.g. above mentioned amidation under these conditions. Indeed, our multistep enzymatic approach including treatment with Pronase E, leucine aminopeptidase and carboxypeptidase Y showed no degradation of reference amide-AGEs.

Table 2 summarizes mono- and bivalent lysine derived AGEs, monovalent arginine derived AGEs and bivalent lysine-arginine crosslinks which are relevant *in vivo*. For each compound the known mechanism of formation and the analytical detection in different matrices are listed without subdivision into protein bound modifications and free adducts. Obviously, there is no claim of completeness.

TABLE 2. Advanced glycation endproducts (AGEs) relevant *in vivo*.

AGE compound	**mechanism of formation**	**quantitation *in vivo***
monovalent lysine modifications		
N^6-carboxymethyl lysine (CML) [a]	isomerization, unknown ox. fragmentation	various tissues, plasma, blood cells, urine, faecis, lens, reviewed in (THORPE and BAYNES 2002, DELGADO-ANDRADE 2016)
N^6-carboxyethyl lysine (CEL) [a]	isomerization	plasma (HENNING et al. 2011, EUPEN et al. 2013, GOPAL et al. 2014, LIEUW-A-FA et al. 2004); plasma, blood cells, urine (THORNALLEY et al. 2003, AHMED et al. 2005); lens (SMUDA et al. 2015, AHMED et al. 1997)
glycolic acid lysine amide (GALA) [b]	isomerization	lens (SMUDA et al. 2015)
N^6-formyl lysine [b]	amine induced β-cleavage	plasma (HENNING et al. 2011), lens (SMUDA et al. 2015)
N^6-acetyl lysine [b]	amine induced β-cleavage	plasma (HENNING et al. 2011), lens (SMUDA et al. 2015)
N^6-oxalyl lysine [b]	ox. α-cleavage, amine induced β-cleavage	lens (SMUDA et al. 2015)
N^6-lactoyl lysine [b]	amine induced β-cleavage	plasma (HENNING et al. 2011); lens (SMUDA et al. 2015)
N^6-glycerinyl lysine [b]	amine induced β-cleavage	plasma (HENNING et al. 2011)
N^6-threonyl lysine [b]	ox. α-cleavage	lens (SMUDA et al. 2015)
N^6-xylonyl lysine [b]	amine induced β-cleavage	lens (SMUDA et al. 2015)
N^6-lyxonyl lysine [b]	amine induced β-cleavage	lens (SMUDA et al. 2015)
pyrraline [b]	cyclization	plasma (PORTERO-OTÍN et al. 1995, MIYATA and MONNIER 1992), serum (ODANI et al. 1999b); urine (TAMURA et al. 2006, ASO et al. 2004, PORTERO-OTÍN et al. 1996); lens (SMUDA et al. 2015, NAGARAJ and SADY 1996) various tissue (MIYATA and MONNIER 1992)

bivalent lysine modifications		
glyoxal lysine amide (GOLA) [b]	isomerization	lens (GLOMB and PFAHLER 2001, SMUDA et al. 2015)
glyoxal lysine dimer (GOLD) [a]	isomerization	serum (ODANI et al. 1999b); lens (FRYE et al. 1998); serum, lens (CHELLAN and NAGARAJ 1999)
methylglyoxal lysine dimer (MOLD) [a]	isomerization	serum (ODANI et al. 1999b, NAGARAJ et al. 1996); plasma, blood cells, urine (THORNALLEY et al. 2003, AHMED et al. 2005); lens (FRYE et al. 1998) ; serum, lens (CHELLAN and NAGARAJ 1999)
vesperlysine (LM-1) [a]	unknown	lens (TESSIER et al. 1999)
monovalent arginine modifications		
N^7-carboxymethyl arginine [b]	isomerization	serum (ODANI et al. 2001), lens (SMUDA et al. 2015)
N^7-carboxyethyl arginine [b]	isomerization	lens (SMUDA et al. 2015)
methylglyoxal imidazolinone (MG-H1) [b]	isomerization	plasma, blood cells, urine (THORNALLEY et al. 2003, AHMED et al. 2005); lens (AHMED et al. 2003, SMUDA et al. 2015)
methylglyoxal imidazolinone (MG-H3) [b]	isomerization	lens (SMUDA et al. 2015)
3-deoxyglucosone imidazolinone (3DG-H) [b]	isomerization	plasma, blood cells, urine (THORNALLEY et al. 2003, AHMED et al. 2005);
argpyrimidine (AP) [a]	isomerization	plasma, blood cells, urine (THORNALLEY et al. 2003, AHMED et al. 2005); lens (AHMED et al. 2003, SMUDA et al. 2015)
tetrahydropyrimidine (THP) [b]	isomerization	plasma (EUPEN et al. 2013); lens (SMUDA et al. 2015)
bivalent lysine-arginine modifications		
glucosepane [b]	cyclization	lens (SMUDA et al. 2015); serum, lens (BIEMEL et al. 2002a); collagen (MONNIER et al. 2013)
pentosidine [a]	cyclization	plasma (SANAKA et al. 2002, GOPAL et al. 2014);serum (ODANI et al. 1999b); urine (TAMURA et al. 2006, ASO et al. 2004); urine, serum (YOSHIHARA et al. 1998); plasma, blood cells, urine (THORNALLEY et al. 2003, AHMED et al. 2005); collagen (SELL and MONNIER 1990, SELL et al. 1992, YU et al. 2006); lens (NAGARAJ et al. 1991, AHMED et al. 2003)

[a] acid stable; [b] total degradation under strong acidic conditions

CML and the crosslink pentosidine represent the most investigated AGEs. CML was the first AGE discovered (AHMED et al. 1986) and is of significant quantitative importance. This fact and its acid stability allowed the quantitation in many different matrices (DELGADO-ANDRADE 2016). Pentosidine represents an acid stable fluorophore and thus enabled very sensitive detection in a variety of human tissues even before the coupling of liquid chromatography with mass spectrometry became a common analytical tool (SELL and MONNIER 1989b, Sell and MONNIER 1989a, SELL et al. 1991).

A novel class of important glycation products are amide-AGEs. Their quantitative relevance in lens protein (GLOMB and PFAHLER 2001, SMUDA et al. 2015) and human plasma (publication A) (HENNING et al. 2011) was already established.

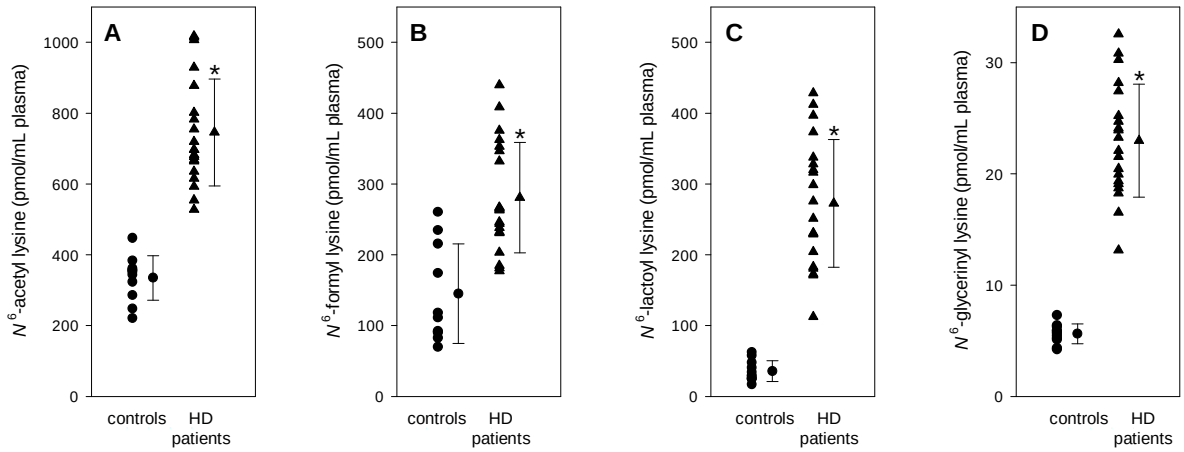

FIGURE 13. *N^6-acetyl lysine (A), N^6-formyl lysine (B), N^6-lactoyl lysine (C) and N^6-glycerinyl lysine (D) levels of plasma from healthy subjects (controls) and non-diabetic hemodialysis patients (HD patients). Data are expressed as mean ± SD. * P < 0.001 vs. control (A, C, D), P < 0.01 vs. control (B). (HENNING et al. 2011)*

In publication A plasma from patients undergoing hemodialysis vs. healthy subjects was used. Including only non-diabetic subjects, the impact of differences in glucose plasma concentrations was avoided. The comparison of in vivo results with glucose-lysine model experiments gave a very diverse picture. While all target amides in vivo were significantly increased in uremia (Fig. 13) only N^6-formyl and N^6-glycerinyl lysine responded to aeration in the glucose-lysine model (Fig. 14). Incubations under aerated conditions should simulate the situation in uremia. In addition, the ratio of concentrations between the individual amide compounds was completely different. In vivo, N^6-acetyl, N^6-formyl and N^6-lactoyl lysine were within the same range whereas N^6-glycerinyl lysine exhibiting 10-fold lower concentrations.

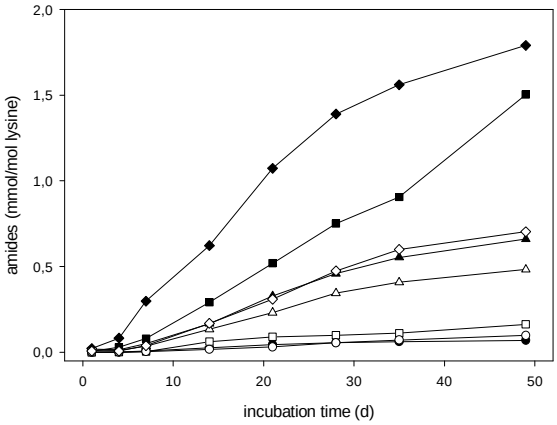

FIGURE 14. *Formation of AGE-amides in incubation mixtures of 200 mM glucose with 42 mM N^1-t-BOC-lysine in phosphate buffer 0.1 M, pH 7.4, at 37 °C under aerated (closed symbols) and deaerated conditions (open symbols): N^6-acetyl lysine (▲), N^6-formyl lysine (◆), N^6-lactoyl lysine (●) and N^6-glycerinyl lysine (■). (HENNING et al. 2011)*

In contrast, glucose incubations showed the smallest concentrations for N^6-lactoyl lysine. Given the fact that N^6-glycerinyl lysine indeed follows the patterns of the model incubation this suggests that the glucose-based pathways shown in Fig. 15 are supplemented by various additional formation reactions of N^6-acetyl, N^6-formyl and N^6-lactoyl lysine.

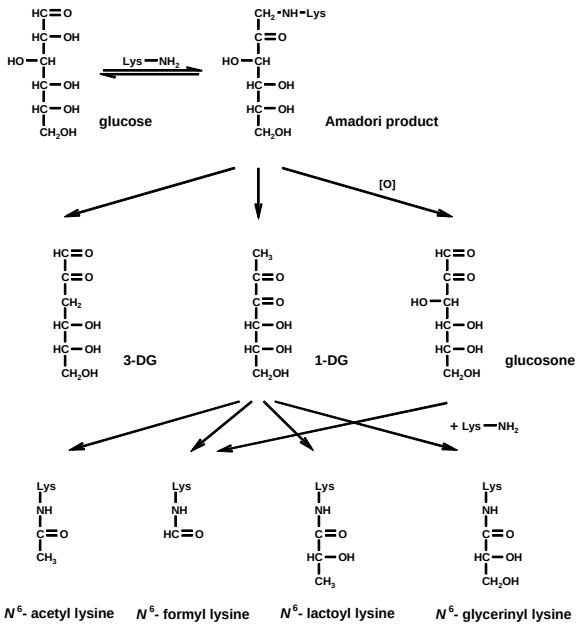

FIGURE 15. *Non-enzymatic degradation pathway of glucose leading to amide-AGEs. (HENNING et al. 2011)*

An alternative mechanism leading to N^6-lactoyl lysine in vivo is the reaction of methylglyoxal with ε-amino lysine residues corresponding to the identification of GALA in glyoxal-lysine model incubations based on rearrangement reactions (GLOMB and PFAHLER 2001). Indeed, incubations of methylglyoxal with N^1-t-BOC-lysine yielded significant amounts of N^6-lactoyl lysine independent from the presence of oxygen. In vivo, this also explains the 7-fold increase in uremia, as a four-fold increase in methylglyoxal in such subjects was determined (61 ± 7 vs. 219 ± 129 pmol/mL plasma) (HENNING et al. 2014). Concentrations of N^6-acetyl lysine were highest in vivo and responded to renal failure by a two-fold increase. This might be explained by increased cell death triggered by the strong inflammatory processes observed under uremia resulting in accelerated release of N^6-acetyl lysine from acetylated histone proteins. In addition, the increased concentration of methylglyoxal in presence of peroxynitrite might also contribute to the acetylation of ε-amino lysine residues in vivo. Massari et al. described a mechanism of L-lysine acetylation by a methylglyoxal-peroxynitrite system in vitro (MASSARI et al. 2010). Although the direct reaction of methylglyoxal with proteins probably dominates over that with peroxynitrite, the post-translational acetylation of proteins by radical mechanisms in the presence of methylglyoxal might be a plausible second non-enzymatic pathway to acetyltransferase-catalyzed reactions. Moreover, although much less reactive, H_2O_2 could replace peroxynitrite in the acetyl generating reaction from methylglyoxal. The idea to explain the two-fold increase of N^6-formyl lysine in uremia based on the in vitro models is supported by the fact that 1-DG and glucosone have recently been detected in vivo (publication B) (HENNING et al. 2014). An alternative mechanism might be the with inflammation increased oxidative DNA breakdown to give 3'-formylphosphate-ended DNA fragments as a potential precursor, however such species have not been identified in vivo so far (JIANG et al. 2007).

Investigations on the mechanistic reaction pathways *in vivo* often raise the question, if the observed AGEs stem mainly from glucose degradation or if ascorbic acid has to be considered as the dominant precursor. The discovery of ascorbic acid specific amide-AGEs N^6-threonyl lysine and N^6-xylonyl/lyxonyl lysine in *in vitro* studies (SMUDA and GLOMB 2013b) and their quantitation in human lens (SMUDA et al. 2015) were a first attempt to clarify this issue. Their low abundance raises questions about the role of ascorbic acid as the predominant precursor of AGEs in lens proteins.

Another interesting aspect which needs critical reflection is the chemical stability of AGEs *in vivo*. Common lysine modifications like CML, CEL, pyrraline, GOLD, MOLD etc. are thought to be stable under physiological conditions. However, *in vitro* experiments clearly showed that monovalent arginine modifications except CMA and argpyrimidine are only of limited chemical stability with half-lives from one to several weeks, strongly dependent on incubation conditions like pH value, the choosen buffer and buffer strenght (KLÖPFER et al. 2010, GLOMB and LANG 2001, AHMED et al. 2002a, BIEMEL et al. 2002a). Consequently, they are not likely to show a time-dependent accumulation in long-lived proteins, e.g. lens protein, over a time scale of years. However, our findings in normal human lenses contradict this assumption (SMUDA et al. 2015). All target AGEs like CML, CEL, CMA, CEA, MG-H1, MG-H3, THP, glucosepane and most of the amide-AGEs correlated with donor age. Obviously, the formation rate of arginine modifications with limited stability has to be higher than their degradation rate. On the other hand, for lysine-amide-AGEs exclusively N^6-acetyl lysine and the C_4-lysine amides (N^6-threonyl and N^6-erythronyl lysine) showed no age-related accumulation. This did not reflect the *in vitro* situation, where all amide-AGEs proved to be stable under physiological conditions. Indeed, formation of N^6-acetyl lysine is known to be paralleled by non-Maillard reactions (ALLFREY et al. 1964) but alternative mechanisms for C_4-lysine amide synthesis/degradation *in vivo* are still unclear.

4 Markers for protein oxidation

It is a matter of common knowledge, that AGE formation *in vivo* is promoted by a general increase in oxidative stress associated with many chronic diseases. This is not surprising, considering that important degradation pathways in Maillard chemistry like the oxidative α-dicarbonyl cleavage or the formation of dehydroascorbic acid require oxygen. Consequently, to evaluate the stress status *in vivo*, one promising approach is the quantitation of specific advanced protein oxidation products. To use AGEs like CML which originate from oxidative reaction pathways as analytical probes is therefore an obvious idea. However, most AGEs or precursors thereof can stem from various sources. Therefore, direct oxidation products of protein side chains independent of Maillard chemistry are often discussed as markers for oxidative stress. Methionine sulfoxide is the oxidation product of methionine residues (LEVINE et al. 2000, SWAIM and PIZZO 1988, YU et al. 2006). *o*-Tyrosine, the isomer of the natural occurring *p*-tyrosine, is formed via attack of hydroxyl radicals at phenylalanine (LEEUWENBURGH et al. 1999). 3-Nitrotyrosine (GAUT et al. 2002) and *o,o'*-dityrosine (LEEUWENBURGH et al. 1999) are the oxidation products of tyrosine residues. In this concept a new marker based on the redox-equilibrium formed by protein bound glycolaldehyde and glyoxal *was introduced in publication C (HENNING et al. 2018).* Glyoxal represents the oxidized form of glycolaldehyde. The respective imines were stabilized as N^6-hydroxyethyl lysine derivatives by reduction with sodium borodeuteride *(Fig. 16). Glyoxal-imine and glycolaldehyde-imine yield double deuterized HEL-2D and mono deuterized HEL-1D, respectively. This enabled us to differentiate between glyoxal- and glycolaldehyde-imines.*

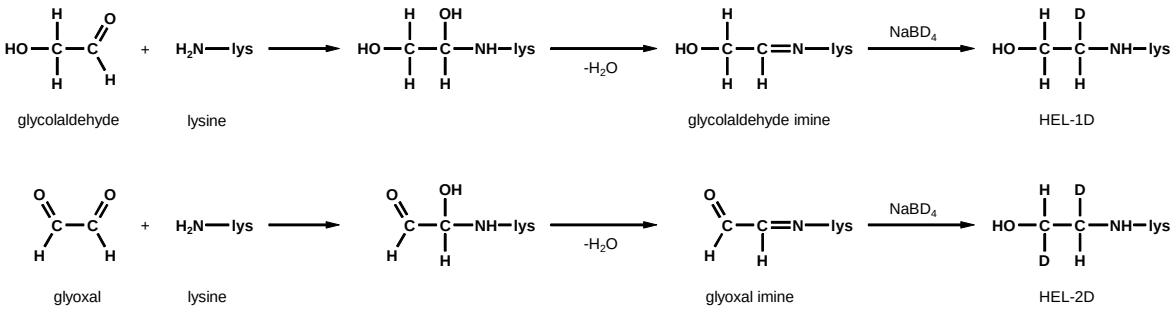

FIGURE 16. *Reaction pathway of glycolaldehyde and glyoxal to form N^6-(2-hydroxy-1-d-ethyl)lysine (HEL-1D) and N^6-(2-hydroxy-1,2-d-ethyl)lysine (HEL-2D), respectively. (HENNING et al. 2018)*

The potential of this approach was proven in a clinical trial with uremic patients versus healthy controls. Uremia is characterized by a state of inflammatory stress resulting from increased oxidation of carbohydrates and lipids (MIYATA et al. 1997a, MIYATA et al. 1997b, MIYATA et al. 1998, CANESTRARI et al. 1994, DASGUPTA et al. 1992, ODETTI et al. 1996, ROSELAAR et al. 1995, WITKO-SARSAT et al. 1996). Indeed, a clear shift towards the factor of 17 of the ratio of glyoxal derived N^6-hydroxyethyl lysine to the glycolaldehyde derived derivative was observed. In healthy human subjects glyoxal and glycolaldehyde are present in approximately equal amounts *(Fig. 17). In hemodialysis patients (HD patients), the results were in line with the established parameters described above but promised significantly enhanced sensitivity.*

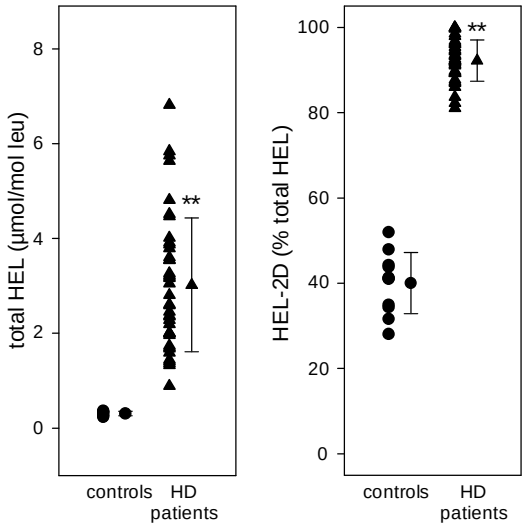

FIGURE 17. *Plasma levels of total N^6-(2-hydroxyethyl)lysine (total HEL) and percentage of N^6-(2-hydroxy-1,2-d-ethyl)lysine (HEL-2D) of total HEL from controls and HD patients. (HENNING et al. 2018)*

It has to be noted that also the total HEL content in sodium borodeuterate-reduced plasma protein was significantly elevated in HD patients vs. controls. Thus, formation of glycolaldehyde and glyoxal are stimulated in uremia. The only known other source of glycolaldehyde in vivo besides the Maillard reaction (GLOMB and MONNIER 1995) is the degradation of the amino acid L-serine in presence of hypochlorous acid formed from superoxide and chloride by myeloperoxidase at sites of inflammation (ANDERSON et al. 1999). Glyoxal does not only stem from oxidation of glycolaldehyde in Maillard driven systems, but alternatively is formed by DNA oxidation, lipid peroxidation (FU et al. 1996)

and sugar autoxidation (WELLS-KNECHT et al. 1995b). However, glyoxal can be detoxified by the glyoxalase system with glutathione as a cofactor (THORNALLEY 1998). Depletion of glutathione under conditions of oxidative stress therefore may lead to enhanced glyoxal-levels.

The relevance of glyoxal and glycolaldehyde to modify and to crosslink proteins in the course of the Maillard reaction has been already studied extensively (GLOMB and PFAHLER 2001, GLOMB and LANG 2001, ACHARYA and MANNING 1983). However, the exact mechanistic relationship of both structures is still unknown. To monitor glyoxal- and glycolaldehyde-amino acid adducts in vitro, N^1-t-BOC-lysine and N^1-t-BOC-arginine were incubated in presence of glyoxal and glycolaldehyde.
In contrast to published data (HOFMANN et al. 1999) it was unequivocally shown that glycolaldehyde is oxidized to glyoxal. This redox reaction only proceeded from glycolaldehyde to glyoxal. Glyoxal incubations never produced any glycolaldehyde adducts (Fig. 18).

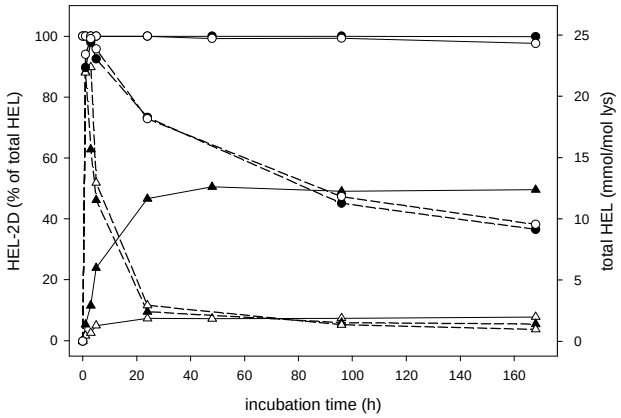

FIGURE 18. *Time dependent formation of total N^6-(2-hydroxyethyl)lysine (total HEL, dashed lines) and N^6-(2-hydroxy-1,2-d-ethyl)lysine (HEL-2D) relative to total content HEL (solid lines) in incubations of 40 mM N^1-t-BOC-lysine with 40 mM glycolaldehyde (▲) and 40 mM glyoxal (●) under aerated (full symbols) and deaerated (open symbols) conditions.*

The oxidation was dependent on the presence of oxygen and, more important, on the catalytic action of the ε-amino moiety of lysine. When aerated glyoxal- and glycolaldehyde-arginine incubations were monitored for imidazolinone as a probe for the reaction of glyoxal with the guanidine function of arginine, there was a 5-fold increase in

concentration from glycolaldehyde to glyoxal (Fig. 19 A). In contrast, glyoxal- and glycolaldehyde-lysine incubations yielded similar amounts of CML as a probe for glyoxal-lysine modifications after 168 h (Fig. 19 B).

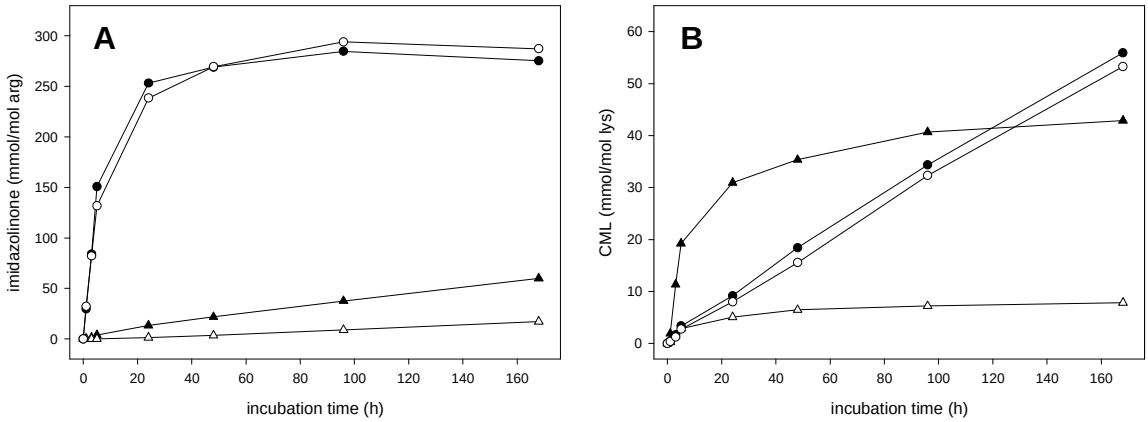

FIGURE 19. ***(A)*** *Time-dependent formation of imidazolinone in incubations of 40 mM N^1-t-BOC-arginine with 40 mM glycolaldehyde (▲) and 40 mM glyoxal (●) under aerated (full symbols) and deaerated (open symbols) conditions.* ***(B)*** *Time-dependent formation of N^6-carboxymethyl lysine (CML) in incubations of 40 mM N^1-t-BOC-lysine with 40 mM glycolaldehyde (▲) and 40 mM glyoxal (●) under aerated (full symbols) and deaerated (open symbols) conditons. (HENNING et al. 2018)*

Even though glyoxal- and glycolaldehyde-lysine incubations produced similar quantities of CML in the long term (168 h), the rate of formation was very different at very short incubation times (< 96 h). Reduction experiments showed clearly that the glycolaldehyde-imine was much more reactive than the glyoxal-imine, resulting in higher concentrations of CML at the beginning of the reaction. Conversion of glyoxal-imines to CML requires a rearrangement step such as enolization or Cannizarro-type reactions whereas conversion of glycolaldehyde-imines must involve an additional oxidation step by definition. This suggests that CML is formed via totally different mechanisms in glycolaldehyde versus glyoxal reactions, respectively. Hayashi et al. (HAYASHI et al. 1977) already established a reaction pathway of glycolaldehyde with an amine leading to glyoxal via a 1,4-dialkylpyrazinium radical cation including an oxidative step, the so-called Namiki pathway. The relevance of this radical intermediate was investigated in-depth by Hofmann et al. (HOFMANN et al. 1999) with regard to the formation of colored compounds during Maillard reactions. This group identified glycolaldehyde as the most effective precursor of

radical formation, tracked by means of electron spin resonance spectroscopy. Notably, the observed radical formation already reached its maximum after 5 minutes in incubations of glycolaldehyde and ethyl amine at 95 °C. However, more importantly, they were able to extend the complex reaction cascades of the intermediate pyrazinium radical cation to the generation of diquaternary and hydroxylated pyrazine species.

It has been hypothesized that the 1,4-dialkylpyrazinium radical cation is also a precursor of CML (GLOMB and MONNIER 1995). In publication C this hypothesis was verfied by using ethyl amine as a substitute for lysine to simplify the experimental design and incubation of 1,4-diethylpyrazine diquaternary salt in the presence of traces of water. Indeed, it was possible to unequivocally verify the formation of N-ethyl glycine, the ethyl amine pendant of CML. Based on this finding a novel radical-assisted pathway of CML formation in line with above mechanistic studies was proposed (Fig. 20).

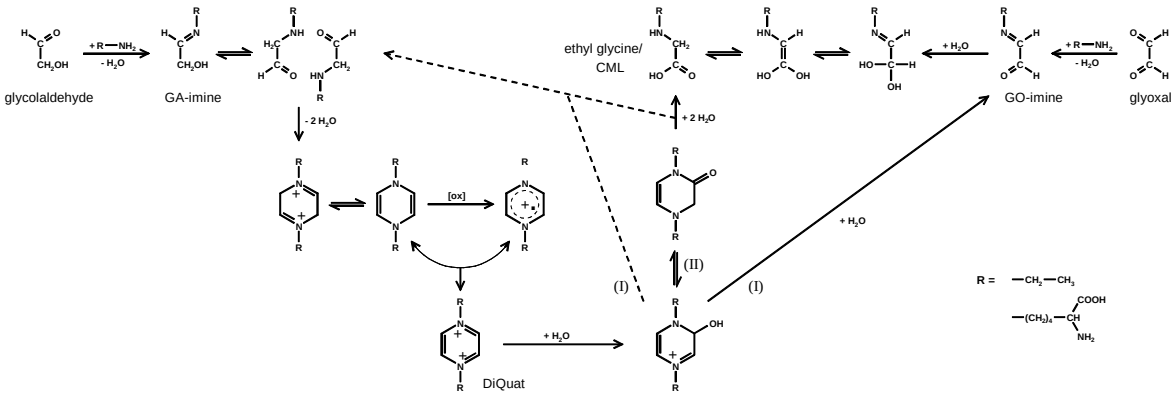

FIGURE 20. *Reaction pathway of glycolaldehyde and glyoxal leading to N-ethyl glycine or N^6-carboxymethyl lysine, respectively, depending on the amine-compound used (ethyl amine or lysine). (HENNING et al. 2018)*

After rearrangement to the corresponding α-aminoaldehydes two molecules condense to yield 1,4-dialkyl-1,4-dihydropyrazine. As shown by Hayashi et al. and confirmed by Hofmann et al. this structure is immediately oxidized to the 1,4-dialkylpyrazinium radical cation. The high potential of these pyrazine species for single electron transfer reactions is further enlightened by a disproportionation to give the corresponding diquaternary salt in a redox cycling equilibrium. The diquat represents an extremely electron deficient compound, which leads to spontaneous attack of nucleophils as water to give the 2-hydroxypyrazinium cation already verified in literature. This intermediate can now be stabilized via two pathways. (I) Hydrolysis leads to one molecule glycolaldehyde-imine

and one molecule of glyoxal-imine, which relatively slow after hydration and rearrangement results in ethyl glycine/ CML. (II) More effectively, alternative direct enolization should give the corresponding 1,4-dialkyl-3,4-dihydro-2-pyrazinone, which hydrolyzes to release glycolaldehyde-imine and instantaneously ethyl glycine/ CML.

The crucial oxidative step in this CML formation cascade was enlightened by the detection of significant lower amounts of CML in glycolaldehyde-lysine incubations under deaeration (Fig. 19 B). On the other hand, as depicted in Fig. 18, deaeration only led to a slight delayed degradation of glycolaldehyde-imines in the initial reaction phase up to 3 h when monitored as content of total HEL. Thus, there must be significant additional unknown degradation mechanisms to CML formation, which do not require oxidative conditions.

5 Summary

In the present Ph.D. thesis the significance of the various routes for carbohydrate fragmentation in Maillard chemistry under physiological conditions was investigated. The results of the in vitro experiments were matched with the in vivo situation.

First, it has to be stated, that the conventional retro-aldol fragmentation pathway is not a significant route for carbohydrate fragmentation in Maillard chemistry under physiological conditions. Second, there is no convincing mechanistic basis for the often suggested hydrolytic α-cleavage. In contrast, the β-dicarbonyl cleavage route must be considered as the major carbohydrate fragmentation pathway in general. The hydrolytic pathway is paralleled in the presence of amino acids or proteins by an analogous amine catalyzed cleavage mechanism. Indeed, acylation is a well-known regulatory concept *in vivo* (ALLFREY et al. 1964). The posttranslational acylation of amino acids in the polypeptide chain neutralizes the positive charge and, thus, changes protein function in various ways (KOUZARIDES 2000, YANG 2004). Non-enzymatic acylation as described above parallels enzymatic regulatory mechanisms by affecting the same targets. However, the Maillard derived protein modifications might be irreversible and lead to accumulation and adverse effects on protein characteristics. A further fragmentation route *in vivo*, which is of great mechanistic importance is based on oxidative α-dicarbonyl scission to yield carboxylic acids and related amine acylation. The quantitative aspects of this route still have to be evaluated.

A formation mechanism for the novel amide-AGEs N^6-acetyl, N^6-formyl, N^6-lactoyl and N^6-glycerinyl lysine via amine induced β-cleavage degradation of 1-deoxyglucosone in the presence of lysine was already established in vitro by Glomb's group. Now, human plasma levels of the respective compounds as free adducts were determined by means of LC-MS/MS, N^6-formyl, N^6-lactoyl and N^6-glycerinyl lysine for the first time. They were significantly higher in uremic patients undergoing hemodialysis than in healthy subjects. Plasma concentration levels were in the same range like other established lysine modifications, i.e. N^6-carboxymethyl lysine and N^6-carboxyethyl lysine. Model reactions with N^1-t-butoxycarbonyl-lysine under physiological conditions confirmed 1-deoxyglucosone as an immediate precursor of amide-AGEs. In addition, glucosone and methylglyoxal were identified as alternative precursors for formyl and lactoyl lysine, respectively. Comparison of the in vivo results with the model experiments provided the

possibility to elucidate possible formation pathways linked to Maillard chemistry. The results strongly suggest a major participation of non-enzymatic Maillard mechanisms on amide-AGE formation pathways in vivo, which, in the case of N^6-acetyl lysine, parallels enzymatic processes.

To understand the complex mechanisms of advanded glycation endproducts (AGE) formation it is crucial to extend the knowledge on plasma concentrations of reactive intermediates. In general, as already mentioned above, α-dicarbonyl compounds (α-DCs) are the key players during Maillard directed degradation processes. With the present work a highly sensitive LC-MS/MS multi-method for human blood plasma based on derivatization with o-phenylenediamine under acidic conditions was introduced. The method was validated with special attention on artifact formation and the mechanistic relationship between the individual target analytes. Included were the α-dicarbonyl compounds 1-deoxyglucosone, 3-deoxyglucosone, glucosone, Lederer's glucosone, dehydroascorbic acid, 2,3-diketogulonic acid, 1-deoxypentosone, 3-deoxypentosone, 3,4-dideoxypentosone, pentosone, 1-deoxythreosone, 3-deoxythreosone, threosone, methylglyoxal, glyoxal, the α-keto-carboxylic acids pyruvic acid and glyoxylic acid and the dicarboxylic acid oxalic acid. The method was then applied to the analyses of 15 healthy subjects and 24 uremic patients undergoing hemodialysis. The comparison of the results revealed a clear shift in the product spectrum. In most cases, the plasma levels of target analytes were significantly higher in the uremic group. In general, the elevated levels of α-DCs found explain the elevated levels of AGEs in uremia. Thus, this is the first time that a complete spectrum of α-dicarbonyl compounds relevant in vivo was established.

The generation and accumulation of these compounds derived from both, oxidative and non-oxidative chemistry must be regarded as one of the major sources of carbonyl stress. AGE formation in vivo is furthermore promoted by a general increase in oxidative stress which is considered as tissue damage resulting from an imbalance between an excessive generation of highly reactive prooxidative compounds, e.g. reactive oxygen species (ROS), and insufficient antioxidant defense mechanisms. Thus, developing novel therapeutic interventions to reduce oxidative stress, i.e. by administration of antioxidants or by attenuation of chronic inflammation, is an important field of research. A necessary prerequisite in such studies is the accurate and reliable quantitation of the stress status. Here, oxidized biomolecules, e.g. proteins, are often used as targets. The present work introduced a novel marker for oxidative stress in human plasma protein and its possible

clinical relevance in chronic uremia. The basis of this approach is the amount of protein bound glycolaldehyde in relation to the oxidized counterpart glyoxal. Reduction of the respective imines with sodium borodeuteride and subsequent protein hydrolysis lead to the N^6-hydroxyethyl lysine (HEL) derivatives. Glycolaldehyde-imines yield mono deuterized HEL (HEL-1D) whereas glyoxal-imines react to double deuterized HEL (HEL-2D). Thus, a shift towards HEL-2D in relation to the total amount of HEL (sum of HEL-1D and HEL-2D) indicates increased protein oxidation. In hemodialysis patients, the results were in line with established parameters like methionine sulfoxide, 3-nitrotyrosine, o-tyrosine and o,o'-dityrosine but promised significantly enhanced sensitivity. The quantitative importance of glycolaldehyde as a potent glycating agent in human plasma was established for the first time, and was in the same range as glyoxal in normal subjects. The significance as a reactive intermediate in Maillard reaction pathways was further emphasized by a novel and very effective mechanism leading to CML incorporating single-electron transfer reactions.

6 Zusammenfassung

In der vorliegenden Dissertation wurde die Bedeutung der verschiedenen Wege des Kohlenhydratabbaus im Rahmen der Maillard-Reaktion unter physiologischen Bedingungen untersucht. Ein Schwerpunkt der Arbeit lag darüber hinaus auf der Übertragung von in vitro gewonnenen mechanistischen Erkenntnissen auf die in vivo Situation.

Betrachtet man die ablaufenden Maillard-induzierten Fragmentierungsreaktionen von Kohlenhydraten, so muß zunächst festgestellt werden, daß unter physiologischen Bedingungen die klassische Retro-Aldolspaltung kaum eine Rolle spielt. Darüber hinaus gibt es keinen sicheren experimentellen Nachweis für die in der Literatur oft ins Feld geführte hydrolytische α-Spaltung. Die β-Dicarbonylspaltung muß dagegen als der bedeutendste Abbauweg von Kohlenhydraten unter den gewählten Bedingungen gelten. Die hydrolytische β-Spaltung wird dabei in Gegenwart von Aminosäuren oder Proteinen ergänzt um einen analog ablaufenden Amin-induzierten Weg. Die bekannte post-translationale Acylierung von Aminosäuren in der Polypeptid-Kette von Proteinen spielt in vielfältigen Regulationsmechanismen in vivo eine Rolle. Hierbei werden positive Ladungen am Protein eliminiert und so die Proteinfunktion verändert. Die hier beschriebene nicht-enzymatische Acylierung tritt dabei in Konkurrenz zu den enzymatisch gesteuerten Prozessen, da dieselben Zielstrukturen angegriffen werden. Die Proteinmodifikationen, welche aus der Maillard-Reaktion hervorgehen, können allerdings irreversibel sein, sich anreichern und so die Proteincharakterisitik entscheidend beeinflussen. Einen weiteren mechanistisch bedeutenden Abbauweg in vivo stellt die oxidative α-Dicarbonylspaltung dar, welche zu Carbonsäuren führt. Auch hier ist ein analoger Amin-induzierter Weg möglich. Die quantitative Bedeutung dieser Reaktionsroute muß aber noch untersucht werden.

Kürzlich wurde durch die Arbeitsgruppe Glomb eine neue Klasse von „Advanced Glycation Endproducts“ (fortgeschrittene Produkte der Maillard-Reaktion, AGEs) eingeführt, die Amid-AGEs. Für vier dieser Strukturen, N^6-Acetyl-, N^6-Formyl-, N^6-Lactoyl- und N^6-Glycerinyllysine, wurde als Bildungsweg bereits eine Lysin-induzierte β-Dicarbonylspaltung von 1-Desoxyglucoson in Modellexperimenten nachgewiesen. In der vorliegenden Arbeit wurden die genannten Strukturen, drei davon erstmalig, über eine LC-MS/MS-Analytik in humanem Blutplasma als freie Aminosäureaddukte nachgewiesen.

Die ermittelten Konzentrationen waren dabei im Plasma von Urämiepatienten signifikant höher als in der nicht-urämischen Kontrollgruppe. Die Gehalte sind vergleichbar mit etablierten Lysinmodifikationen wie N^6-Carboxymethyllysin oder N^6-Carboxyethyllysin. Dies legt einen ähnlichen Einfluß auf die Pathophysiologie im menschlichen Organismus nahe. Modelluntersuchungen mit N^1-t-Butoxycarbonyllysin unter physiologischen Bedingungen bestätigten 1-Desoxyglucosone als direkte Vorläuferstruktur von Amid-AGEs. Darüber hinaus wurden die weiteren α-Dicarbonylverbindungen Glucoson und Methylglyoxal als alternative Vorläufer für N^6-Formyllysin bzw. N^6-Lactoyllysin identifiziert. Der Vergleich der in vivo Ergebnisse mit den Modellexperimenten erlaubte es, mögliche Maillard-Bildungswege in vivo aufzudecken. Dabei wurde deutlich, daß ein wesentlicher Einfluß nicht-enzymatischer Mechanismen auf die Bildung von Amid-AGEs in vivo besteht, welche im Falle von N^6-Acetyllysin in Konkurrenz zu enzymatisch katalysierten Reaktionen treten.

Um die komplexen Reaktionskaskaden verstehen zu können, auf denen AGEs in Blutplasma gebildet werden, ist es unabdingbar, auch die Konzentration von reaktiven Zwischenprodukten zu bestimmen. Wie bereits weiter oben angesprochen sind α-Dicarbonylverbindungen die Schlüsselintermediate der Maillard-Reaktion. Daher wurde im Rahmen der vorliegenden Arbeit erstmals eine empfindliche und umfangreich validierte LC-MS/MS-Multimethode entwickelt, die alle relevanten Strukturen umfaßt. Als Derivatisierungsreagenz kam hierbei o-Phenylendiamin unter sauren Bedingungen zum Einsatz. Insbesondere wurde die Stabilität der entstehenden Derivate und die Artefaktbildung untersucht. Die Methode umfaßt die α-Dicarbonylverbindungen 1-Desoxyglucoson, 3-Desoxyglucoson, Glucoson, Lederer Glucoson, Dehydroascorbinsäure, 2,3-Diketogulonsäure, 1-Desoxypentoson, 3-Desoxypentoson, 3,4-Didesoxypentoson, Pentoson, 1-Desoxythreoson, 3-Desoxythreoson, Threoson, Methylglyoxal, Glyoxal, die α-Ketocarbonsäuren Brenztraubensäure und Glyoxylsäure und die α-Dicarbonsäure Oxalsäure. Die Ergebnisse einer Studie mit 24 hämodialyse-pflichtigen Urämiepatienten und 15 nicht-urämischen Kontrollen zeigten eine deutliche Verschiebung im Produktspektrum, wobei in den meisten Fällen die Plasmakonzentrationen der Zielstrukturen in der urämischen Gruppe signifikant höher ausfielen. Im allgemeinen ließen sich so erhöhte Werte bekannter AGEs in Urämiepatienten mit den Konzentrationsanstiegen der einzelnen Dicarbonylstrukturen erklären.

Während die Bildung und Anreicherung von α-Dicarbonylstrukturen, die sowohl auf oxidativem wie auch auf nicht-oxidativem Wege gebildet werden können, die Hauptquelle für Carbonylstress im Organismus darstellen, wird die AGE-Bildung in vivo darüber hinaus durch eine Erhöhung des generellen oxidativen Stresses angeregt. Dieser resultiert aus einer Störung des Gleichgewichts zwischen der Bildung von prooxidativ wirkenden Verbindungen wie z.B. reaktiver Sauerstoffspezies (reactive oxygen species, ROS) und antioxidativen Mechanismen. Die Balance wieder herzustellen, z.B. durch Gabe von Antioxidantien oder Entzündungshemmern, ist ein wichtiges medizinisches Forschungsgebiet. Eine wichtige Voraussetzung, um hier untersuchende Studien durchführen zu können, ist die genaue und zuverlässige Bestimmung des Stress-Status. Oft werden oxidierte Biomoleküle wie z.B. Proteine zu diesem Zwecke genutzt. Mit vorliegender Arbeit wurde ein neuer Marker für oxidativen Stress in menschlichem Plasma eingeführt, der auf dem Verhältnis von proteingebundenem Glycolaldehyd in Relation zu dem oxidiertem Pendant Glyoxal beruht. Die Reduktion der entsprechenden Imin-Strukturen mit Natriumbordeuterid und anschließender Proteinhydrolyse führt für Glycolaldehyd zu einfach deuteriertem N^6-Hydroxyethyllysin (HEL) während für Glyoxal zweifach deuteriertes HEL erhalten wird. Bei Hämodialyse-Patienten waren die so gewonnenen Ergebnisse vergleichbar mit anderen etablierten Protein-Oxidationsmarkern wie Methioninsulfoxid, 3-Nitrotyrosin, o-Tyrosin und o,o‘-Dityrosin. Es wurde allerdings eine deutlich höhere Empfindlichkeit erreicht. Die qualitative und insbesondere auch die quantitative Bedeutung von Glycolaldehyd als potentes Glykierungreagenz in menschlichem Blutplasma konnte hier erstmals dargestellt werden. In der Kontrollgruppe lagen die Konzentrationen für Glycolaldehyd im selben Bereich wie jene für Glyoxal. Die Bedeutung des Glycolaldehyds als reaktives Intermediat der Maillard-Reaktion wurde darüber hinaus durch den Nachweis eines neuen und sehr effektiven Reaktionsweges zu N^6-Carboxymethyllysin unterstrichen, bei welchem Ein-Elektronen-Transfer-Reaktionen eine wichtige Rolle spielen.

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10 Appendix

10.1 *Publication A*: Journal of Biological Chemistry (2011), vol. 286, 44350-44356

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Molecular Basis of Maillard Amide-Advanced Glycation End Product (AGE) Formation *in Vivo*

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Background: Advanced glycation end products (AGEs) play a prominent role in various pathophysiologies.
Results: A new class of lysine amide modifications was established *in vivo*.
Conclusion: Non-enzymatic Maillard mechanisms participate on amide-AGE formation pathways *in vivo*.
Significance: Plasma levels of the amide-AGEs were in the same range similar to other established lysine modifications and suggest a comparable impact of non-enzymatic biochemistry on pathophysiologies in the human organism.

The Maillard reaction *in vivo* entails alteration of proteins or free amino acids by non-enzymatic glycation or glycoxidation. The resulting modifications are called advanced glycation end products (AGEs) and play a prominent role in various pathologies, including normoglycemic uremia. Recently, we established a new class of lysine amide modifications *in vitro*. Now, human plasma levels of the novel amide-AGEs N^6-acetyl lysine, N^6-formyl lysine, N^6-lactoyl lysine, and N^6-glycerinyl lysine were determined by means of LC-MS/MS. They were significantly higher in uremic patients undergoing hemodialysis than in healthy subjects. Model reactions with N^1-*t*-butoxycarbonyl-lysine under physiological conditions confirmed 1-deoxy-D-*erythro*-hexo-2,3-diulose as an immediate precursor. Because formation of N^6-formyl lysine from glucose responded considerably to the presence of oxygen, glucosone was identified as another precursor. Comparison of the *in vivo* results with the model experiments enabled us to elucidate possible formation pathways linked to Maillard chemistry. The results strongly suggest a major participation of non-enzymatic Maillard mechanisms on amide-AGE formation pathways *in vivo*, which, in the case of N^6-acetyl lysine, parallels enzymatic processes.

The Maillard reaction (non-enzymatic reactions of reducing sugars with amines) *in vivo* is associated with long term complications of diabetes, uremia, atherosclerosis, and Alzheimer disease as well as with pathophysiologies linked to aging in general. The stable products of this reaction are referred to as advanced glycation end products (AGEs).[2] Numerous studies have documented the accumulation of AGEs in tissue proteins but also in the circulation of patients with renal failure, irrespective of the presence of diabetes (1–3). In general, AGEs can accumulate as protein modifications or as AGE-free adducts (4). These free adducts are formed by cellular proteolysis of glycated proteins, direct glycation of amino acids, and digestion of glycated proteins in food. They have high renal clearance and are the major form in which AGEs are excreted from the body in urine and in dialysate in renal replacement therapy (5).

We reported on two groups of Maillard amide-AGEs. A first set N^6-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine and N^6-glycoloyl lysine have been established for the reaction of glyoxal with lysine via rearrangement reactions *in vitro* and *in vivo*. The formation pathways are directly linked to N^6-carboxymethyl lysine (CML) via glyoxalimine structures (6). More recently, we identified a second class of lysine amide modifications N^6-acetyl lysine, N^6-formyl lysine, N^6-lactoyl lysine, and N^6-glycerinyl lysine *in vitro* based on β-dicarbonyl fragmentation of native and oxidized reductone structures. Incubation experiments confirmed the α-dicarbonyl compound 1-deoxy-D-*erythro*-hexo-2,3-diulose (1-deoxyglucosone, 1-DG) as a direct precursor (7). The essential role of α-dicarbonyls as central intermediates of high reactivity in the complex Maillard chemistry *in vitro* and *in vivo* has been manifested over the years. The α-dicarbonyl compounds glyoxal, methylglyoxal and 3-deoxy-D-*erythro*-hexos-2-ulose (3-deoxyglucosone, 3-DG) were already detected in human plasma (8–11). Even at the low concentrations found in physiological systems, α-dicarbonyls retain potent glycating activity (12).

Acylation of amino acids *in vivo* within the polypeptide chain was first detected in cell nuclei as a rapid and reversible incorporation of radioactive labeled acetate into histones H3 and H4 (13). This posttranslational acetylation neutralizes the positive charge of the amino acid, changing protein function in diverse ways (14, 15). The modification of core histone tails by histone acetyltransferases or histone deacetylases plays a key role in the regulation of gene expression (16, 17). Despite great biochemical and clinical interest in lysine acetylation, the knowledge of *in vivo* acetylation sites is limited and subject to current research (18). Piraud *et al.* (19) presented an ion-pair liquid chromatography/electrospray ionization mass spectrometric analysis for N^6-acetyl lysine in human plasma and urine. Tsutsui *et al.* (20) detected N^6-acetyl lysine in the plasma of ddY

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[2] The abbreviations used are: AGE, advanced glycation end product; CML, N^6-carboxymethyl lysine; 1-DG, 1-deoxy-d-*erythro*-hexo-2,3-diulose (1-deoxyglucosone); 3-DG, 3-deoxy-d-*erythro*-hexos-2-ulose (3-deoxyglucosone); HD patients, patients undergoing hemodialysis; Amadori product, 1-deoxy-1-(N^6-lysino)-D-fructose; glucosone, D-*arabino*-hexos-2-ulose; CEL, N^6-carboxyethyl lysine; Boc, butoxycarbonyl; DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; LOD, limit of detection; LOQ, limit of quantification.

TABLE 1
Mass spectrometric parameters for amide-AGE, CML, and CEL quantification

		Precursor ion		Product ion 1[a]			Product ion 2[b]			Product ion 3[b]		
	Retention time	*m/z*	DP	*m/z*	CE	CXP	*m/z*	CE	CXP	*m/z*	CE	CXP
	min	*amu*	*V*	*amu*	*eV*	*V*	*amu*	*eV*	*V*	*amu*	*eV*	*V*
N^6-acetyl lysine	11.7	189.2	30.0	126.1	18.0	10.0	84.2	31.0	5.0	143.1	14.0	10.0
N^6-formyl lysine	8.2	175.1	25.0	112.1	20.0	13.0	84.1	35.0	7.0	129.1	15.0	13.0
N^6-lactoyl lysine	12.1	219.2	32.0	156.2	20.0	8.0	84.1	35.0	9.0	173.1	17.0	8.0
N^6-glycerinyl lysine	6.7	235.3	48.0	84.2	37.0	6.0	172.3	23.0	30.0	189.3	17.0	15.0
CML	6.9	205.1	50.0	130.1	17.0	9.5	84.1	46.0	13.0			
CEL	11.5	219.1	54.0	130.1	18.0	11.0	84.1	33.0	7.00	173.0	18.0	15.0

[a] MRM transition used for quantification (quantifier).
[b] MRM transition used for confirmation (qualifier).

strain mice, which naturally develop diabetes with age. The lysine adduct was discussed as one of several potential biomarker candidates related to diabetes mellitus.

N^6-formyl lysine is formed *in vitro* by reaction of albumin with trichloroethylene oxide, a major metabolite of 1,1,2-trichloroethylene, one of the most common compounds found in chemical waste dumps (21). In addition, it could be identified in incubation mixtures of various sugars, l-ascorbic acid or l-dehydroascorbic acid with poly-l-lysine and β-lactoglobulin, respectively (22). Jiang *et al.* (23) observed transfer of formyl groups from 3′-formylphosphate-ended DNA, arising from oxidation of the 5′-position of deoxyribose and subsequent DNA strand breakage by the enediyne antibiotic neocarzinostatin, to histone proteins in human TK6 cells to give N^6-formyl lysine.

The aim of the present work was to elucidate formation and relevance of the four amide-AGEs N^6-acetyl lysine, N^6-formyl lysine, N^6-lactoyl lysine, and N^6-glycerinyl lysine (structures are shown in Fig. 5) *in vivo* with regard to non-enzymatic mechanisms within the Maillard reaction. Therefore, the free adducts were analyzed in human blood plasma by means of an LC-MS/MS technique. In model experiments, formation from glucose, the physiological most important sugar, and from various α-dicarbonyl structures as direct precursors was confirmed. Comparison of *in vivo* with *in vitro* results allowed us to assess the impact of Maillard chemistry on amide-AGE formation *in vivo*.

EXPERIMENTAL PROCEDURES

Plasma Samples—Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of the Medical Faculty of the Martin Luther University Halle-Wittenberg. Blood samples were obtained from 11 healthy subjects (controls) with normal renal function and 18 non-diabetic patients undergoing hemodialysis (HD patients) using disodium ethylenediaminetetraacetic acid as an anticoagulant. In dialysis patients, samples were obtained predialysis before the mid-week treatment session. Blood samples were centrifuged (2500 × *g*, 10 min, 4 °C) within 30 min of collection, and the plasma was frozen immediately at −80 °C. Hemodialysis was performed three times weekly for 4–5 h using polysulfone dialyzers and dialysate containing bicarbonate buffer. HbA1c, creatinine, and C-reactive protein were measured by routine methods at the central laboratory of Martin Luther University Clinical Center (Halle/Saale).

Materials—Chemicals of the highest grade available were obtained from Sigma-Aldrich and Thermo Fisher Scientific unless otherwise indicated. 1-deoxy-1-(N^6-lysino)-D-fructose (Amadori product) (24), 3-DG (25), D-*arabino*-hexos-2-ulose (glucosone) (26), 1-DG and 2-ethyl-3-methylquinoxaline (27), CML (28), N^6-carboxyethyl lysine (CEL) (29), and N^6-lactoyl lysine and N^6-glycerinyl lysine (7) were synthesized according to the literature. The identities of target compounds was verified by nuclear magnetic resonance experiments. Furthermore, the elemental composition was confirmed by accurate mass determination.

TABLE 2
CV, LOD, and LOQ (all steps of the analysis included) of plasma samples

	CV			
	Controls[a]	HD patients[b]	LOD	LOQ
	%		*pmol/ml*	
N^6-acetyl lysine	1–10	6–14	2.1	6.2
N^6-formyl lysine	1–7	8–24	4.1	12.3
N^6-lactoyl lysine	1–13	6–16	1.7	5.1
N^6-glycerinyl lysine	1–20	10–18	1.3	4.0
CML	1–13	8–26	5.6	16.9
CEL	1–12	7–20	5.0	14.9

[a] 11 subjects, replicate analyses ($n = 2$).
[b] 18 subjects, replicate analyses ($n = 6$).

Model Reactions—In general, incubations were conducted in 0.1 M phosphate buffer, pH 7.4, after sterile filtration in a shaker incubator (New Brunswick Scientific, Nürtingen, Germany) at 37 °C. The reactant concentrations are mentioned in the legends to the corresponding figures and tables. Deaeration and inhibition of metal-catalyzed oxidation chemistry was achieved by using phosphate buffer containing 1 mM diethylenetriaminepentaacetic acid and gassing with argon. Buffer was degassed with helium and stored under argon prior to sample preparation. At various time points aliquots of the reaction mixtures were diluted with 6 M HCl to a final HCl concentration of 3 M. For quantitative removal of the Boc protection group, the samples were kept at room temperature for 30 min. After dilution to appropriate concentrations, the solutions were subjected LC-MS/MS analysis.

Assay of Amide-AGEs, CML, and CEL in Plasma—200 μl of plasma was transferred into a centrifugal filter containing a modified polyethersulfone membrane with 3 kDa molecular weight cut-off (VWR International, Darmstadt, Germany). Centrifugation was carried out at 14,000 × *g* and 4 °C. The filtrate was diluted on a scale of 1:20 with 0.1% HCl and administered to LC-MS/MS analysis.

Assay of Methylglyoxal in Plasma—Methylglyoxal in plasma samples was analyzed according to a modified method by McLellan *et al.* (8). Briefly, 500 μl of plasma were incubated

with *o*-phenylenediamine at room temperature in the dark for 24 h under acidic conditions. 2-Ethyl-3-methylquinoxaline was used as internal standard. The filtered supernatant was subjected to LC-MS/MS analysis.

High Performance LC-MS/MS—The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a pump (PU-2080 Plus) with degasser (LG-2080-02) and quaternary gradient mixer (LG-2080-04), a column oven (Jasco Jetstream II), and an autosampler (AS-2057 Plus). Mass spectrometric detection was conducted on an API 4000 QTrap LC-MS/MS system (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with a turbo ionspray source using electrospray ionization in positive mode: sprayer capillary voltage, 4.0 kV; nebulizing gas flow, 50 ml/min; heating gas, 60 ml/min at 550 °C; and curtain gas, 40 ml/min.

For the detection of the amide-AGEs, CML and CEL, chromatographic separations were performed on a stainless steel column packed with RP-18 material (Vydac CRT, no. 218TP54, 250 × 4.0 mm, RP-18, 5 μm, Hesperia, CA) using a flow rate of 1.0 ml/min. The mobile phase used was water (solvent A) and methanol/water (7:3 (v/v), solvent B). To both solvents (A and B), 1.2 ml/liter heptafluorobutyric acid was added. Analysis was performed at 35 °C column temperature using isocratic elution at 98% A/2% B. For mass spectrometric detection, the multiple reaction monitoring mode was used, utilizing collision-induced dissociation of the protonated molecules with compound specific orifice potentials and fragment-specific collision energies (Table 1).

Quantification was performed using the standard addition method. More precisely, increasing concentrations of authentic reference compounds at factors of 0.5, 1, 2, and 3× the concentration of the analyte in the sample were added to separate aliquots of the sample after workup procedure. The aliquots were analyzed, and a regression of response *versus* concentration was used to determine the concentration of the analyte in the sample. Spikes were run one in approximately every 30 samples. Calibration with this method resolves potential matrix interferences.

To obtain fragmentation spectra of amide-AGEs in plasma workup solutions, target material was first enriched by repeated collection from the above HPLC system. After solvent evaporation in a vacuum concentrator (Savant SpeedVac Plus SC 110 A combined with a Vapor Trap RVT 400, Thermo Fisher Scientific GmbH), the residue was dissolved in water and reinjected, using a collision-induced dissociation experiment. The fragmentation spectra of the authentic references were obtained with the same parameters. For N^6-lactoyl lysine, the following parameters were used: declustering potential (DP), 33 V; collision energy (CE), 23 eV; collision cell exit potential (CXP), 8 V; and scan range (*m/z*), 50–220 (2 s).

For the detection of methylglyoxal quinoxaline, chromatographic separations were performed on a stainless steel column (Knauer, Eurospher 100 C18, 5 μm, 250 × 4.6 mm, Berlin, Germany) using a flow rate of 1.0 ml/min. The mobile phase used was water (solvent A) and methanol/water (7:3 (v/v), solvent B). To both solvents (A and B), 0.6 ml/liter heptafluorobutyric acid was added. Analysis was performed at 35 °C column temperature using gradient elution: 20 (0) to 30 (35) to 100 (65–70) to 20 (75–85); % B (*t*/min). For mass spectrometric detection, the multiple reaction monitoring mode was used, utilizing a collision-induced dissociation of the protonated molecules with MS parameters as follows: *m/z* 229.2 → 77.0 (DP, 50 V; CE, 41.0 eV; CXP, 5.0 V, quantifier), *m/z* 229.2 → 118.1 (DP, 50 V; CE, 30.5 eV; CXP, 7.0 V; qualifier), *m/z* 229.2 → 65.0 (DP, 50 V; CE, 45.0 eV; CXP, 5.0 V, qualifier). Methylglyoxal quinoxaline was detected at the retention time of t_R = 58.1 min. Quantification was performed using the standard addition method with pure authentic reference compounds.

TABLE 3
Profile of subjects examined in this study

	Healthy subjects	Hemodialysis patients
No. of participants	11	18
Age (years)	66 ± 6	69 ± 11
HbA1c (%)	5.6 ± 0.2	6.3 ± 1.7[a]
Serum creatinine (μmol/liter)	81 ± 8	706 ± 222[b]
C-reactive protein (mg/liter)	1.7 ± 1.1 (<1.0–4.5)	23 ± 19 (6–70)

[a] Not significant.
[b] $p < 0.001$ *versus* healthy subjects.

All plasma workup samples and incubations were analyzed in single batches to exclude interassay variations. Intra-assay coefficients of variation were determined by repeated analyses of controls and HD patients. In addition, as shown in Table 2, limit of detection (LOD) and limit of quantification (LOQ) with all steps of the analysis included were estimated according to DIN 32645 (n = 6, confidence level, p = 0.95, k = 3) (30). In model experiments, coefficients of variation < 10% (n = 3) for all target compounds was achieved, and LOD/LOQ was 0.0004/0.001 mmol/mol lysine for acetyl lysine, 0.0013/0.004 mmol/mol lysine for formyl lysine, 0.0007/0.002 mmol/mol lysine for lactoyl lysine, and 0.0135/0.041 mmol/mol lysine for glycerinyl lysine.

Statistical Evaluation—Data are expressed as mean ± S.D. The Student's *t* test was used for statistical evaluation of significant differences between both groups.

RESULTS

Amide-AGEs in Human Blood Plasma of Uremic Patients versus Healthy Subjects—Plasma was obtained from 11 healthy subjects (controls) with normal renal function and no proteinuria and 18 HD patients. In dialysis patients, samples were obtained predialysis before the mid-week treatment session. Details on the study population are summarized in Table 3. Normal renal function was defined as a serum creatinine level below 102 μmol/liter. After appropriate dilution and separation from the protein residue by a 3 kDa molecular weight cutoff membrane, the plasma samples were subjected to liquid chromatography coupled with mass spectrometric detection using multiple reaction monitoring.

As shown in Fig. 1, plasma levels of all four carboxylic acid amides were significantly higher in HD patients than those in controls (acetyl lysine, 746 ± 151 *versus* 335 ± 63 pmol/ml plasma; formyl lysine, 281 ± 78 *versus* 145 ± 70 pmol/ml plasma; lactoyl lysine, 273 ± 90 *versus* 36 ± 15 pmol/ml plasma; glycerinyl lysine, 23.0 ± 5.1 *versus* 5.6 ± 0.9 pmol/ml plasma). To compare with well established AGE structures, we measured plasma concentrations of CML and CEL. In accordance with the literature (4), the levels were significantly elevated in

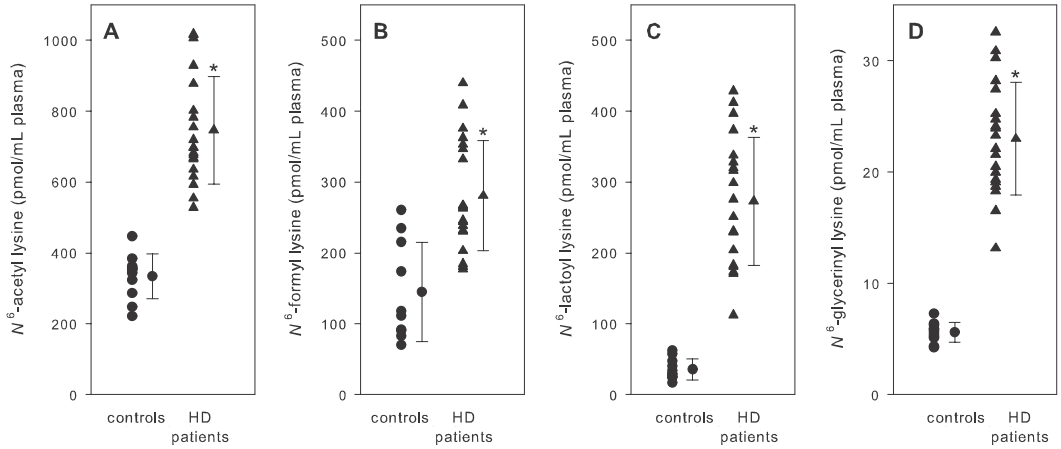

FIGURE 1. **N^6-acetyl lysine (*A*), N^6-formyl lysine (*B*), N^6-lactoyl lysine (*C*), and N^6-glycerinyl lysine (*D*) levels of plasma from healthy subjects (controls) and HD patients.** Data are expressed as mean ± S.D., *, $p < 0.001$ *versus* control (*A*, *C*, and *D*), $p < 0.01$ *versus* control (*B*).

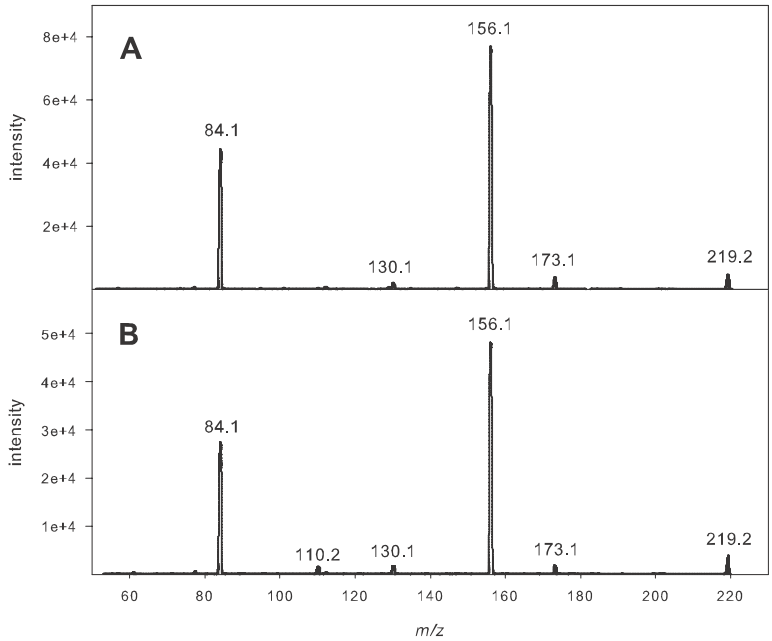

FIGURE 2. **Verification of N^6-lactoyl lysine by collision-induced dissociation of *m/z* 219.2 $[M + H]^+$, authentic reference (*A*), and plasma workup of HD patients (*B*).**

HD patients (CML, 2051 ± 760 *versus* 129 ± 59 pmol/ml plasma; CEL, 2063 ± 785 *versus* 157 ± 78 pmol/ml plasma).

In selected cases, the identity of the detected compounds was confirmed by comparison of the retention time and fragmentation pattern with authentic reference standards. The results for lactoyl lysine are shown in Fig. 2. The quasi molecular ion $[M + H]^+$ at *m/z* 219.2 is expected to undergo dehydration with immediate decarbonylation to the ion at *m/z* 173. The subsequent deamination of the immonium fragment renders the most abundant ion at *m/z* 156. Cyclization of lactoyl lysine to a six-membered ring and elimination of N^{ϵ} functionality yields the ion at *m/z* 130. *m/z* 84 presents the pyrrolinium ion.

Formation of Amide-AGEs in α-Dicarbonyl/N^1-t-Boc-lysine Incubations—To identify the direct precursors of the carboxylic acid amides, 1-DG, 3-DG, D-*arabino*-hexos-2-ulose (glucosone) and methylglyoxal were incubated with N^1-*t*-Boc-lysine under physiological conditions (pH 7.4, 37 °C). To study the impact of oxygen, incubations were conducted under aera-

Amide-AGEs in Vivo

TABLE 4

Comparison of amide concentrations in incubation mixtures of 200 mM glucose, 42 mM 1-desoxyglucoson (1-DG), 42 mM glucosone, and 42 mM methylglyoxal in the presence of 42 mM N^1-*t*-Boc-lysine and in incubation mixtures of 42 mM Amadori product under aerated and deaerated conditions

	Glucose[a]		Amadori product[b]		1-DG[b]		Glucosone[b]		Methylglyoxal[b]	
	Aerated	Deaerated	Aerated	Deaerated	Aerated	Deaerated	Aerated	Deaerated	Aerated	Deaerated
	mmol/mol lysine		*mmol/mol Amadori product*		*mmol/mol lysine*		*mmol/mol lysine*		*mmol/mol lysine*	
N^6-acetyl lysine	0.46	0.35	0.004	0.007	0.78	0.75	0.05	0.04	< LOD	< LOD
N^6-formyl lysine	1.39	0.48	0.37	0.05	1.47	1.08	2.60	0.89	< LOD	< LOD
N^6-lactoyl lysine	0.06	0.06	< LOD	< LOD	0.25	0.26	< LOQ	< LOQ	0.35	0.34
N^6-glycerinyl lysine	0.75	0.10	0.20	< LOQ	0.15	0.09	0.06	< LOQ	< LOD	< LOD

[a] At 28 days.
[b] At 72 h.

tion and deaeration. After acidic treatment to remove the Boc protection group, the samples were subjected to LC-MS/MS. Amides were found to be stable under these workup conditions, and artifact formation was excluded by immediate assay of the incubation mixtures at 0 h. The results at 72 h are provided in Table 4. 3-DG is not listed because none of the acylation products could be detected. The data for 1-DG were published in our previous work (7). With methylglyoxal, lactoyl lysine was the sole amide generated and the formation did not respond to aeration. In contrast, aeration revealed significant higher amounts of formyl lysine in glucosone incubations, whereas the levels of acetyl lysine, lactoyl lysine, and glycerinyl lysine were negligible compared with the amounts produced in 1-DG incubations.

Formation of Amide-AGEs in Glucose/N^1-t-Boc-lysine Incubations—Because glucose is a major source of glycation and glycoxidation *in vivo*, incubations of glucose with N^1-*t*-Boc-lysine and of 1-deoxy-1-(N^6-lysino)-D-fructose, the Amadori product of both, were performed under physiological conditions (pH 7.4, 37 °C) for comparison. The time course of formation of target amides in the glucose-lysine system over a time period of 4 weeks is shown in Fig. 3 and Table 4.

Acetyl lysine and formyl lysine were the dominant amide modifications under deaerated conditions. Both lactoyl lysine and glycerinyl lysine showed considerably lower levels of formation. In contrast to formyl and glycerinyl lysine, the impact of aeration on formation of acetyl and lactoyl lysine was negligible. However, the ratio of glycerinyl to formyl lysine changed dramatically with aeration: 1.50 *versus* 1.79 mmol/mol lysine compared with 0.16 *versus* 0.70 mmol/mol lysine with deaeration (incubation time, 48 days). This led to the conclusion that there must be additional oxygen-dependent mechanisms for glycerinyl lysine independent from formation of formyl lysine.

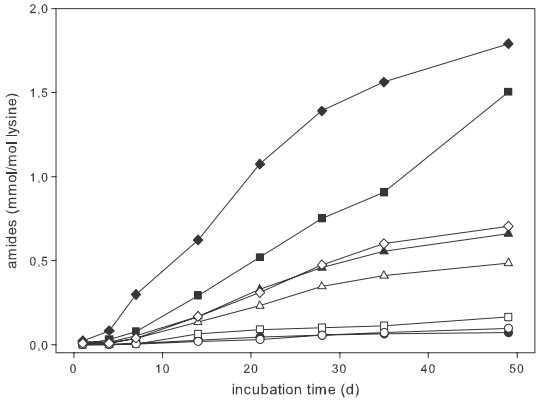

FIGURE 3. **Formation of AGE-amides in incubation mixtures of 200 mM glucose with 42 mM N^1-*t*-Boc-lysine in 0.1 M phosphate buffer, pH 7.4, at 37 °C under aerated (*closed symbols*) and deaerated conditions (*open symbols*): N^6-acetyl lysine (▲), N^6-formyl lysine (♦), N^6-lactoyl lysine (●), and N^6-glycerinyl lysine (■).**

DISCUSSION

Performing several model incubations, we tried to gain insights into possible formation pathways linked to Maillard chemistry *in vivo*. N^6-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine and N^6-glycoloyl lysine are formed via rearrangement reactions of glyoxal with lysine in contrast to the novel amide-AGEs involving β-dicarbonyl fragmentations (Fig. 4). We already investigated the degradation of the α-dicarbonyl 1-DG in the presence of N^1-*t*-Boc-lysine, identifying 1-DG as a direct precursor of the amide-AGEs (7). Now, glucosone was found to be another potential precursor of formyl lysine, while degradation of 3-DG did not result in any acylation. This is expected because 3-DG in contrast to 1-DG and glucosone cannot be converted to an α-oxoenediol, which is a prerequisite for β-dicarbonyl fragmentations. As shown in Fig. 5, all three C6-dicarbonyl structures are known Maillard degradation products of glucose. Although 1-DG and 3-DG are generated non-oxidatively via enolization and dehydration from the Amadori product, glucosone is formed via autoxidation of glucose or oxidation of the Amadori product or the intermediate Schiff base (31).

The results of incubations of glucose with N^1-*t*-Boc-lysine and of the Amadori product of both revealed a very similar formation pattern for acetyl lysine and lactoyl lysine in reaction mixtures with glucose and 1-DG, respectively (Table 4 and Fig. 3), confirming 1-DG as the direct precursor independent from oxidative processes via hydrolytic β-dicarbonyl fragmentation. Although the formation of formyl lysine from 1-DG responded to oxidative conditions, the difference was far more pronounced from glucose or the Amadori product. This pointed to the existence of another precursor emerging from oxidative pathways, which was identified as glucosone. Here, formyl lysine was detected in significant amounts, whereas formation of all other target amides was negligible. These findings were further supported by the same ratio of acetyl to formyl lysine in 1-DG *versus* glucose incubations under deaerated conditions, *i.e.* glucosone contributes only under oxidative conditions to formation of formyl lysine from glucose, whereas 1-DG is the

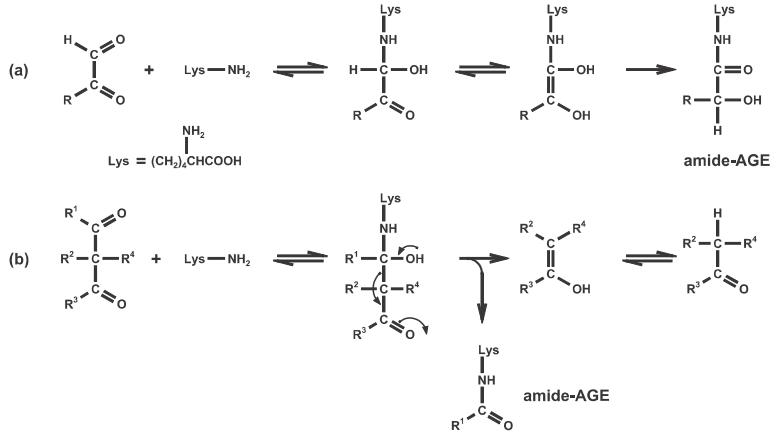

FIGURE 4. **Mechanism of amide-AGE formation via rearrangement (*a*) and β-dicarbonyl cleavage (*b*).**

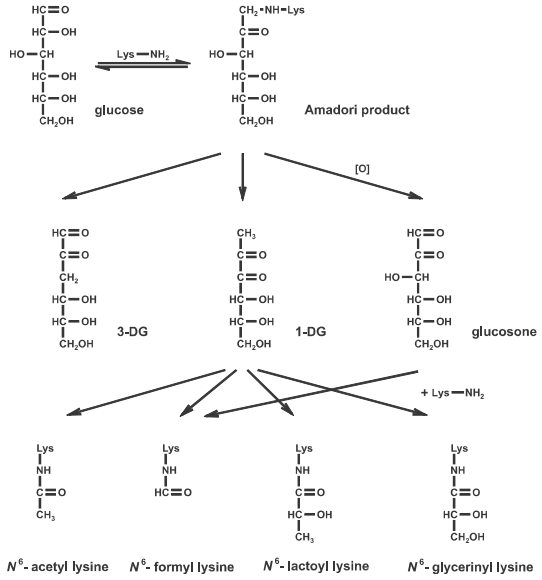

FIGURE 5. **Non-enzymatic degradation pathway of glucose.**

sole precursor in absence of oxygen. As formyl lysine, glycerinyl lysine showed a significant discrepancy in glucose *versus* 1-DG incubations induced by aeration. From the present results the additional reaction pathway leading to glycerinyl lysine from glucose remained unclear as none of the other Maillard intermediates responded. However, it is obvious that oxidation is required and that glucosone is not a relevant precursor.

Comparing the results of *in vivo* samples with above model experiments, incubations under aerated conditions should simulate the situation in uremia. Uremia has been described as a state of inflammatory stress resulting from either increased oxidation of carbohydrates and lipids (oxidative stress) or inadequate detoxification or inactivation of reactive carbonyl compounds derived from both carbohydrates and lipids by oxidative and non-oxidative chemistry (carbonyl stress) (3). Including only non-diabetic subjects in the present study, the impact of differences in glucose plasma concentrations was avoided.

The comparison gave a very diverse picture. Although all target amides *in vivo* were significantly increased in uremia (Fig. 1), only formyl and glycerinyl lysine responded to aeration in the glucose-lysine model. In addition, the ratio of concentrations between the single amides was completely different. *In vivo*, acetyl, formyl, and lactoyl lysine were within the same range, with glycerinyl lysine showing 10-fold lower concentrations. In contrast, glucose incubations showed the smallest concentrations for lactoyl lysine. Given that glycerinyl lysine indeed follows the patterns of the model incubations, this suggests additional pathways for the formation of acetyl, formyl, and lactoyl lysine. An alternative mechanism leading to lactoyl lysine *in vivo* is the reaction of methylglyoxal with ϵ-amino lysine residues corresponding to our identification of N^6-glycoloyl lysine in glyoxal lysine model incubations based on rearrangement reactions. Indeed, when we incubated methylglyoxal with N^1-*t*-Boc-lysine, we found significant amounts of lactoyl lysine independent from the presence of oxygen (Table 4). *In vivo*, this also explains the 7-fold increase in uremia, as we measured a 4-fold increase in methylglyoxal in such subjects (138 ± 39 *versus* 496 ± 132 pmol/ml plasma). Concentrations of acetyl lysine were highest *in vivo* and responded to renal failure by a 2-fold increase. This might be explained by increased cell death triggered by the strong inflammatory processes observed under uremia resulting in accelerated release of acetyl lysine from acetylated histone proteins. In addition, the increased concentration of methylglyoxal in presence of peroxynitrite might also contribute to the acetylation of ϵ-amino lysine residues *in vivo*. Massari *et al.* (32) described a mechanism of L-lysine acetylation by a methylglyoxal-peroxynitrite system *in vitro*. Although the direct reaction of methylglyoxal with proteins probably dominates over that with peroxynitrite, the post-translational acetylation of proteins by radical mechanisms in the presence of methylglyoxal might be a plausible

Amide-AGEs in Vivo

second non-enzymatic pathway to acetyltransferase-catalyzed reactions. Moreover, although much less reactive, H_2O_2 could replace peroxynitrite in the acetyl-generating reaction from methylglyoxal. The idea to explain the 2-fold increase of formyl lysine in uremia based on the *in vitro* models is undermined by the fact that neither 1-DG nor glucosone have been detected *in vivo* so far. An intriguing alternative mechanism might be the with inflammation increased oxidative DNA breakdown to give 3′-formylphosphate-ended DNA fragments as a potential precursor, but again, such species have not been identified *in vivo* (23).

As a first attempt to assess the importance of the novel amide-AGEs as clinical markers for kidney failure, plasma levels of the well established lysine modifications CML and CEL were measured additionally. In healthy human subjects, concentrations were within the same range. The AGE levels decreased in the following order: acetyl lysine > CEL ≈ formyl lysine ≈ CML > lactoyl lysine > glycerinyl lysine. In uremia, we observed a 13- to 16-fold increase of CEL and CML. Amide-AGEs showed only a 2- to 8-fold increase. Thus, amide-AGEs might be of major importance when compared with other AGE structures identified *in vivo* so far, even though the impact of uremia on plasma concentrations of CML and CEL is more pronounced.

In conclusion, we previously proposed a formation mechanism for the novel amide-AGEs acetyl, formyl, lactoyl, and glycerinyl lysine via degradation of 1-DG in the presence of lysine (7). Now, glucosone and methylglyoxal were identified as alternative precursors for formyl and lactoyl lysine, respectively. All four amide-AGEs were unequivocally detected in human plasma, formyl, lactoyl, and glycerinyl lysine for the first time. The results strongly suggest a major participation of non-enzymatic Maillard mechanisms on amide-AGE formation pathways *in vivo* paralleled by enzymatic processes. The pathophysiologic consequences of two to 7-fold increased levels of amide-AGE free adducts in plasma of HD patients is not yet understood and requires further investigation. Also, the question which quantities of AGE-free adducts are derived from the breakdown of AGE-modified proteins or from direct synthesis remains unclear. However, absolute plasma concentration levels were in the same range similar to other established lysine modifications, *i.e.* CML and CEL, and suggest a comparable impact of non-enzymatic biochemistry on pathophysiologies in the human organism.

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Extending the Spectrum of α-Dicarbonyl Compounds *in Vivo*[*]

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Background: α-Dicarbonyls are central intermediates in the formation of advanced glycation end products (AGEs).
Results: A quantitation method for the complete spectrum relevant *in vivo* was established.
Conclusion: Non-enzymatic chemistry of glucose and L-ascorbic acid as precursors and α-dicarbonyl intermediates play an important role *in vivo*.
Significance: Knowledge of plasma levels of α-dicarbonyls is crucial to understand the complex pathways of AGE formation *in vivo*.

Maillard α-dicarbonyl compounds are known as central intermediates in advanced glycation end product (AGE) formation. Glucose is the primary source of energy for the human body, whereas L-*threo*-ascorbic acid (vitamin C) is an essential nutrient, involved in a variety of enzymatic reactions. Thus, the Maillard degradation of glucose and ascorbic acid is of major importance *in vivo*. To understand the complex mechanistic pathways of AGE formation, it is crucial to extend the knowledge on plasma concentrations of reactive key α-dicarbonyl compounds (*e.g.* 1-deoxyglucosone). With the present work, we introduce a highly sensitive LC-MS/MS multimethod for human blood plasma based on derivatization with *o*-phenylenediamine under acidic conditions. The impact of workup and reaction conditions, particularly of pH, was thoroughly evaluated. A comprehensive validation provided the limit of detection, limit of quantitation, coefficients of variation, and recovery rates. The method includes the α-dicarbonyls 1-deoxyglucosone, 3-deoxyglucosone, glucosone, Lederer's glucosone, dehydroascorbic acid, 2,3-diketogulonic acid, 1-deoxypentosone, 3-deoxypentosone, 3,4-dideoxypentosone, pentosone, 1-deoxythreosone, 3-deoxythreosone, threosone, methylglyoxal, glyoxal; the α-keto-carboxylic acids pyruvic acid and glyoxylic acid; and the dicarboxylic acid oxalic acid. The method was then applied to the analyses of 15 healthy subjects and 24 uremic patients undergoing hemodialysis. The comparison of the results revealed a clear shift in the product spectrum. In most cases, the plasma levels of target analytes were significantly higher. Thus, this is the first time that a complete spectrum of α-dicarbonyl compounds relevant *in vivo* has been established. The results provide further insights into the chemistry of AGE formation and will be helpful to find specific markers to differentiate between the various precursors of glycation.

Advanced glycation of proteins has been investigated in aging, diabetes, and nutrition (1–3). The Maillard reaction, a non-enzymatic process, is initiated when proteins are exposed to glucose or other carbohydrates. Through a series of reactions, it eventually yields the irreversible advanced glycation end products (AGEs).[2] There are multiple sources and mechanisms of AGE formation *in vivo*, involving oxidative and non-oxidative chemistry of reducing sugars, Schiff bases, Amadori adducts, ascorbic acid and metabolic intermediates (4, 5). Because they are correlated to the severity of diabetic and uremic complications their clinical relevance was established (6–8).

AGE concentrations *in vivo* are markedly amplified in diabetes but also in non-diabetic uremia with a significant loss of renal clearance (9). Thus, besides substrate concentration, carbonyl stress, as described by Baynes *et al.* (10), is an alternative explanation for the increase of chemical protein modifications in various diseases. Carbonyl stress is caused by a generalized increase in the concentration of reactive carbonyl precursors of AGEs. Among those, α-dicarbonyl compounds (α-DCs) play a very prominent role. Their relevance as key intermediates in AGE formation was extensively investigated in *in vitro* model systems (11–15).

Endogenous formation is supposed to be the predominant source of α-DCs in human circulation (16). However, information on the physiological importance of exogenous dicarbonyl intake is scarce and subject to recent investigations (17–19). Regarding this topic, foods and peritoneal dialysis fluids are considered to release considerable amounts of reactive α-DCs (20–24).

For a comprehensive understanding of the complex formation pathways of AGEs *in vivo*, the analytical assessment of the complete α-DC spectrum is crucial. Nemet and Monnier (25) examined the L-*threo*-ascorbic acid (ASA, vitamin C) degradation products dehydroascorbic acid (DHA), 2,3-diketogulonic acid

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[2] The abbreviations used are: AGE, advanced glycation end products; α-DC, α-dicarbonyl compound; ASA, L-*threo*-ascorbic acid; DHA, dehydroascorbic acid; 2,3-DKG, 2,3-diketogulonic acid (L-*threo*-2,3-hexodiulosonic acid); 3-DT, 3-deoxythresosone (4-hydroxy-2-oxobutanal); GO, glyoxal; MGO, methylglyoxal (2-oxopropanal); 3-DG, 3-deoxyglucosone (3-deoxy-D-*erythro*-hexos-2-ulose); OPD, *o*-phenylenediamine; threosone, 3,4-dihydroxy-2-oxobutanal; 1-DG, 1-deoxyglucosone (1-deoxy-D-*erythro*-hexo-2,3-diulose); DHA, dehydroascorbic acid; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 1-DP, 1-deoxypentosone; 3-DP, 3-deoxypentosone; 3,4-DDP, 3,4-dideoxypentosone (3,4-dideoxypentos-2-ulose); 1-DT, 1-deoxythresosone (1-hydroxy-2,3-butanedione); Q, quinoxaline; HD patients, patients undergoing hemodialysis; 3-DGal, 3-deoxygalactosone; EtOAc, ethyl acetate; 3,4-DGE, 3,4-dideoxyglucosone-3-ene; MeOH, methanol; CID, collision-induced dissociation; glucosone, D-*arabino*-hexos-2-ulose; Lederer's glucosone, N^6-(3,6-dideoxyhexos-2-ulos-6-yl)-L-lysine; pentosone, pentos-2-ulose.

(2,3-DKG), threosone, 3-deoxythresosone (3-DT), and xylosone in human lens. In human blood plasma, however, only glyoxal (GO), methylglyoxal (MGO), 3-deoxyglucosone (3-DG), and DHA have been described in detail so far (26–33). The implemented analytical methods vary not only in the inevitable derivatization procedure but also in the chromatographic technique used. The common alternative approach for the quantitation of DHA is the measurement of the difference of ASA before and after a reduction step (34, 35). Consequently, concentrations of published plasma levels of healthy subjects differ in a wide range and are thus not comparable (*e.g.* for GO from 220 to 1150 pmol/ml, for MGO from 120 to 650 pmol/ml, and for DHA from 550 to 6800 pmol/ml (mean values)).

Glomb and Tschirnich compared common derivatization approaches (36). According to them, the use of aromatic *o*-diamines (*e.g. o*-phenylenediamine (OPD)) is prerequisite for the analysis of highly reactive α-DCs, such as 1-deoxyglucosone (1-DG). The detection of these short lived intermediates is limited by the rate of condensation of the reagent with the carbonyl moiety. However, these trapping reagents impose high oxidative stress on the system investigated and could lead to artifact formation, especially of α-DCs that originate from oxidative pathways (*e.g.* D-*arabino*-hexos-2-ulose (glucosone)).

The work group of Thornalley (31) developed a reliable method for the detection of MGO as 6,7-dimethoxy-2-methylquinoxaline in human plasma. They stressed the importance of pH control to avoid the degradation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G3P) to MGO, both central intermediates in several metabolic pathways in living organisms.

Although many methods for the detection of a few selected α-DCs were described already, a comprehensive method including all relevant compounds *in vivo* has been missing up to now. The particular analytical challenge in this regard is the very diverging polarity of the target substances but also the baseline separation of isomeric pairs with identical molecular masses (*e.g.* 1-/3-deoxypentosone (1-/3-DP)).

We developed and validated a highly sensitive LC-MS/MS multimethod suitable for routine analysis based on the derivatization with OPD, including 15 α-dicarbonyl compounds and two α-ketocarboxylic acids. Both substance categories are hereafter referred to as α-DCs for reasons of simplicity. In detail, the method covers 1-DG, 3-DG, glucosone, N^6-(3,6-dideoxyhexos-2-ulos-6-yl)-L-lysine (Lederer's glucosone), DHA, 2,3-DKG, 4,5-dihydroxy-2,3-pentanedione (1-DP), 4,5-dihydroxy-2-oxopentanal (3-DP), 3,4-dideoxypentosone (3,4-DDP), pentos-2-ulose (pentosone), 1-deoxythreosone (1-DT), 3-DT, threosone, MGO, ethanedial (glyoxal, GO), 2-oxopropanoic acid (pyruvic acid), oxoethanoic acid (glyoxylic acid), and ethanedioic acid (oxalic acid) and was used to screen an initial set of plasma samples from 15 healthy human subjects and 24 uremic patients undergoing hemodialysis (HD patients). The results are discussed with respect to the sources and mechanistic relationships of the α-DCs detected.

EXPERIMENTAL PROCEDURES

Materials and Plasma Samples—Chemicals of the highest grade available were obtained from Sigma-Aldrich and Fisher unless otherwise indicated. NMR solvents were purchased from ARMAR Chemicals (Leipzig/Doettingen, Germany). The quinoxaline derivatives of the α-DCs will be marked hereafter with the suffix "-Q". 1-DG-Q, 3-DG-Q, glucosone-Q, Lederer's glucosone-Q, 1-DP-Q, 3-DP-Q, 3,4-DDP-Q, pentosone-Q, 1-DT-Q, 3-DT-Q, threosone-Q, pyruvic acid-Q, glyoxylic acid-Q, oxalic acid-Q, and 3-DG were synthesized according to our previous work (37–39). The identities of target compounds were verified by nuclear magnetic resonance (NMR) experiments.

Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of the Medical Faculty of the Martin Luther University Halle-Wittenberg. Blood samples were obtained from 15 healthy subjects with normal renal function and 24 non-diabetic patients undergoing hemodialysis using EDTA as an anticoagulant (2 mg/ml whole blood). In HD patients, samples were obtained predialysis before the midweek treatment session. Hemodialysis was performed three times weekly for 4–5 h using polysulfone dialyzers. All patients were treated with bicarbonate hemodialysis (acid concentrate type 257, 8.4% sodium bicarbonate type 200, MTN Neubrandenburg GmbH, Neubrandenburg, Germany) with ultrapure water quality (by reverse osmosis and sterile filters). Plasma was derived by centrifugation (3000 × *g*, 10 min, 4 °C) within 20 min of collection and immediately subjected to the assay procedure described below. HbA1c, creatinine, and C-reactive protein were measured by routine methods at the central laboratory of Martin-Luther-University Clinical Center, Halle (Saale, Germany).

2-(2′(R),3′(R),4′-Trihydroxybutyl)quinoxaline (3-DGal-Q)—3-Deoxy-D-threo-hexos-2-ulose (3-deoxygalactosone; 3-DGal) was synthesized according to the literature (40) with the exception of the 3-DGal bis(benzoyl hydrazone) cleavage. Here, instead of benzaldehyde, sodium nitrite was used, following the procedure of Henseke and Bauer (41). Purification of crude 3-DGal-Q was achieved by preparative high performance liquid chromatography with ultraviolet detection (HPLC-UV). NMR results were in line with those of Hellwig *et al.* (40).

3-(D-erythro-Glycerol-1-yl)-quinoxaline-2-carboxylic Acid o-Aminoanilide (DHA Precursor-Q)—DHA (250 mg; 1.44 mmol) was suspended in methanol (25 ml), and OPD was added (308.5 mg; 2.85 mmol). The reaction mixture was heated for 2 h at 40 °C and allowed to cool. The precipitated solid was isolated by filtration; washed with water, ethanol, and ether; and dried (yield: 282.6 mg, 76%). Recrystallization from ethanol gave yellow needles.

^{1}H NMR (500 MHz, DMSO-d_6): δ (ppm) = 3.44–3.55 (m, 2H), 4.03 (m, 1H), 5.42 (m, 1H), 6.63 (m, 1H), 6.8 (dd, J = 8.0, 1.4 Hz, 1H), 7.0 (m, 1H), 7.36 (dd, J = 8.0, 1.5 Hz, 1H), 7.96 (m, 2H), 8.17 (m, 1H), 8.23 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 63.3, 72.0, 74.5, 116.4, 116.6, 122.9, 126.4, 127.2, 128.9, 129.5, 131.0, 132.0, 139.6, 141.2, 143.2, 147.4, 156.0, 165.1. HR-MS: *m/z* 393.09645 (found); *m/z* 393.09596 (calculated for $C_{18}H_{18}O_4N_4K$ $[M + K]^+$).

3-(D-erythro-Glycerol-1-yl)-quinoxaline-2-carboxylic γ-Lactone (DHA-Q)—A suspension of precursor DHA-Q (500 mg) in water (10 ml) was treated with 0.1 M hydrochloric acid (25 ml) and stirred at room temperature. The reaction process was followed by thin layer chromatography (TLC; EtOAc, UV detection). The reaction mixture was extracted with EtOAc (3 ×

35 ml), the organic layers were combined, and the solvent was evaporated. The residue was purified by column chromatography (silica gel 60, 63–200 μm (Merck), EtOAc). Fractions including the compound with R_f 0.24 (TLC: EtOAc, UV detection) were collectively concentrated *in vacuo* to afford 180 mg (66%) of light yellow needles. NMR results were in line with those of Nemet and Monnier (25). HR-MS: *m/z* 269.05301 (found); *m/z* 269.05328 (calculated for $C_{12}H_{10}O_4N_2Na$ $[M + Na]^+$).

2,3-DKG—2,3-DKG as sodium salt was synthesized according to the method of Otsuka *et al.* (42). The product was obtained as a white hygroscopic precipitate. It was characterized by the LC-MS/MS method mentioned below after derivatization with OPD and directly utilized for quinoxaline synthesis.

3-((1S,2R)-1,2,3-Trihydroxypropyl)-quinoxaline-2-carboxylic Acid (2,3-DKG-Q)—The corresponding quinoxaline of 2,3-DKG was obtained according to Nemet and Monnier (25). Purification of the quinoxaline solution was done by preparative HPLC-UV (t_R = 62 min). Fractions including the pure compound were collectively evaporated under reduced pressure, dissolved in water, and lyophilized to afford 2,3-DKG-Q as a light yellow powder in quantitative yield. NMR data were in line with the literature (25). HR-MS: *m/z* 263.06749 (found); *m/z* 263.06735 (calculated for $C_{12}H_{11}O_5N_2$ $[M - H]^+$).

Isotopically Labeled 2-Hydroxy-3-methyl-2,3-$^{13}C_2$-quinoxaline (Pyruvic Acid-$^{13}C_2$-Q)—Pyruvic acid-$^{13}C_2$-Q was synthesized according to the method of Arun *et al.* (43). Briefly, a solution of OPD in distilled water (306.9 mg in 8 ml of ultrapure water, 2.84 mM) was added to 1,2-$^{13}C_2$-pyruvic acid (250.0 mg in 8 ml ultrapure water, 2.84 mM) dropwise with constant stirring. The precipitated pale yellow colored compound was filtered, washed with water, and lyophilized. The crude sample was recrystallized from 50% ethanol absolute (404.7 mg, 88%). NMR data were in line with the literature. HR-MS: *m/z* 163.0779 (found); *m/z* 163.0776 (calculated for $C_7{}^{13}C_2H_8ON_2$ $[M + H]^+$).

Time Course of the OPD Reaction of 3-DG, MGO, GO, Glyoxylic Acid, Pyruvic Acid, Oxalic Acid, DHA Precursor-Q, and 2,3-DKG-Q under Assay Conditions—All compounds (20 μM) were incubated under assay conditions in amber glass vials under argon atmosphere at 22 °C in 0.4 M formate buffer (pH 3.0) containing 0.55 mM OPD and 3.4 mM EDTA. Vials were tightly sealed by a screw cap with integrated polytetrafluoroethylene/silicone septum. At various time points, aliquots of the reaction mixtures were subjected to LC-UV analysis as described below. After 24 h, trifluoroacetic acid (TFA; 0.4 M) was added, and the incubation was continued for 1 h. Then pH 3.0 was adjusted again with 4 M ammonium hydroxide. The injection volume was adjusted to account for the dilution of the reaction mixture. The yields were determined against the authentic reference α-DC-Q standards.

Time Course of the OPD Reaction of ASA, DHA, 2,3-DKG, and 3-DG-Q under Assay Conditions—All compounds (20 μM) were incubated as described above over a prolonged time period. No TFA was added after 24 h. At various time points, aliquots of the reaction mixtures were subjected to LC-UV analysis as described below. The yields were determined against the authentic reference α-DC-Q standards. Due to the lack of authentic reference for the quinoxaline of 3,4-dideoxyglucosone-3-ene (3,4-DGE-Q), quantitation was done with 3-DGal-Q under the assumption of an equal extinction coefficient.

De Novo Formation of MGO-Q from G3P and DHAP with OPD under Various Assay Conditions—G3P and DHAP (50 and 100 μM, which corresponds to a plasma concentration of 100 and 200 μM, respectively) were incubated under assay conditions in amber glass vials under argon atmosphere at 22 °C in various buffer solutions containing 0.55 mM OPD and 3.4 mM EDTA. The buffers used were potassium phosphate buffer (0.4 M, pH 7.0), sodium formate buffer (0.4 M, pH 3.0), and perchloric acid (0.4 M, pH <0). Vials were tightly sealed by a screw cap with integrated polytetrafluoroethylene/silicone septum. Reaction mixtures were subjected to LC-UV analysis as described below not only after 24 h but immediately after the addition of OPD and after a 1-h incubation time to account for possible MGO impurities of the starting materials. The yield of MGO was determined against the authentic reference α-DC-Q standards.

Assay of α-DCs in Plasma—Sodium formate buffer (2 M, pH 3.0, 200 μl), the internal standard pyruvic acid-$^{13}C_2$-Q (1.25 μM, 100 μl) and the derivatizing agent OPD (2.75 mM, 200 μl) were added to 500 μl of blood plasma. The sample was incubated for 24 h in the dark at room temperature under argon atmosphere. TFA (2 M, 250 μl) was added, and the incubation continued for 1 h under the same conditions. The pH of the sample was then adjusted to pH 3.0 with ammonium hydroxide (4 M, 415 μl). Water was added (85 μl) to give a total sample volume of 1750 μl. The protein precipitate was separated by centrifugation (16,000 × *g* and 20 °C). The supernatant (storage at −80 °C) was administered to LC-MS/MS analysis.

HPLC-UV—A Besta HD 2-200 pump (Wilhelmsfeld, Germany) was used at a flow rate of 15 ml/min. Elution of material was monitored by a UV detector (Jasco UV-2075 with a preparative flow cell (Gross-Umstadt, Germany)). The detection wavelength was 320 nm. Chromatographic separations were performed on a stainless steel column (KNAUER, 250 × 20 mm, Eurospher-100 C18, 10 μm (Berlin, Germany)). The mobile phase used consisted of water (solvent A) and methanol/water (7:3 (v/v), solvent B). To both solvents (A and B) 0.8 ml/liter formic acid was added. Samples were injected at 5% B for 2,3-DKG-Q and at 35% B for 3-DGal-Q and purified under isocratic conditions.

NMR Spectroscopy—NMR spectra were recorded on a Varian VXR 400 spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C or on a Varian Unity Inova 500 instrument operating at 500 MHz for ^1H and 125 MHz for ^{13}C, respectively. Chemical shifts are given relative to external $SiMe_4$.

Accurate Mass Determination (High Resolution MS)—The high resolution positive and negative ion electrospray ionization mass spectra (electrospray ionization-high resolution MS) were taken on a Bruker Apex III Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with an Infinity cell, a 7.0-tesla superconducting magnet, a radio frequency-only hexapole ion guide, and an external off-axis electrospray source (Apollo; Agilent, Santa

TABLE 1
LC-MS/MS parameters for quinoxaline quantitation

Quinoxaline and letter assignment (see Fig. 2)	Retention time	Precursor ion		Product ion 1[a]			Product ion 2[b]			Product ion 3[b]		
		m/z	*DP*[c]	*m/z*	*CE*[d]	*CXP*[e]	*m/z*	*CE*	*CXP*	*m/z*	*CE*	*CXP*
	min	*amu*[f]	*V*	*amu*	*eV*	*V*	*amu*	*eV*	*V*	*amu*	*eV*	*V*
2,3-DKG (*a*)	9.5	265.1	40	185.1	23.0	11.0	221.1	17.0	17.0	161.0	27.0	11.0
Glucosone (*b*)	9.9	251.1	55	173.1	24.0	16.0	161.1	30.0	13.0	233.2	19.0	16.0
Oxalic acid (*c*)	10.5	162.9	70	90.1	42.0	15.0	145.1	25.0	10.0	117.1	31.0	7.0
Pentosone (*d*)	11.2	221.1	50	173.1	22.0	14.0	161.0	27.0	13.0	132.1	45.0	10.0
1-DG (*e*)	11.9	235.2	55	175.1	28.0	12.0	187.1	27.0	11.0	217.2	18.0	16.0
DHA (*f*)	12.0	247.1	55	229.0	18.0	20.0	185.3	20.0	15.0	171.1	33.0	10.0
3-DG (*g*)	13.9	235.2	55	199.1	24.0	17.0	145.0	31.0	9.0	157.1	12.0	30.0
Threosone (*h*)	14.0	191.2	45	173.1	17.5	14.0	144.1	37.0	11.0	161.2	24.0	12.0
1-DP (*i*)	16.8	205.1	50	187.1	21.0	14.0	145.1	27.0	12.0	169.2	30.0	11.0
Glyoxylic acid (*j*)	17.1	147.1	65	129.0	26.5	9.0	119.1	30.0	10.0	102.1	39.0	8.5
3-DP (*k*)	17.1	205.1	50	187.2	19.5	10.0	158.1	34.0	14.0	174.1	32.5	13.0
Lederer's glucosone	18.4	363.3	65	187.1	27.0	16.0	159.1	29.0	10.0	219.1	24.0	12.0
3-DT (*l*)	19.2	175.1	55	157.2	24.0	10.0	129.2	35.0	9.0	145.0	25.5	10.0
DHA precursor (*m*)	19.5	355.3	52	109.2	19.0	8.0	247.2	15.0	16.0	337.2	15.0	12.0
Pyruvic acid (*n*)	19.5	160.9	60	133.1	28.0	10.0	91.9	40.0	7.0	64.9	54.0	11.0
Pyruvic acid-$^{13}C_2$	19.5	163.1	60	134.0	28.0	10.0	92.1	40.0	7.0	64.9	54.0	11.0
1-DT (*o*)	19.6	175.1	45	157.1	22.0	7.0	156.1	35.0	15.0	89.1	55.0	5.0
3,4-DDP (*p*)	20.5	189.1	55	171.2	25.0	15.0	145.1	25.0	11.0	102.1	52.0	16.0
GO (*q*)	20.9	131.1	32	77.0	40.0	6.0	104.0	30.0	8.0			
MGO (*r*)	22.3	145.0	50	77.0	41.0	5.0	118.1	30.5	7.0	65.0	45.0	5.0

[a] MRM transition used for quantitation (quantifier).
[b] MRM transition used for confirmation (qualifier).
[c] *DP*, declustering potential.
[d] *CE*, collision energy.
[e] *CXP*, collision cell exit potential.
[f] amu, atomic mass units.

Clara, CA). Nitrogen was used as a drying gas at 150 °C. The samples were dissolved in methanol, and the solutions were introduced continuously via a syringe pump at a flow rate of 120 μl/h. The data were acquired with 256,000 data points and zero-filled to 1,024,000 by averaging 32 scans.

High Performance Liquid Chromatography with Coupled Ultraviolet-Mass Spectrometry Detection (LC-UV-MS/MS)—The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a pump (PU-2080 Plus) with degasser (LG-2080-02) and quaternary gradient mixer (LG-2080-04), a column oven (Jasco Jetstream II), an autosampler (AS-2057 Plus), and a UV detector (UV-2075). Mass spectrometric detection was conducted on an API 4000 QTrap LC-MS/MS system (Applied Biosystems/MDS Sciex, Concord, Canada) equipped with a turbo ion spray source using electrospray ionization in positive mode: sprayer capillary voltage, 4.5 kV; nebulizing gas flow, 50 ml/min; heating gas, 60 ml/min at 550 °C; and curtain gas, 40 ml/min.

Chromatographic separation was performed on a stainless steel column packed with RP-18 material (KNAUER, 250 × 4.6 mm, Eurospher-100 C18, 5 μm (Berlin, Germany)) using a flow rate of 1.0 ml/min. The mobile phase used was water (solvent A) and methanol/water (7:3 (v/v), solvent B). To both solvents (A and B), 0.6 ml/liter heptafluorobutyric acid was added. Analysis was performed at 25 °C column temperature using gradient elution: 5% B (0–0.1 min) to 35% B (0.5 min) to 47% B (12 min) to 100% B (17–25 min). For mass spectrometric detection, the scheduled multiple-reaction monitoring mode was used, utilizing collision-induced dissociation (CID) of the protonated molecules with compound-specific orifice potentials and fragment-specific collision energies (Table 1). Quantitation was performed using the standard addition method. More precisely, increasing concentrations of authentic α-DC-Q reference compounds at factors of 0.5, 1, 2, and 3 times the concentration of the analyte in the sample were added to separate aliquots of the sample after the workup procedure. The aliquots were analyzed, and a regression of response *versus* concentration is used to determine the concentration of the analyte in the sample. Spikes were run for one of approximately every 30 samples. Calibration with this method resolves potential matrix interferences. Potential losses during the workup procedure and intrabatch changes of instrument sensitivity were corrected with pyruvic acid-$^{13}C_2$ as an internal standard.

To obtain fragmentation spectra of α-DCs-Q in plasma workup solutions, target material was first enriched by repeated collection from the above HPLC system. After solvent evaporation in a vacuum concentrator (Savant SpeedVac Plus SC 110 A combined with a Vapor Trap RVT 400, Thermo Fisher Scientific GmbH (Dreieich, Germany)), the residue was dissolved in water and reinjected, using a CID experiment. The fragmentation spectra of the authentic references were obtained with the same parameters. For 1-DG-Q, the following parameters were used: declustering potential, 50 V; collision energy, 30 eV; collision cell exit potential, 10 V; scan range, *m/z* 130–250 (4 s). All plasma workup samples and incubations were analyzed in single batches to exclude interassay variations.

Validation of the Quantitation Method—Intraassay coefficients of variation were determined by repeated workup and analysis of a plasma sample ($n = 6$). In addition, the limit of detection and limit of quantitation with all steps of the analysis included were estimated according to DIN 32645 ($n = 6$, confidence level $p = 0.95$, $k = 3$) (44). To determine recovery rates, the respective alpha-DCs-Q were added at four different concentrations to three parallel sets of blood samples of one subject. The native and the spiked plasma sample were subjected to the assay of α-DCs in plasma as described above. The recovery rate was estimated as the quotient of (spiked α-DC-Q amount − amount α-DC-Q in native plasma sample)/amount of added α-DC-Q × 100%.

RESULTS

α-Dicarbonyl Compounds in Blood Plasma of Healthy Human Subjects and Hemodialysis Patients—Plasma was obtained from 15 healthy subjects with normal renal function and no proteinuria and 24 uremic patients undergoing hemodialysis (HD patients). Details are given in Table 2. Normal renal function was defined as a serum creatinine level below 102 μmol/liter. To ensure the absence of inflammatory reactions, C-reactive protein had to be 5 μmol/liter or below for all healthy subjects.

After derivatization of target α-DCs to the respective quinoxalines and separation from the protein residue, the plasma samples were subjected to the described LC-MS/MS method. All plasma workup samples of controls and HD patients were each analyzed in single batches to exclude interassay variations. Intraassay coefficients of variation were determined by independent analyses of three different blood samples for each subject and were <10% in all cases. The results are presented in Table 3.

In selected cases, the identity of the detected compounds in plasma was confirmed by comparison of the native fragmentation spectrum with authentic reference standards. The results were in full compliance and are exemplarily shown for 1-DG-Q in Fig. 1. The protonated molecular ion $[M + H]^+$ at m/z 235.2 is expected to undergo dehydration to the ion at m/z 217.2 and 199.2. Subsequent decay leads to signals at m/z 191.0, 187.1, 175.1, 171.2, and 158.1.

TABLE 2
Profile of subjects examined in this study

	Healthy subjects	HD patients
No. of participants	15	24
Sex, female/male	6/9	6/18
Age (years)	24–34	30–85
HbA1c (mmol/mol)	37 ± 2	40 ± 17
Serum creatinine (μmol/liter)	82 ± 13	861 ± 322
C-reactive protein (mg/liter)	<5	11 ± 21

Development of an LC-MS/MS Method for 17 Relevant Dicarbonyl Compounds as Their Corresponding Quinoxalines—The complex matrix and the low concentration of most target analytes hamper the use of UV detectors for qualitative and quantitative analysis. Tandem mass spectrometric detection in the multiple-reaction monitoring mode was prerequisite for the detection of the 17 relevant α-DCs in blood plasma. To achieve highly sensitive detection utilizing collision-induced dissociation of the protonated molecules, the compound-specific orifice potentials and fragment-specific collision energies had to be determined by authentic reference material (Table 1).

HPLC separation was based on a RP-18 phase with methanol as the organic eluent. Optimization of gradient, column temperature, and ion pair reagent led to almost baseline separation of all critical quinoxalines possessing the same molecular weight, which therefore could not be distinguished via MS detection (1-DG-Q *versus* 3-DG-Q *versus* 4-DG-Q *versus* 3-DGal-Q, 1-DP-Q *versus* 3-DP-Q, 1-DT-Q *versus* 3-DT-Q). Fig. 2 shows the typical chromatogram of a plasma sample after derivatization with OPD.

Verification of the Derivatization Procedure—The reactivity of α-DCs required conversion into stable quinoxaline derivatives. After the blood sample is drawn from the living subject, the complex system blood is prone to significant alterations regarding its biological activity and chemical composition, especially under exposure to oxygen. Immediate plasma generation and instant start of the derivatization procedure under well defined conditions is therefore prerequisite for reproducible quantitative results.

To prevent underestimation of rather slow reacting compounds, OPD was chosen as the derivatization agent. It has a comparatively high conversion rate of α-DCs to the respective quinoxaline derivatives. To ensure complete derivatization, selected native α-DCs (GO, MGO, glyoxylic acid, pyruvic acid, oxalic acid, and 3-DG) were incubated with OPD under stan-

TABLE 3
Levels of all relevant α-dicarbonyl compounds in human blood plasma

Values shown are the mean ± S.D. of 15 healthy subjects and 24 patients undergoing hemodialysis (HD patients), replicate analyses, $n = 3$. Student's t test was used for statistical evaluation of significant differences between both groups: *, $p < 0.01$ *versus* healthy subjects; **, $p < 0.001$ *versus* healthy subjects.

	Healthy subjects		HD patients	
Quinoxaline	Mean ± S.D.	Range	Mean ± S.D.	Range
	pmol/ml		*pmol/ml*	
Glucosone	46 ± 11	28–67	96 ± 49**	57–276
1-DG	22 ± 3	16–28	30 ± 16	12–82
3-DG	43 ± 5	35–56	65 ± 20**	36–125
Lederer's glucosone	<*LOD*		7.0 ± 2.5[a]	<*LOD*–13
Pentosone	15 ± 10[a]	<*LOD*–32	11 ± 5[a]	<*LOD*–23
1-DP	3.6 ± 1.2[a]	2.6–7.8	6.2 ± 2.6[a]**	3.2–15
3-DP	11 ± 2	9–17	33 ± 9**	20–53
Threosone	5.4 ± 0.7[a]	4.2–6.6	9.0 ± 3.5[a]**	4.2–19
1-DT	3.1 ± 0.3	2.6–3.8	3.8 ± 1.0*	2.0–6.6
3-DT	10 ± 2	8–14	45 ± 11**	28–75
MGO	61 ± 7	51–76	219 ± 129**	42–617
GO	491 ± 47	405–564	1273 ± 980**	400–4914
Pyruvic acid	7250 ± 2549	2818–12,038	35,874 ± 19,080**	8399–97,935
Glyoxylic acid	1264 ± 353	783–1942	2031 ± 608**	1026–3514
Oxalic acid	40 ± 21	18–82	36 ± 12	16–70
3,4-DDP	6.0 ± 1.0	4.4–7.7	19 ± 8**	6–42
DHA precursor	687 ± 351[b]	337–1303	1925 ± 1106[b]**	308–4829
DHA	15,400 ± 4019[b]	6809–23,967	12,538 ± 7183[b]	2737–26,680
2,3-DKG	1741 ± 674[c]	567–2669	411 ± 238[c]**	145–937

[a] $LOD < \times < LOQ$.
[b] The sum of DHA-Q and DHA precursor-Q account for approximately 38% of the true DHA content.
[c] Values adjusted as described under "Disussion."

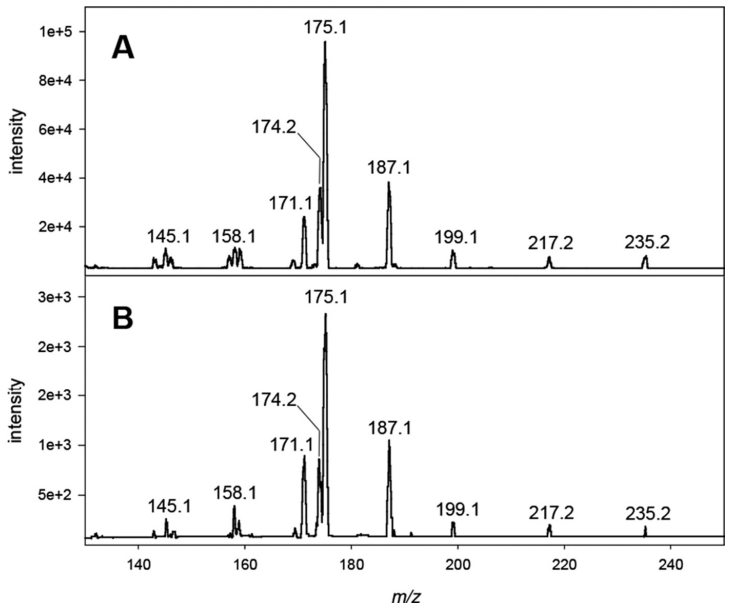

FIGURE 1. **Verification of 1-DG by CID of *m/z* 235.2 $[M + H]^+$.** *A*, authentic reference; *B*, plasma workup.

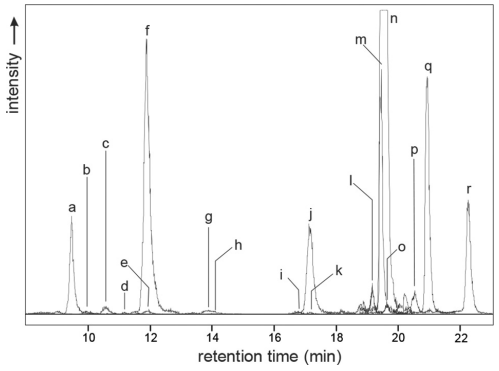

FIGURE 2. **LC-MS/MS chromatogram of a plasma sample after standard workup procedure.** For clarity, only the quantifier mass transition for each analyte of the scheduled multiple-reaction monitoring is shown. For *letter* assignments (*a–r*), see Table 1.

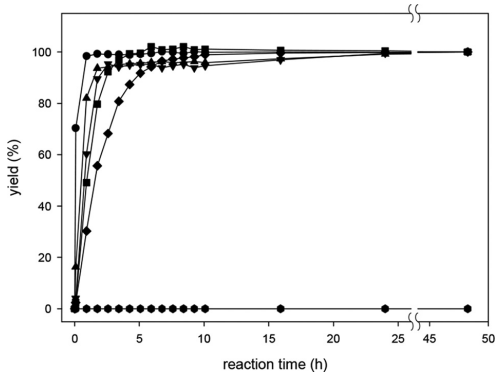

FIGURE 3. **Reactions of selected dicarbonyl compounds with OPD in formate buffer (0.4 M, pH 3.0) at room temperature: 3-DG (♦), MGO (●), GO (■), pyruvic acid (▼), glyoxylic acid (▲), and oxalic acid (*hexagons*).**

dard assay conditions, and the formation of respective quinoxalines was monitored between 0.5 and 48 h. The results presented in Fig. 3 revealed complete reaction (±5%) within 11 h, with the short-chained substances reacting much more rapidly, which is in accordance with our previous findings (36). The exception was oxalic acid, which showed no reaction with OPD. Furthermore, the stability of all quinoxaline compounds included in our method was monitored with authentic references under the derivatization conditions for 24 h. No degradation was observed.

In addition, the derivatization of a plasma sample was monitored by repeated injection of aliquots and LC-MS/MS analyses of the complete α-DC spectrum. Besides the risk of underestimation of α-DCs in trace amounts, avoiding overestimation of certain dicarbonyl quinoxalines is an even bigger challenge. For example, *de novo* formation of glucosone-Q and GO-Q during derivatization with OPD are described in the literature (22, 36, 45). The main reasons are the strong oxidative conditions imposed on the system by the addition of OPD in combination with oxygen and trace amounts of metal ions. However, with our derivatization procedure, a stable plateau for all analytes was reached within the relevant incubation time, indicating no or negligible *de novo* formation in the plasma matrix. In this regard, it is important to note that the incubation took place under argon atmosphere to minimize the impact of oxygen. Furthermore, EDTA as a metal ion-chelating agent was present in the assay.

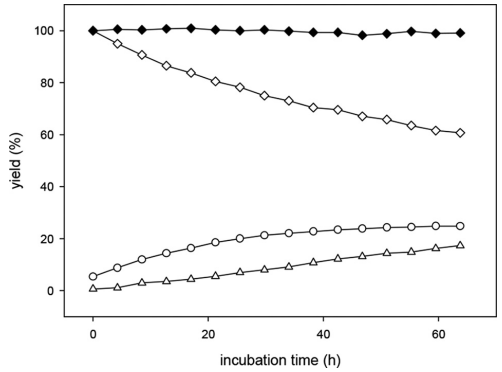

FIGURE 4. **Degradation of 3-DG-Q at pH 0.6 (*open symbols*) and pH 3.0 (*closed symbols*): 3-DG (♦), 3-DGal (●), and 3,4-DGE (▲).**

The plasma samples were diluted in formate buffer (pH 3.0). McLellan and Thornalley (31) applied different derivatization procedures to biological systems and discovered significant interferences from G3P and DHAP by spontaneous elimination of phosphate to form MGO during sample processing, depending on the chosen pH value. The rate of reaction increased with increasing pH. To avoid the degradation of G3P and DHAP, we conducted the derivatization step under strong acidic conditions with perchloric acid. However, under these harsh conditions, we discovered significant degradation of 3-DG-Q, 1-DP-Q, 3-DT-Q, and threosone-Q (data not shown). Especially 3-DG-Q with a half-life of 80 h was rapidly degraded to form the diastereomer 3-DGal-Q (Fig. 4). Mittelmaier *et al.* (46) already investigated the formation of 3-DGal in peritoneal dialysis fluids under sterilization conditions. They postulated a reaction mechanism that includes a reversible dehydration of free 3-DG leading to 3,4-dideoxyglucosone-3-ene (3,4-DGE). Subsequently, 3,4-DGE undergoes hydration to form 3-DGal. Obviously, this type of reaction also takes place with the corresponding quinoxalines under harsh acidic assay conditions. Whereas with the native α-DC, the carbonyl moiety is the driving force, with the quinoxaline, it is the extension of conjugated unsaturation.

We monitored the degradation of 3-DG-Q in sodium formate solution at different pH values (data not shown). Based on our findings, we chose more gentle conditions and employed a formate buffer (0.4 M, pH 3.0). Here, no degradation of 3-DG-Q at 21 °C for 48 h was observed. In addition, formation of MGO caused by oxidative degradation of nucleic acids and related compounds during the derivatization process is accelerated by strong acids like perchloric acid but negligible with milder acidic treatment (47, 48).

To ensure reliable MGO quantitation with the method described herein, we conducted control experiments at different pH conditions with 100 and 200 μM concentrations of G3P and DHAP, respectively (Table 4). The chosen concentrations were based on the experiments conducted by McLellan and Thornalley (31). The conversion rate at pH 3.0 after 24 h in mol % was below 2% for G3P and below 8% for DHAP, independent from the starting concentration of both. The minor interferences found were inevitable, and the results were comparable with the well established method of McLellan. To ensure the absence of contaminants in water or chemicals used in the assay possibly leading to overestimation, a reactant blank was incubated along with the plasma samples.

TABLE 4

Conversion rate in mol % of G3P and DHAP, respectively, to MGO over an incubation period of 24 h in the indicated medium in presence of 0.55 mM OPD and 3.4 mM EDTA

	G3P		DHAP	
	100 μM	200 μM	100 μM	200 μM
	mol %		*mol %*	
pH <0; 0.4 M perchloric acid	21.3	21.2	8.5	8.3
pH 3.0; 0.4 M sodium formate buffer	1.4	1.5	7.7	7.6
pH 7.0; 0.4 M potassium phosphate buffer	18.3	19.8	96.6	98.6

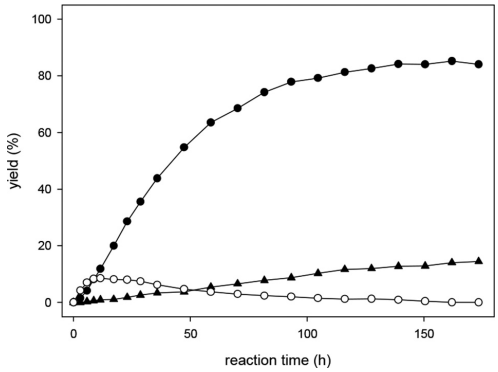

FIGURE 5. **Reaction of DHA with OPD in formate buffer (0.4 M, pH 3.0) at room temperature yields DHA precursor-Q (○), DHA-Q (●), and 2,3-DKG-Q (▲).**

Mechanistic Relationship of DHA, 2,3-DKG, and Their Corresponding Quinoxalines under Assay Conditions—Investigation of the derivatization reaction of DHA in sodium formate buffer (0.4 M, pH 3.0) with OPD (0.55 mM) gave rather surprising results shown in Fig. 5 and in the scheme in Fig. 6. Initially, a precursor compound (DHA precursor-Q) with two molecules of OPD was formed. The precursor then converted to DHA-Q. However, after 24 h, no plateau was reached, and the precursor compound was still detectable in significant amounts. A quantitative reaction of DHA with OPD was only achieved after more than 6 days, which is not feasible for the present analytical assessment. In addition, oxidative conditions imposed by OPD lead to formation of DHA from ASA. When 20 μM ASA was incubated under assay conditions, 50 mol % of the ASA was converted to DHA-Q after 6 days, whereas after 24 h, *de novo* formation of DHA-Q from ASA was negligible (<2%).

In contrast, conversion of 2,3-DKG to its corresponding quinoxaline is completed after 8 h (data not shown). Obviously, DHA is the least reactive of all α-DCs assessed (with the exception of oxalic acid). Consequently, it is important to note that the plasma levels of DHA-Q and DHA precursor-Q given in Table 3 have to be added and account for ~38% of the true DHA plasma content.

To monitor the conversion of DHA precursor-Q to DHA-Q, the precursor was synthesized independently. The authentic

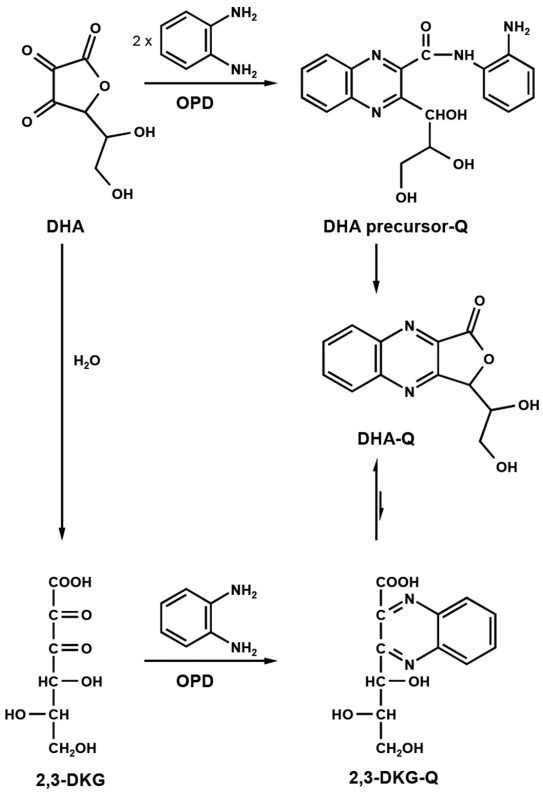

FIGURE 6. **Mechanistic relationship of DHA, 2,3-DKG, and their corresponding derivatization products.**

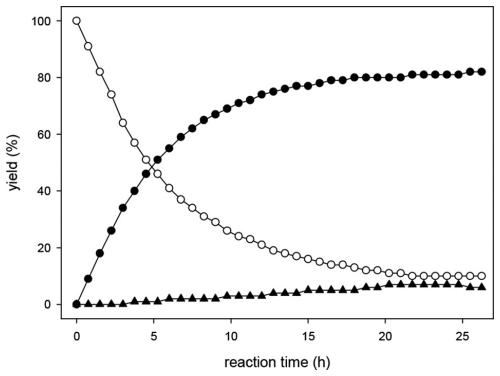

FIGURE 7. **Formation of DHA-Q (●) and 2,3-DKG-Q (▲) starting from DHA precursor-Q (○) under standard workup conditions.**

reference DHA precursor-Q was then incubated under assay conditions (Fig. 7). A half-life of 5 h was observed. After 24 h, 81% of DHA precursor-Q was converted to DHA-Q. Residual amounts of DHA precursor-Q (10% based on initial concentration) remained and had to be considered for the calculation of DHA concentration. Interestingly, also small amounts of 2,3-DKG-Q were detected (7%). A reverse reaction of the quinoxalines to form free DHA with subsequent hydrolytic opening of the lactone ring system to yield 2,3-DKG and its corresponding quinoxaline 2,3-DKG-Q can be excluded. Thus, DHA-Q has to be hydrolyzed slowly to form 2,3-DKG-Q directly. To further investigate the relationship of the two quinoxalines, 2,3-DKG-Q was incubated under assay conditions, including the protein precipitation step with TFA after 24 h. The results in Fig. 8 show conversion of 2,3-DKG-Q to DHA-Q, which was significantly triggered at the lower pH during the 1-h precipitation step. 2,3-DKG-Q yields ~25% DHA-Q after the complete workup procedure. Obviously, there is a steady state between DHA-Q and 2,3-DKG-Q with preferred DHA-Q formation as the more stable product under acidic conditions.

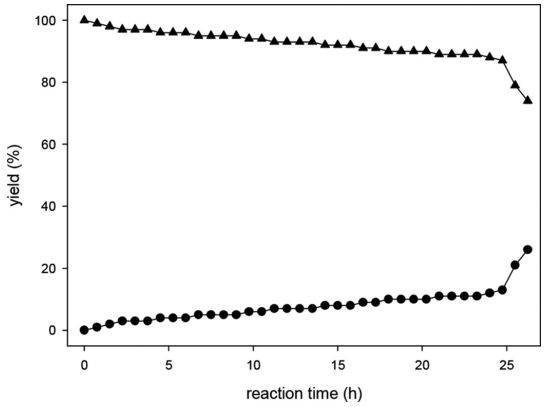

FIGURE 8. **Degradation of 2,3-DKG-Q (▲) under standard workup conditions yields DHA-Q (●).**

Validation of the Proposed Method—The limit of detection and limit of quantitation with all steps of the analytical method included were calculated as described under "Experimental Procedures." The limit of detection differs from 0.5 to 42.2 pmol/liter. The accuracy of the method was determined as the recovery rate at three different concentration levels and ranged between 82 and 120%. Repeatability was expressed as the coefficient of variation and was 10% or better. The complete validation data are shown in Table 5.

DISCUSSION

Up to now, only GO, MGO, 3-DG, and DHA were quantitated in human plasma using established methods, although the spectrum of α-DCs *in vivo* is supposed to be far more complex. Major endogenous sources are the degradation of blood glucose and ascorbic acid. As a result, the corresponding degradation products already identified in model experiments should also be present *in vivo*. Beyond that, lipid peroxidation as well as enzymatic and non-enzymatic pathways irrespective of Maillard reactions contribute to the content of α-DCs. It is speculated that even exogenous sources like foods add to the plasma level of α-DCs.

Because derivatization with OPD is prone to both underestimation of α-DC levels and *de novo* formation during the derivatization procedure, recovery levels for each compound

TABLE 5
Coefficient of variation (*CV*), recovery rates, limit of detection (*LOD*), and limit of quantitation (*LOQ*) (all steps of the analysis included) of plasma samples

Quinoxaline	*CV*[a]	Recovery[b]	*LOD*[b]	*LOQ*[b]
	%	%	*pmol/ml*	*pmol/ml*
Glucosone	6	109	6.1	18.3
1-DG	4	112	2.2	6.6
3-DG	5	97	5.4	16.2
Lederer's glucosone	—[c]	114	3.5	10.5
Pentosone	7	98	8.3	24.9
1-DP	9	82	2.5	7.5
3-DP	8	93	3.5	10.5
Threosone	3	90	4.2	12.6
1-DT	3	91	0.5	1.5
3-DT	3	98	2.0	6.0
MGO	8	106	3.0	9.0
GO	9	98	6.9	20.7
Pyruvic acid	2	99	14.1	42.3
Glyoxylic acid	4	92	42.2	126.6
Oxalic acid	7	89	6.8	20.4
3,4-DDP	3	110	0.8	2.4
DHA precursor	—[d]	—[d]	2.9	8.7
DHA	10	91	22.9	68.7
2,3-DKG	6	119	6.1	18.3

[a] Repeatability conditions, $n = 6$.
[b] Replicate analyses, $n = 3$.
[c] Estimation not possible because analyte was below the limit of detection.
[d] Estimation not possible because compound is the intermediate in DHA-Q formation.

were thoroughly evaluated, and conversion conditions were kept strictly constant. A blank was prepared for each derivatization set to monitor the quality of all incorporated reagents. The derivatization period was adjusted to provide quantitative conversion of all α-DCs except DHA and oxalic acid, which will be discussed below. The sample deproteinization step by the addition of TFA was kept as short as possible. Immediately after protein separation, the pH was adjusted to pH 3.0 again. Under these conditions, even critical quinoxaline structures like 3-DG-Q proved to be stable, and oxidative degradation of nucleic acids and related compounds as well as degradation of G3P and DHAP, which will lead to overestimation of MGO, were avoided.

α-DCs are known to exhibit a high reactivity toward N-terminal amino acids and amino acid residues of proteins, especially to the sulfhydryl group of cysteine, the guanidino group of arginine, and the ϵ-amino group of lysine. Because these amino functionalities are readily available in excess in human blood plasma, we reason that nearly all α-DCs are bound reversibly or irreversibly to these residues. In the case of MGO, *in vitro* experiments of Lo *et al.* (49) showed that only 1% exists in a free form. The reversibly bound MGO is in a dynamic equilibrium with its free form and can be measured by trapping with OPD (50, 51). Therefore, our approach included the separation of the high molecular plasma fraction only after the derivatization procedure to cover the total amount of free and reversibly bound α-DCs in a reliable way. Although, the definition of "reversibly bound α-DCs" remains the object of critical discussion (48) because some reversible reactions under assay conditions might be instead irreversible under physiological conditions, the good validation results proved the reliability of the newly developed workup and derivatization procedure.

The formation of MGO in physiological systems was studied extensively in the literature, reviewed recently by Rabbani and Thornalley (16). The enzymatic and spontaneous degradation of the triose phosphates G3P and DHAP are known sources for MGO. Oxidation of acetone in the catabolism of ketone bodies, oxidation of aminoacetone in the catabolism of threonine, degradation of proteins glycated by glucose, and degradation of ASA also contribute to the total plasma content of MGO. GO is formed by DNA oxidation, lipid peroxidation (52), sugar autoxidation (53), and oxidative degradation of glycated proteins (11). GO and MGO are both detoxified mainly by the glyoxalase system with glutathione as a cofactor to give glycolate and lactate, respectively (55). Because C_2- and C_3-fragments originate by various non-enzymatic and enzymatic pathways besides the breakdown of glucose and ASA, they must be evaluated as very vague parameters from the mechanistic point of view. The same applies for pyruvic acid as a central intermediate in several metabolic processes, which explains its high concentration in plasma.

The C_6-dicarbonyl compound glucosone and its analogues 1-DG and 3-DG do not arise from ascorbate degradation and therefore are markers for glucose-derived α-DCs in the context of Maillard chemistry (see scheme in Fig. 9). 3-DG is formed non-oxidatively from the Amadori product of glucose via 1,2-enolization and dehydration, whereas 2,3-enolization yields 1-DG (56). Oxidation of the Amadori compound leads to glucosone (37). Besides from the Maillard reaction, an important endogenous route leading to 3-DG formation from glucose is the enzymatic polyol pathway (57). Although the bioconversion of glucose into glucosone by pyranose oxidase for synthetic purposes is described in the literature (58), no such enzymatic pathway has been identified *in vivo* so far.

3-DG is reviewed in the literature as the most abundant C_6-dicarbonyl *in vivo* (59) but possesses only a very limited glycating reactivity (37). Thus, the chemistry of 3-DG has to be considered as of minor relevance regarding Maillard processes under physiological conditions. This is supported by the fact that 3-DG-derived AGEs (*e.g.* N^{δ}-[5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl]ornithine (3-DG-H1), 6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-l-norleucine (pyrraline), and N^{ϵ}-{2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-5-[(2*S*,3*R*)-2,3,4-trihydroxybutyl]-3,5-dihydro-4H-imidazol-4-ylidene}-l-lysinate (DODIC or DOGDIC)) are only of minor quantitative importance in blood plasma. 1-DG and glucosone were identified as the central intermediates leading to C_4- and C_5-fragments, respectively (60). Their reductone structure with an α-oxo-enediol moiety boasts significantly higher reactivity. More generally, this applies to all analog C_4- and C_5-dicarbonyls. As reported before, the half-life of 1-DG is about 0.5 h under physiological conditions (61) *versus* 8 h for glucosone (37) and 40 h for 3-DG (36). Hence, 1-DG is by far the most reactive and thus important α-DC intermediate regarding glucose-derived AGEs. In particular, amine-induced β-cleavage in the presence of lysine leads directly to carboxylic acid amides, a novel class of amide-AGEs (14, 62) that are of quantitative importance *in vivo* (9).

Lederer's glucosone was not detected in the plasma of healthy subjects, although it is a relative stable non-reductone structure like 3-DG. However, unlike 3-DG, the required enolization along the entire carbon backbone makes this α-DC susceptible to multiple degradation processes. The existence of

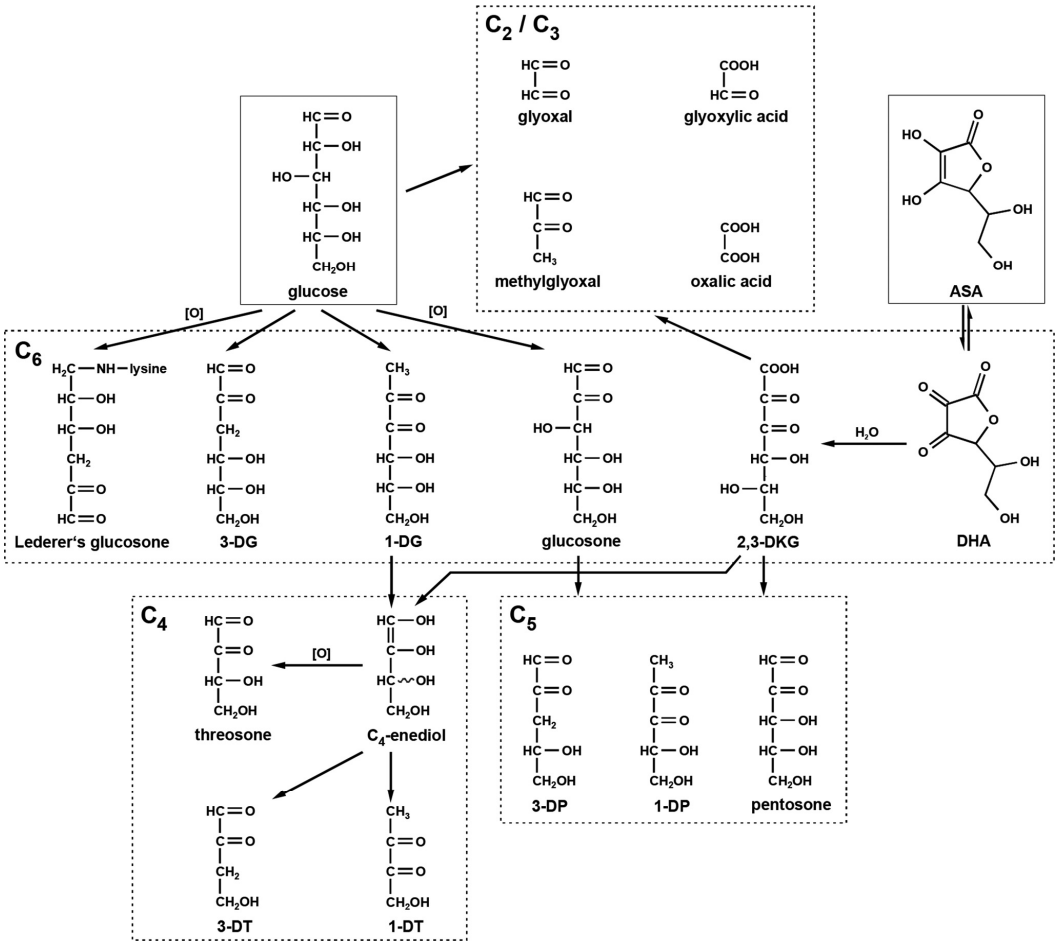

FIGURE 9. **Formation pathways of α-DCs from glucose and ascorbic acid.**

Lederer's glucosone *in vivo* is evident from the detection of its AGE follow-up structure glucosepane at low levels in human blood and extracellular matrix (63, 64).

DHA is a C_6-dicarbonyl structure exclusively assigned to the degradation of ASA, formed by oxidation. As mentioned above, the DHA level in plasma can only be roughly estimated by the analytical assay described herein, mainly because of the incomplete conversion to its corresponding quinoxaline in the given time period. However, DHA in aqueous solution hydrolyzes irreversibly to 2,3-DKG, which is the central intermediate of ASA degradation (65). Therefore, it is important to differentiate between DHA as part of the redox equilibrium ASA-DHA, mediated *in vivo* by enzymatic pathways, and 2,3-DKG as the direct precursor of fragmentation products with a carbon backbone smaller than C_6.

The yields of 2,3-DKG-Q from DHA in Fig. 5 and from DHA precursor-Q in Fig. 7 after 24 h were almost equal, which certainly excludes *de novo* formation of 2,3-DKG via hydrolyzation of DHA. In addition, we confirmed that ASA and DHA did not yield any C_4- or C_5-dicarbonyl degradation compounds during the incubation period. Indeed, the plasma level of 2,3-DKG can be estimated fairly accurately, taking into account the comprehensively investigated relationship of DHA-Q and 2,3-DKG-Q under assay conditions. Based on our results, 6.5% of DHA-Q was converted to 2,3-DKG-Q during the derivatization time of 24 h (Figs. 7 and 8). On the other hand, 25% of the formed 2,3-DKG-Q was converted to DHA-Q. The values given in Table 3 for 2,3-DKG are already adjusted accordingly. The adjustments described above are only valid if DHA-Q is in significant excess, which is given by the situation in blood plasma. Only in this case, the change in DHA-Q level due to the conversion is negligible compared with that of 2,3-DKG-Q.

α-DCs with a carbon skeleton smaller than C_6 arise from the degradation of glucose as well as of ASA. As established in previous papers of our group (39, 66), the C_4-dicarbonyls threosone, 1-DT, and 3-DT are formed from both 1-DG and

2,3-DKG via β-dicarbonyl cleavage with an C_4-enediol as the reactive intermediate. Oxidation of the latter leads to threosone, whereas dehydration at C3 results in 3-DT. The enediol may also undergo isomerization to give 1-DT in an equivalent reaction. As a consequence under deaeration, which represents the situation *in vivo*, 3-DT was the prominent structure because the reductone 1-DT has to be considered a much more reactive and, thus, short lived intermediate.

It has to be noted that differentiation between l-*threo*-pentos-2-ulose (arising from ASA and often referred to as xylosone) and the C4-stereoisomer D-*erythro*-pentos-2-ulose (arising from glucose) was not possible. For the applied LC-MS/MS method, MRM parameters were determined with L-*threo*-pentos-2-ulose. In model incubations of glucose and lysine, a signal with retention time and mass transitions identical to those of L-*threo*-pentos-2-ulose was detected (data not shown). By definition, with glucose as precursor, the detected compound has to be D-*erythro*-pentos-2-ulose. Thus, both structures coelute and are therefore summarized under the term pentosone regardless of its origin. Yet, the plasma levels of pentosone were comparatively high and did not fit into the picture. Decarboxylation is a well established mechanism of ASA degradation and leads to C_5-compounds, including pentosone (65, 67), but under physiological conditions, the formation of C_4-dicarbonyls from 2,3-DKG is favored (25, 39). Glucose-derived pentosone stems from glucosone by the same mechanism of hydrolytic β-cleavage as threosone from 1-DG as the precursor. After isomerization and hydration, formic acid is cleaved off and gives an C_5-1,2-enediol as the reactive intermediate. Oxidation leads to pentosone, whereas water elimination yields 3-DP or, after 2,3-enolization, 1-DP (60). Considering the need for an oxidation step in order to obtain pentosone from both 2,3-DKG or glucosone, formation of 1-DP and 3-DP should be favored. The results therefore strongly suggest an additional alternative source for pentosone formation *in vivo*.

In Maillard chemistry, glyoxylic acid is assigned to disaccharide chemistry (38) but can also arise from oxidation of glyoxal (68) and degradation of DHA (65, 69). However, this cannot account solely for the plasma levels, which were 2.5-fold higher than GO and in the same range as 2,3-DKG. An alternative source is the degradation of hydroxyproline with the subsequent glyoxylate metabolism in the human organism, which is rather complex, involving several enzymatic and non-enzymatic reactions, and has been subject to recent investigation (54).

Oxalic acid can originate via β-dicarbonyl fragmentation as well as via oxidative α-DC cleavage from 2,3-DKG and is the main degradation product of the latter (39). In addition, oxalate is also part of the glyoxylate metabolism mentioned above. However, under assay conditions, the dicarboxylic acid oxalic acid is not converted to its corresponding quinoxaline. This is expected, because at the chosen workup pH, the carboxylic acid groups do not show sufficient carbonyl activity to react with OPD. Consequently, there must be alternative precursors for oxalic acid-Q formation to oxalic acid itself. 3,4-DDP is a known intermediate of maltose degradation but was found in neither the glucose nor ASA reaction systems (38). Hence, the origin of the detected 3,4-DDP-Q remains unknown.

The presented LC-MS/MS method provides for the first time the opportunity to identify and quantitate the complete spectrum of relevant α-DCs in plasma of healthy human subjects in a single chromatographic run. For 14 compounds, the plasma levels were determined. Three compounds were below the limit of quantitation but were unequivocally identified. 10 substances have not been reported for human plasma samples before. To evaluate the clinical relevance of the assay described herein, an initial set of 24 uremic patients undergoing hemodialysis was analyzed. Uremia is related to an increase in oxidative and carbonyl stress and thus should lead to a clear shift in the dicarbonyl spectrum. Indeed, most α-DCs were considerably higher in HD patients. Glucose-derived glucosone exhibited a 2-fold increase, which is expected under conditions of elevated oxidative stress. In contrast, 1-DG does not require an oxidation step for its formation and remained nearly at the level of healthy subjects. Interestingly, plasma levels of 2,3-DKG were considerably decreased in HD patients. This must be explained by a significantly accelerated degradation via oxidative pathways. GO and MGO are further compounds of published interest in regard to certain chronic diseases like uremia. A 3-fold elevation of GO and a 4-fold increase of MGO were observed in uremic plasma. This is in line with the literature (27, 29, 32), although the absolute values differ significantly, depending on the respective study. The assessment of α-DC plasma levels depends strongly on the analytical approach, specifically on workup conditions, derivatization procedure, and chromatographic method. Most importantly, the derivatization was conducted in the presence of protein to assess both free and reversibly bound α-DCs. Therefore, a direct comparison of the plasma levels of the present study and those reported previously is not possible.

Nevertheless, validation of the present method for the detection of α-DC compounds in plasma has been carried out extensively regarding the formation of artifacts and mechanistic relationships to exclude false quantitative data. In general, the elevated levels of α-DCs found explain the elevated levels of AGEs in uremia. The newly developed method has now to be extended to follow-up studies with patients with various complications. The results for a wide range of highly reactive carbonyl intermediates will help us to understand the complex mechanisms and factors that influence α-DC formation and consequently open new perspectives regarding the formation and relevance of AGE chemistry *in vivo*.

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10 Appendix

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Analysis and Chemistry of Novel Protein Oxidation Markers in Vivo

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ABSTRACT: Proteins continually undergo spontaneous oxidation reactions, which lead to changes in structure and function. The quantitative assessment of protein oxidation adducts provides information on the level of exposure to reactive precursor compounds with a high oxidizing potential and reactive oxygen species (ROS). In the present work, we introduce N^6-(2-hydroxyethyl)lysine as a novel marker based on the ratio of glycolaldehyde and its oxidized form glyoxal. The high analytical potential was proven with a first set of patients undergoing hemodialysis versus healthy controls, in comparison with well-established parameters for oxidative stress. In vitro experiments with N^1-*t*-BOC-lysine and N^1-*t*-BOC-arginine enlightened the mechanistic relationship of glycolaldehyde and glyoxal. Oxidation was strongly dependent on the catalytic action of the ε-amino moiety of lysine. Investigations on the formation of N^6-carboxymethyl lysine revealed glycolaldehyde-imine as the more reactive precursor, even though an additional oxidative step is required. As a result, a novel and very effective alternative mechanism was unraveled.

KEYWORDS: *carbohydrate chemistry, oxidative stress, Maillard reaction, glyoxal, glycolaldehyde, carboxymethyl lysine, hydroxyethyl lysine, high-performance liquid chromatography, mass spectrometry, plasma, hemodialysis*

INTRODUCTION

Glycation is an unavoidable process of post-translational protein modification, relevant in aging and complications of certain diseases. A complex series of reactions, collectively referred to as the "Maillard reaction", leads to a variety of stable reaction products (advanced glycation end products, AGEs).

AGE formation in vivo is promoted by a general increase in oxidative stress, on the one hand, and the availability of reactive precursor compounds derived from both oxidative and nonoxidative chemistry (carbonyl stress), on the other hand.[1] We already investigated one of the major sources of carbonyl stress, the generation and accumulation of reactive α-dicarbonyl compounds with special attention on uremia compared to healthy human subjects.[2] In the present study, we focused on oxidative stress, which is often considered as tissue damage resulting from an imbalance between an excessive generation of highly reactive prooxidative compounds and insufficient antioxidant defense mechanisms.[3] If chronically present, e.g., in pathological situations, such as uremia, oxidative stress can lead to protein oxidation, entailing complications like atherosclerosis and β_2-microglobulin amyloidosis.[4,5] This is generally due to a combination of inflammatory processes, a comprised metabolism by the uremic milieu, a significantly impaired glomerular filtration rate, and furthermore to an abnormal nutrition.[6] Especially the latter is of importance because a controlled diet can not only have preventive effects but may also reduce adverse long-term effects. Dietary antioxidative capacity in foods is thus an area of intense research. Nutritional antioxidative compounds are vitamin C and E, polyphenols and phenolic acid derivatives, carotenoids, and certain trace elements.[7] Here, one must differentiate between the effect in foods and the health benefits in vivo, as described by Yang et al.[8] Blocking oxidative reactions prolongs not only the shelf lives of food products but also might suppress formation of potentially noxious substances. For example, oxidized tyrosine or AGEs like CML in the diet are likely to induce an oxidative stress response in the living organism.[9−11] Other major considerations in this respect are bioavailability, synergistic effects with other nutritional components or endogenous enzyme systems, and dose dependence.[12−14] Indeed, at higher doses many antioxidants exert a pro-oxidative effect which is referred to as "the antioxidant paradox".[15]

Thus, developing novel therapeutic approaches to reduce protein oxidative stress, i.e., by dietary interventions or administration of antioxidants, is a promising task. A necessary prerequisite in such studies is the accurate and reliable quantitation of the stress status in vivo. Here, oxidized biomolecules, e.g., proteins, are often used as targets. A term frequently used in protein oxidation research is "advanced oxidation protein products (AOPPs)" expressed as the ability of proteins to oxidize iodide to iodine.[16] As a major drawback of this approach, the molecular nature of AOPPs is not further specified, and potential mechanistic backgrounds remain unclear. A promising but analytically much more complex alternative is the detection of specific well-defined oxidation marker compounds.

Especially, methionine residues exposed on protein surfaces are readily oxidized to methionine sulfoxide (MetSO) by many reactive species. MetSO reductase in tissues serves as a recovery system and maintains the balance in this redox system. The process is also thought to act as a protective mechanism for other redox active sites on proteins and to preserve functionality.[17,18]

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3-Nitrotyrosine (3-NT) is a marker of nitration damage to proteins. The nitrating species are formed enzymatically via peroxidases or in a direct reaction between nitric oxide and superoxide, which yields peroxynitrite.[19]

o-Tyrosine, the isomer of the natural occurring *p*-tyrosine, is formed via oxidation of protein-bound phenylalanine residues by hydroxyl radicals. Hydroxyl radicals can also induce the cross-linking of tyrosine residues. The resulting oxidation product *o,o′*-dityrosine is alternatively produced from the enzymatic reaction of free or protein-bound tyrosine with tyrosyl radicals generated by myeloperoxidase or other heme enzymes from superoxide and *p*-tyrosine.[20] Myeloperoxidase-catalyzed oxidative pathways in renal failure were further investigated by Himmelfarb et al.[21] and Gozdzikiewicz et al.[22]

N^6-Carboxymethyl lysine (CML) is a widely studied AGE, derived from the reaction of glyoxal (GO) with the ϵ-amino moiety of lysine but also from the oxidative cleavage of the Amadori product of the latter with reducing sugars.[23,24]

5-(4,5-Dihydroxy-2-imino-1-imidazolidinyl)norvaline, the early product of the reaction of GO with arginine residues, is slowly degraded to N^5-[[(carboxymethyl)amino]-(imino)-methyl]ornithine (N^5-carboxymethyl arginine, CMA), the corresponding arginine-derived AGE to CML. Both GO-arginine adducts are converted to 5-(2-imino-5-oxo-1-imidazolidinyl)norvaline (imidazolinone) under conditions of acid protein hydrolysis.[25]

As a novel sensitive marker for oxidative stress, we introduce N^6-(2-hydroxyethyl)lysine (HEL). The potency of HEL is based on the redox system formed by glycolaldehyde (GA) and GO. The mechanistic background of HEL is discussed in detail and compared to the established parameters of the oxidative protein damage in vivo mentioned above to evaluate the suitability of HEL as a probe of plasma protein oxidation.

MATERIALS AND METHODS

Materials. Chemicals of the highest grade available were obtained from Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Nidderau, Germany) unless otherwise indicated. *o,o′*-Dityrosine,[26] 5-(2-imino-5-oxo-1-imidazolidinyl)norvaline (imidazolinone, G-H3),[25] N^6-(2-hydroxyethyl) lysine (HEL), and N^6-carboxymethyl lysine (CML)[23] were synthesized according to the literature. The identity of the target compounds was verified by nuclear magnetic resonance (NMR) experiments. Furthermore, the elemental composition was confirmed by accurate mass determination.

Plasma Samples. Written informed consent was obtained from all of the patients. The study was approved by the Ethics Committee of the Medical Faculty of the Martin-Luther-University Halle-Wittenberg. Blood samples were obtained from 11 healthy subjects with normal renal function and 40 nondiabetic patients undergoing hemodialysis (HD patients). The samples were anticoagulated with ethylenediaminetetraacetate (EDTA, 2 mg/mL of whole blood). In HD patients, samples were obtained predialysis before the midweek treatment session. Hemodialysis was performed three times weekly for 4−5 h using polysulfone dialyzers. All of the patients were treated with bicarbonate hemodialysis (acid concentrate type 257, sodium bicarbonate 8.4% type 200, MTN Neubrandenburg GmbH, Neubrandenburg, Germany) with ultrapure water quality (by reverse osmosis and sterile filters). Plasma was obtained by centrifugation (3000 × *g*, 10 min, 4 °C) within 20 min of collection, and an aliquot of 500 μL was immediately incubated with sodium borodeuteride (6.7 M in 0.01 M sodium hydroxide solution, 1000 μL) for 5 h on ice. After the addition of ultrapure water (5 mL), the protein content was precipitated (50% trichloracetic acid, 3275 μL), and the precipitate was washed two times with diluted trichloracetic acid solution (5%, 4000 μL), lyophilized, and treated with hydrochloric acid (6 M, 6 mL, degassed with helium for 10 min) in an argon atmosphere for complete protein hydrolysis (20 h at 110 °C). The lyophilized residue was dissolved in 600 μL of hydrochloric acid (0.05 mM). The filtrate (0.45 μm, cellulose acetate) was diluted on a scale of 1:5 with hydrochloric acid (0.05 mM) and administered to liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) analysis and the ninhydrin assay.

HbA1c, creatinine, and C-reactive protein (CRP) were measured by routine methods at the central laboratory of Martin-Luther-University Clinical Center, Halle (Saale).

Ninhydrin Assay. The appropriately diluted plasma workup samples (100 μL) were incubated for 60 min at 80 °C with 100 μL of ninhydrin reagent solution (2.0 mg of ninhydrin, 0.3 mg of hydrindantin, 74 μL of ethylene glycol monomethyl ether, 26 μL of 4 M acetate buffer, pH 5.5). After being cooled to room temperature, the absorbance of the samples and standard solutions (0.5−10 nmol leucine) was determined at 546 nm with a microplate reader (Tecan Infinite M200) using 96-well plates. Each sample was prepared at least three times.

1,4-Diethylpyrazinium Diquaternary Salt. The diquat was synthesized according to the method of Curphey and Prasad.[27] Briefly, a suspension of triethyloxonium tetrafluoroborate (50 mmol) in dichlormethane (20 mL, anhydrous) was stirred under argon, while a solution of pyrazine (20 mmol) in dichlormethane (10 mL, anhydrous) was added dropwise (5 min). The reaction mixture was heated under reflux for 40 min. After being cooled to room temperature, the precipitated pale green-colored compound was obtained by centrifugation. Recrystallization from acetonitrile (anhydrous) by the addition of 2 volumes of dichlormethane yielded a white solid (13 mmol, 65%). NMR data were in line with the literature.[27]

Degradation of 1,4-Diethylpyrazinium Diquaternary Salt in the Presence of Water. The diquat (3 mg) was dissolved in acetonitrile (500 μL, anhydrous), and water (2 μL) was added. The solution was immediately dried under argon and administered to the gas chromatography coupled mass spectrometry (GC-MS) procedure described below.

Gas Chromatography with Coupled Mass Spectrometry (GC-MS). Adopting the method described by Glomb and Tschirnich,[28] the dried sample was dissolved in pyridine (100 μL), and *N,O*-bis(trimethylsilyl)acetamide with 5% trimethylchlorosilane (100 μL) was added. Samples were kept 30 min at 80 °C to yield the corresponding trimethylsilyl derivatives.

Samples were analyzed on a Trace GC Ultra coupled to a Trace DSQ (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The GC column was a DB-5 (30 m × 0.32 mm, film thickness 0.25 μm; Agilent Technologies, Palo Alto, CA, USA). The injector was 220 °C, the split ratio was 1:20, and the transfer line was 250 °C. The oven temperature program was as follows: 60 °C (1 min), 2 °C/min to 110, and 50 °C/min to 270 °C (10 min). Helium 5.0 was used as the carrier gas in the constant flow mode (linear velocity, 28 cm/s; flow, 1 mL/min). Mass spectra were obtained with electron ionization (EI) at 70 eV (source temperature: 210 °C) in full scan mode (mass range m/z 50−350). Identification was achieved by comparison of the retention time and the fragmentation pattern in the EI mass spectrum with the authentic reference compound.

Incubation of N^1-*t*-BOC-Lysine and N^1-*t*-BOC-Arginine with Glyoxal and Glycolaldehyde. Incubations were conducted in 0.2 M phosphate buffer, pH 7.4, after sterile filtration in a shaker incubator (New Brunswick Scientific, Nürtingen, Germany) at 37 °C. The reactant concentrations are mentioned in the legends of the corresponding figures and tables. Deaeration and inhibition of metal-catalyzed oxidation chemistry were achieved by using a phosphate buffer containing 1 mM diethylenetriamine pentaacetic acid (DTPA) and gassing with argon. The buffer was degassed with helium for 10 min and stored under argon prior to sample preparation. At various time points, aliquots of the reaction mixtures were reduced for 1 h at room temperature with a 5-fold excess of sodium borodeuteride in relation to the carbonyl compound used. Addition of 6 M hydrochloric acid to a final concentration of 3 M hydrochloric acid was followed by incubation at 110 °C (arginine) or room temperature (lysine) for 1 h.

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Table 1. LC-MS/MS Parameters for Quantitation of Target Analytes

		precursor ion		product ion 1[a]			product ion 2[b]			product ion 3[b]		
	ret. time (min)	*m/z* (amu)	DP (V)	*m/z* (amu)	CE (eV)	CXP (V)	*m/z* (amu)	CE (eV)	CXP (V)	*m/z* (amu)	CE (eV)	CXP (V)
MetSO	2.6	165.9	33	74.2	20.0	12.0	56.1	30.0	10.0	102.1	19.0	18.0
CML	2.9	205.1	50	130.2	17.0	9.5	84.1	46.0	13.0	56.1	59.0	8.0
HEL-1D	4.2	192.2	42	84.0	28.0	6.0	130.2	18.0	23.0	56.2	56.0	4.0
HEL-2D	4.2	193.2										
imidazolinone	5.1	215.1	48	70.1	38.0	11.3	100.1	20.0	8.0	116.2	20.0	9.0
o-tyrosine	18.0	182.1	35	136.2	18.0	24.0	91.1	42.0	4.5	119.1	27.0	6.2
3-NT	23.3	227.2	50	181.1	18.0	32.0	117.1	35.0	8.0	168.1	23.0	14.0
o,o′-dityrosine	26.7	361.2	25	315.3	23.5	7.5	254.2	33.0	15.0	237.2	34.0	21.0

[a]MRM transition used for quantitation (quantifier). [b]MRM transition used for confirmation (qualifier).

The acidic solvent was removed in a vacuum concentrator. The residue was resuspended, filtered (0.45 μm, cellulose acetate), and diluted appropriately with 0.05 mM hydrochloric acid prior to liquid chromatography mass spectrometry (LC-MS/MS) analysis.

High-Performance Liquid Chromatography with Coupled Mass Spectrometry Detection (LC-MS/MS). The HPLC apparatus (Jasco, Groß-Umstadt, Germany) consisted of a pump (PU-2080 Plus) with a degasser (LG-2080-02) and quaternary gradient mixer (LG-2080-04), a column oven (Jasco Jetstream II), and an autosampler (AS-2057 Plus). Mass spectrometric detection was conducted on an API 4000 QTrap LC-MS/MS system (AB Sciex, Concord, ON, Canada) equipped with a turbo ionspray source using electrospray ionization (ESI) in positive mode. The chromatographic method and the source parameters for mass spectrometric detection were recently published by Smuda et al.[29] Compound specific orifice potentials and fragment specific collision energies are listed in Table 1. Quantitation was performed using the standard addition method. More precisely, increasing concentrations of authentic reference compounds at factors of 0.5, 1, 2, and 3 times the concentration of the analyte in the sample were added to separate aliquots of the sample after the workup procedure. The aliquots were analyzed, and a regression of response versus concentration was used to determine the concentration of the analyte in the sample. Spikes were run one in approximately every 30 samples. Calibration with this method resolves the potential matrix interferences. All of the plasma workup samples and incubations were analyzed in single batches to exclude interassay variations. Each plasma sample was quantitated in duplicate. The limit of detection (LOD) and limit of quantitation (LOQ) with all steps of the analysis included (Table 2) were estimated according to DIN

Table 2. Limit of Detection LOD and Limit of Quantitation LOQ (All Steps of the Analysis Included) of Plasma Samples

	LOD[a] (μmol/mol leu)	LOQ[a] (μmol/mol leu)
MetSO	1.13	3.39
CML	0.31	0.93
HEL	0.07	0.20
imidazolinone	0.07	0.21
o-tyrosine	0.08	0.24
3-NT	0.04	0.12
o,o′-dityrosine	0.01	0.03

[a]Replicate analyses $n = 6$.

32645 ($n = 6$, confidence level $P = 0.95$, $k = 3$).[30] In model experiments, coefficients of a variation <10% ($n = 3$) for all of the target compounds were achieved.

Statistical Evaluation. Data are expressed as mean ± SD. The Student's *t*-test (modified according to Welch) was used for statistical evaluation of significant differences between both study groups (* $p < 0.05$; ** $p < 0.001$).

RESULTS

Kinetic Studies on the Reaction of N^1-*t*-BOC-Lysine with Glyoxal and Glycolaldehyde. To monitor the reaction of GO and GA in the presence of ε-amino residues of lysine, both were incubated with N^1-*t*-BOC-lysine over a time period of 168 h (pH 7.4, 37 °C). To evaluate the influence of oxidative conditions, reaction batches were performed aerated and deaerated in parallel. At specific time points, samples were reduced with sodium borodeuterate and administered to LC-MS/MS.

In the first step, the free α-dicarbonyl structure GO and the α-hydroxyaldehyde GA reversibly bind to the ε-amino group of N^1-*t*-BOC-lysine to yield the corresponding imines. After reduction with sodium borodeuterate, the stable product HEL is obtained (Figure 1). Specifically, GO-imine reacts to N^6-(2-hydroxy-1,2-*d*-ethyl)lysine (HEL-2D), whereas GA-imine yields N^6-(2-hydroxy-1-*d*-ethyl)lysine (HEL-1D). As a result, it became possible to obtain insights into the redox system GA/GO.

Figure 2 shows the time course of the formation of HEL-2D as a percentage of the total HEL (sum of HEL-1D and HEL-2D). Starting from GO, only HEL-2D was detected after 168 h, whereas GA was oxidized to GO under aerated conditions. After 24 h, a plateau with equal amounts of HEL-2D and HEL-1D was reached. In the absence of oxygen, only negligible amounts of HEL-2D were detected after 168 h. This clearly indicated the need for an oxidative step from GA to GO.

Total HEL concentration reached a maximum at about 3 h. Immediate GA-imine degradation in GA incubations led to a steady state ratio of GA- and GO-imines after 24 h at very low concentration levels. In contrast, GO incubations never showed any GA-imines, and degradation proceeded much slower to give 8-fold higher levels of total HEL, even after 168 h. Remarkably, the presence of oxygen had no influence on the total degradation process in both cases.

Formation of CML, generated by the reaction of GO with the ε-amino group of lysine, is illustrated in Figure 3. With GO as a precursor, no oxidation is required. Therefore, no differences between the aerated and deaerated conditions were observed. In contrast, the reaction of GA to CML depends on an additional oxidation step. Indeed, formation of CML only proceeded under aerated conditions. Surprisingly, the formation rate was considerably higher in the first 24 h of the incubation than with GO and lysine.

Investigation of a Single-Electron Transfer assisted Reaction Pathway with 1,4-Dialkylpyrazinium Diquaternary Salt as Intermediate. GA in the presence of an amine compound is a known precursor of 1,4-dialkylpyrazinium

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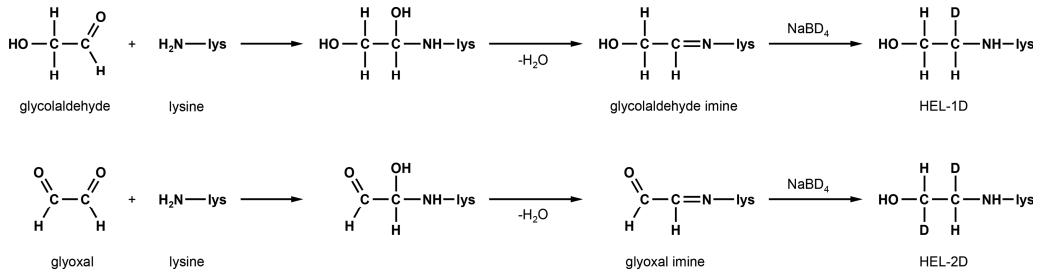

Figure 1. Reaction pathway of glycolaldehyde and glyoxal to form N^6-(2-hydroxy-1-*d*-ethyl)lysine (HEL-1D) and N^6-(2-hydroxy-1,2-*d*-ethyl)lysine (HEL-2D), respectively.

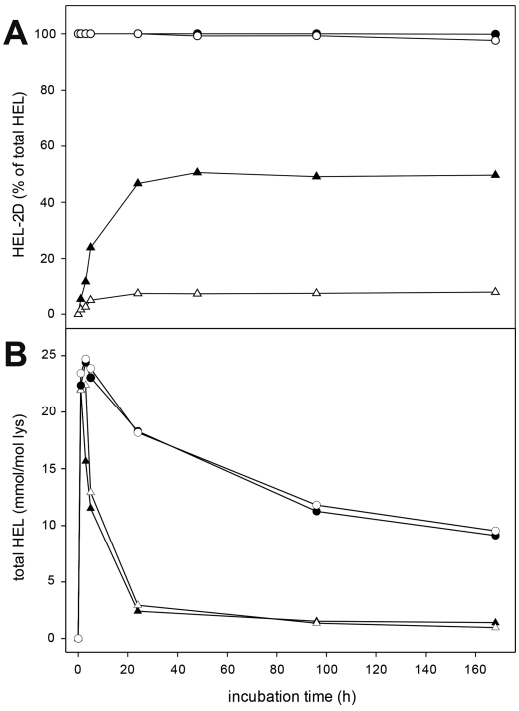

Figure 2. Time dependent formation of N^6-(2-hydroxy-1,2-*d*-ethyl)-lysine (HEL-2D) relative to total content of HEL (A) and the absolute amount of HEL formation (total HEL, sum of HEL-1D and HEL-2D) (B) in incubations of 40 mM N^1-*t*-BOC-lysine with 40 mM glycolaldehyde (▲) and 40 mM glyoxal (●) under aerated (full symbols) and deaerated (open symbols) conditions.

diquaternary salts.[30] Highly unstable in aqueous solutions, we hypothesized the hydration of this early intermediate to yield the 1,4-dialkyl-2-hydroxypyrazinium cation. Hydrolysis then decomposes the pyrazine ring system to give the corresponding *N*-alkyl glycine. If the ε-amino group of lysine acts as the amine in the reaction with GA, the described mechanism might be an alternative route of CML formation. To confirm this hypothesis, we synthesized the diquaternary salt under strictly anhydrous conditions with ethyl amine as the amine compound. The addition of traces of water should produce *N*-ethyl glycine. Indeed, we identified the silylated target

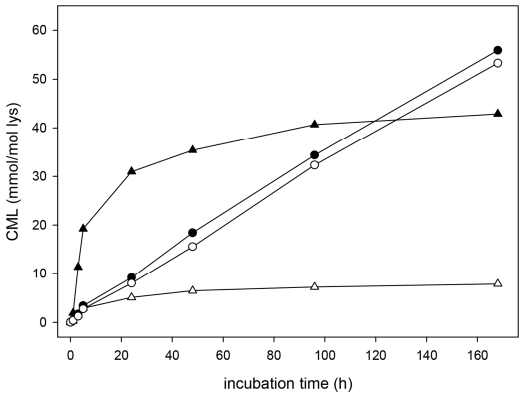

Figure 3. Time-dependent formation of N^6-carboxymethyl lysine in incubations of 40 mM N^1-*t*-BOC-lysine with 40 mM glycolaldehyde (▲) and 40 mM glyoxal (●) under aerated (full symbols) and deaerated (open symbols) conditions.

analyte with GC-MS (Figure 4). The comparison of the EI-spectra at the same retention time of the authentic reference

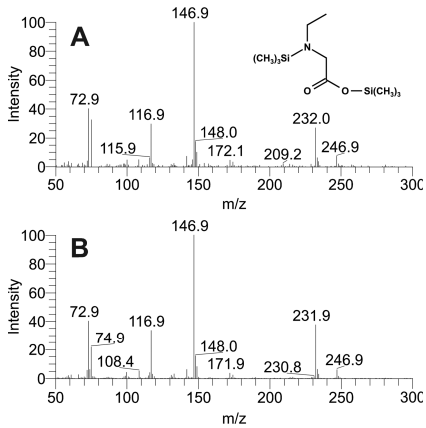

Figure 4. Verification of *N*-ethyl glycine by GC-MS (after silylation, retention time, 27.1 min; *m*/*z*, 247). EI mass spectrum of (A) the authentic reference and (B) 1,4-diethylpyrazinium diquaternary salt incubated in the presence of traces of water.

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with the reaction mixture workup gave the molecular ion peak at m/z 246.9 and the more abundant [M-15] peak, which represents the loss of a methyl group. Other more abundant peaks at m/z 73, 75, or 147 result nonspecifically from the silylation. Thus, both spectra were virtually identical.

Kinetic Studies on the Reaction of N^1-*t*-BOC-Arginine with Glyoxal and Glycolaldehyde. In arginine incubations, the product spectrum of the reaction with GO is more complex than in lysine incubations. From the mechanistic point of view, the early product 5-(4,5-dihydroxy-2-imino-1-imidazolidinyl)-norvaline (DIN) is slowly degraded to N^6-carboxymethyl arginine (CMA).[25] However, under strong acidic conditions, as in protein hydrolysis (6 M HCl, 110 °C, 20 h), both structures are converted to an imidazolinone, also called G-H3. The latter, in turn, is slowly degraded to arginine under the very same conditions. However, if the acidic workup conditions are well controlled as herein, the imidazolinone can be used as a marker of the total extent of GO-arginine modifications.

As depicted in Figure 5, the reaction of GO with the guanidine group of arginine was independent of oxygen,

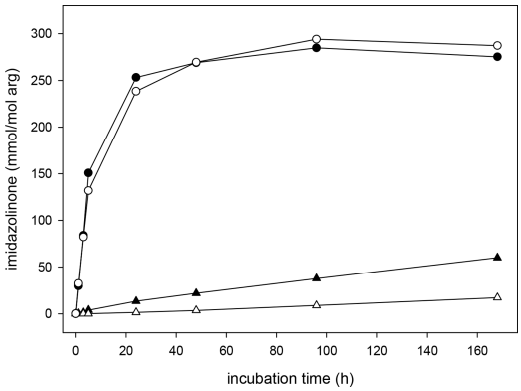

Figure 5. Time-dependent formation of imidazolinone (G-H3, a measure of total GO-arginine modifications) in incubations of 40 mM N^1-*t*-BOC-arginine with 40 mM glycolaldehyde (▲) and 40 mM glyoxal (●) under aerated (full symbols) and deaerated (open symbols) conditions.

whereas GA and arginine under deaerated conditions yielded only negligible amounts of the imidazolinone. In line with literature, this highlights that the products DIN, CMA, and the imidazolinone are specific for the reaction of GO with the guanidine group of arginine, and that starting from GA, an oxidation step to GO is a prerequisite for the progression of the reaction. It has to be noted that the GO reactivity is predominantly arginine-directed as the total amount of GO-arginine modification was 5-fold higher than that of the GO-lysine modification CML.

Markers for Protein Oxidation in Uremic Plasma vs Healthy Controls. Plasma was obtained from 11 healthy subjects (controls) with normal renal function and no proteinuria and from 40 HD patients. Details on the study population are given in Table 3. Normal renal function was defined as a creatinine level below 100 μmol/L, and the absence of inflammatory reactions in controls was ensured by a CRP concentration of 5 μmol/L or below.

Table 3. Profile of Subjects Examined in This Study

	healthy subjects	HD patients
no. of participants	11	40
sex, female/male	2/9	18/25
age (years)	66 ± 6	67 ± 12
HbA1c (mmol/mol)	38 ± 1	40 ± 17
serum creatinine (μmol/L)	81 ± 8	659 ± 284
C-reactive protein (mg/L)	1.7 ± 1.1	12 ± 11

The reduction of plasma samples was performed within 20 min after blood draw to preserve the GA/GO redox status. After protein precipitation and acid hydrolysis samples were subjected to LC-MS/MS. Quantitation with the standard addition method incorporating authentic references guaranteed elimination of matrix interferences. All of the results were normalized to the respective total amino acid content expressed as leucine equivalents (leu). The total amino acid content was determined by a high-throughput ninhydrin assay.

The ratio of HEL-2D/1D, as shown in Figure 6, expressed as a percentage of HEL-2D to the total HEL, clearly shifted to

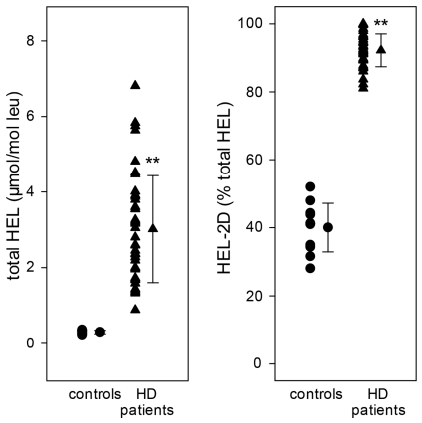

Figure 6. Plasma levels of total N^6-(2-hydroxyethyl)lysine and the percentage of N^6-(2-hydroxy-1,2-*d*-ethyl)lysine of the total from controls and HD patients. Data are expressed as mean ± SD, the significant difference between groups was calculated by Welch's *t*-test (** $p < 0.001$).

GO-imines in HD patients (92 ± 5 vs 40 ± 7% HEL-2D; $p < 0.001$). In addition, the plasma levels of the total HEL content were significantly elevated (3.0 ± 1.4 vs 0.3 ± 0.1 μmol/mol leu).

MetSO, as the most important product in quantitative terms, was significantly higher in HD patients compared to controls (2530 ± 1683 vs 452 ± 35 μmol/mol leu; $p < 0.001$). However, the large deviation range in the patient group was striking. MetSO is part of an enzymatically triggered redox equilibrium, which is believed to serve as a defense mechanism against oxidative protein damage. This implies that levels of MetSO cannot be directly correlated to oxidative stress but to the efficiency of this specific enzyme system in general. Thus, a possible explanation for the individual variations might be the interference of the uremic metabolism with the activity of MetSO reductases. However, all other protein markers formed

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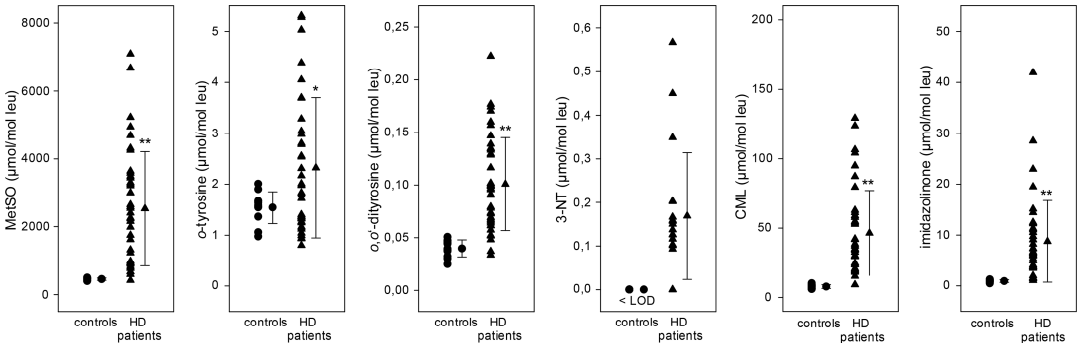

Figure 7. Plasma levels of methionine sulfoxide, *o*-tyrosine, *o,o'*-dityrosine, 3-nitrotyrosine, N^6-carboxymethyl lysine, and imidazolinone from controls and HD patients. Data are expressed as mean ± SD, the significant difference between groups was calculated by Welch's *t*-test (* $p < 0.05$, ** $p < 0.001$).

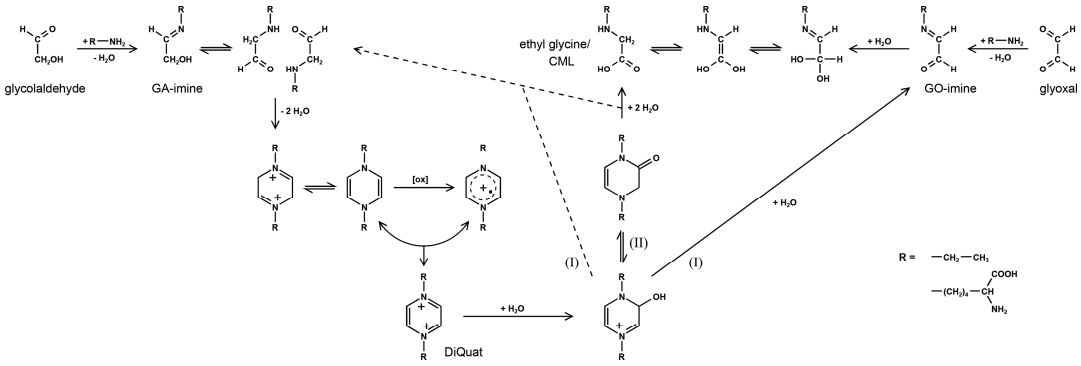

Figure 8. The reaction pathway of glycolaldehyde and glyoxal leading to *N*-ethyl glycine or N^6-carboxymethyl lysine, respectively, depending on the amine-compound used (ethyl amine or lysine).

enzymatically or did not show the same basic distribution pattern (Figure 7).

Mean values of *o*-tyrosine were increased by 50% in HD patients vs controls, but the difference was only of limited significance ($p < 0.05$). *o,o'*-Dityrosine showed a 2.5-fold increase in HD patients (0.10 ± 0.04 vs 0.04 ± 0.01 µmol/mol leu; $p < 0.001$). 3-NT, as a marker for protein nitration, was 0.17 ± 0.14 µmol/mol leu in HD patients but below LOD in controls.

Markers for Maillard-Modified Protein Residues. The lysine derived AGE CML was 6-fold elevated in HD patients (46.4 ± 30.6 vs 7.8 ± 1.3 µmol/mol leu; $p < 0.001$) (Figure 8). GO-imidazolinone, as a cumulative parameter of GO-derived arginine AGEs, was 10-fold elevated in HD patients (8.8 ± 8.0 vs 0.9 ± 0.2 µmol/mol leu; $p < 0.001$). The comparison of CML vs the imidazolinone content gave a reverse picture, as in the above model experiments. In vivo, CML was much more important than the imidazolinone with regard to the quantitative amount.

DISCUSSION

The present study describes a novel marker for oxidative stress and its possible clinical relevance in chronic uremia. The basis of this approach is the amount of protein bound GA in relation to the oxidized counterpart GO. The relevance of GO and GA to modify and to cross-link proteins in the course of the Maillard reaction has been already studied extensively.[31,25,32] However, the exact origin and mechanistic relationship of both structures is still unknown.

To monitor GO- and GA-amino acid adducts in vitro, N^1-*t*-BOC-lysine and N^1-*t*-BOC-arginine were incubated in the presence of GO and GA. In addition, solutions were reduced with sodium borodeuterate. GO-imine and GA-imine yield double deuterized HEL-2D and mono deuterized HEL-1D, respectively. This enabled us to differentiate between GO- and GA-imines.

In contrast to the published data,[33] it was unequivocally shown that GA is oxidized to GO. This redox reaction only proceeded from GA to GO. GO incubations never produced any GA adducts (Figure 2). The oxidation was dependent on the presence of oxygen and, more importantly, on the catalytic action of the ε-amino moiety of lysine. When aerated GO- and GA-arginine incubations were monitored for imidazolinone as a probe for the reaction of GO with the guanidine function of arginine, there was a 5-fold increase in concentration from GA to GO (Figure 5). In contrast, GO- and GA-lysine incubations yielded similar amounts of CML as a probe for GO-lysine modifications after 168 h (Figure 3).

Even though GO- and GA-lysine incubations produced similar quantities of CML in the long term (168 h), the rate of

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formation was very different at very short incubation times (<96 h). Reduction experiments showed clearly that the GA-imine was much more reactive than the GO-imine, resulting in higher concentrations of CML at the beginning of the reaction. Conversion of GO-imines to CML requires a rearrangement step, such as enolization or Cannizarro-type reactions, whereas conversion of GA-imines must involve an additional oxidation step by definition. This suggests that CML is formed via totally different mechanisms in GA versus GO reactions. Hayashi et al. already established a reaction pathway of GA with an amine leading to GO via a 1,4-dialkylpyrazinium radical cation, including an oxidative step, the so-called Namiki pathway.[30] The relevance of this radical intermediate was investigated in-depth by Hofmann et al. with regard to the formation of colored compounds during the Maillard reactions.[33] This group identified GA as the most effective precursor of radical formation, tracked by means of electron spin resonance spectroscopy. Notably, the observed radical formation already reached its maximum after 5 min in incubations of GA and ethyl amine at 95 °C. However, more importantly, they were able to extend the complex reaction cascades of the intermediate pyrazinium radical cation to the generation of diquaternary and hydroxylated pyrazine species.

It has been hypothesized that the 1,4-dialkylpyrazinium radical cation is also a precursor of CML.[23] To verify this hypothesis, we used ethyl amine as a substitute for lysine to simplify the experimental design and incubated 1,4-diethylpyrazine diquaternary salt in the presence of traces of water. Indeed, we were able to unequivocally verify the formation of *N*-ethyl glycine, the ethyl amine pendant of CML. Based on this finding, we propose a novel radical-assisted pathway of CML formation in line with above mechanistic studies, as depicted in Figure 8. After rearrangement to the corresponding α-aminoaldehydes, two molecules condense to yield 1,4-dialkyl-1,4-dihydropyrazine. As shown by Hayashi et al.[30,34] and confirmed by Hofmann et al,.[33] this structure is immediately oxidized to the 1,4-dialkylpyrazinium radical cation. The high potential of these pyrazine species for single electron transfer reactions is further enlightened by a disproportionation to give the corresponding diquaternary salt in a redox cycling equilibrium. The diquat represents an extremely electron deficient compound, which leads to a spontaneous attack of nucleophiles as water to give the 2-hydroxypyrazinium cation already verified in literature.[27,33] This intermediate can now be stabilized via two pathways. (I) Hydrolysis leads to one molecule of GA-imine and one molecule of GO-imine, which is relatively slow after hydration, and the rearrangement results in ethyl glycine/CML. (II) More effectively, the alternative direct enolization should give the corresponding 1,4-dialkyl-3,4-dihydro-2-pyrazinone, which hydrolyzes to release GA-imine and instantaneously ethyl glycine/CML.

The crucial oxidative step in this CML formation cascade was enlightened by the detection of the significant lower amounts of CML in the GA-lysine incubations under deaeration (Figure 3). On the other hand, as depicted in Figure 2, deaeration only led to a slightly delayed degradation of GA-imines in the initial reaction phase up to 3 h, when monitored as the content of the total HEL. Thus, there must be significant additional unknown degradation mechanisms to the CML formation, which do not require oxidative conditions.

In vivo, the situation is far more complex. When the plasma levels of protein-bound MetSO and *o*,*o*′-dityrosine were increased, oxidation products of methionine and tyrosine, respectively, could indeed be explained by increased ROS levels and a decreased antioxidative capacity. However, alternative explanations are an impairment of proteolytic activity, like proteasomes, which preferentially degrade oxidized and misfolded proteins, but also of enzymatic reactions meant to maintain protein integrity. For example, MetSO residues are converted back to methionine by the tissue MetSO reductase system.[35] In contrast, oxidation of tyrosine residues to form *o*,*o*′-dityrosine cross-links was enhanced by the inflammatory myeloperoxidase−superoxide system, as demonstrated by Heinicke et al. in in vitro experiments.[36] This notion is further supported by the fact that products of neutrophil degranulation are elevated in the plasma of chronic HD patients, e.g., Hörl et al. observed the elevation of myeloperoxidase plasma levels by the factor of approximately two during a 3.6 h hemodialysis session.[37]

We detected a 5-fold increase in MetSO plasma levels of HD patients with respect to control subjects. In contrast, Agalou et al. reported only on a 2-fold increase, but with a very limited number of participants, including diabetic subjects as well.[38] In this study, no significant influence of the diabetic state of HD patients was determined. However, in diabetic subjects with normal renal function, the same workgroup detected a 4-fold increase of MetSO compared to nondiabetic controls.[39]

Plasma levels of *o*-tyrosine, which represent another post-translational protein alteration induced by hydroxyl radicals, indicated only a slight increase toward HD patients. Comparing both tyrosine species, concentrations of *o*-tyrosine were 10-fold higher than *o*,*o*′-dityrosine which suggests that the former may stem from other additional sources. Leeuwenburgh et al., who investigated oxidized amino acids in the urine of aging rats, argued the possibility that *o*-tyrosine contained in the diet might be taken up by the intestines and then misincorporated into proteins.[20] Alternative metal-catalyzed pathways with phenylalanine as the precursor appeared to be of minor importance.[40] This line of argumentation can be also found in other studies for different matrices and contexts, like human lens protein,[41] low density lipoprotein isolated from human atherosclerotic plaques,[42] and various tissues of Wistar rats.[43]

The 3-NT residue content of plasma protein as a measure for protein nitration was increased by a factor of at least two in HD patients due to the oxidation of nitric oxide to peroxynitrite, a potent nitrating intermediate.[44] This is in line with the results of Agalou et al.[38] More recently, Ahmed et al. investigated the protein oxidation and nitration biomarkers MetSO, *o*,*o*′-dityrosine, and 3-NT with regard to arthritic disease.[45] The results for the plasma of healthy subjects differ significantly from those published by Agalou; however, a different workup and standardization method was used.

The ratio between GO-imine and GA-imine significantly shifted toward 95/5 in HD patients, while controls gave 40/60, i.e., here, GA-imine was the predominant parameter. Alternative approaches to quantitate GO and GA, e.g., by derivatization of GO and GA with *o*-phenylenediamine[2] and ethoxamine,[33] respectively, share a common problem. The inevitable but different derivatization procedures do not allow a direct quantitative comparison of the individual species and methods. Consequently, in vivo studies on GO levels in plasma of uremic patients gave very different results ranging between 1 and approximately 400 nmol/mL.[2,46,47] On the other hand, to our knowledge, there is no data available regarding plasma concentrations of GA in HD patients so far.

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It has to be noted that also the total HEL content in sodium borodeuterate-reduced plasma protein was significantly elevated in HD patients vs controls. Thus, formation of GA and GO are stimulated in uremia. The only known other source of GA in vivo, besides the Maillard reaction,[23] is the degradation of the amino acid L-serine in the presence of hypochlorous acid formed from superoxide and chloride by myeloperoxidase at sites of inflammation.[48] GO does not only stem from oxidation of GA in Maillard driven systems but alternatively is formed by DNA oxidation, lipid peroxidation,[49] and sugar autoxidation.[50] However, GO can be detoxified by the glyoxalase system with glutathione as a cofactor.[51] Depletion of glutathione under conditions of oxidative stress, therefore, may lead to enhanced GO-levels.

The reactivity of GO toward the guanidino group of arginine is significantly higher than that toward the ϵ-amino group of lysine, which, in vitro, resulted in 5-fold higher levels of GO-imidazolinone as a probe for arginine modifications compared to CML. In vivo, the situation was reversed. This was not surprising since here the alternative pathway, i.e., the oxidative cleavage of the Amadori product of glucose with lysine, is the major source for CML formation.[23,24] CML plasma levels of HD patients were already the subject of several studies.[38,52] An approximately 3-fold elevation in regard to controls was observed, however, again with a very limited number of participants. Here, we found a nearly 6-fold elevation though the variances between individual HD subjects were tremendous. Several outliers at the upper end of the concentration range have to be noticed. Thus, comparison of the median plasma level of HD patients (35.0 μmol/mol leu) with controls gave only a 4-fold increase, which is in line with the literature. Nevertheless, the multifaceted formation mechanisms hamper the use of CML as a specific probe of reactive C2-carbonyl intermediates. GO-imidazolinone, on the contrary, is a specific tool to gain insights into the overall extent of GO-arginine modifications and, consequently, of GO levels in general. Thus, as a confirmation, the 10-fold increase of the total HEL was reflected by a 10-fold increase of imidazolinone.

In conclusion, the relation of HEL-2D to HEL-1D was introduced as a novel potent marker for oxidative stress in human plasma protein. In HD patients, the results were in line with the established parameters but promised significantly enhanced sensitivity. The quantitative importance of GA as a potent glycating agent in human plasma was established for the first time and was in the same range of GO in normal subjects. The importance as a reactive intermediate in the Maillard reaction pathways was further emphasized by a novel and very effective mechanism leading to CML incorporating single-electron transfer reactions.

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Notes

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ABBREVIATIONS USED

ROS, reactive oxygen species; GA, glycolaldehyde; GO, glyoxal; HEL, N^6-(2-hydroxyethyl)lysine; *t*-BOC, *tert*-butoxycarbonyl; CML, N^6-carboxymethyl lysine; AGE, advanced glycation end products; AOPPS, advanced oxidation protein products; MetSO, methionine sulfoxide; 3-NT, 3-nitrotyrosine; CMA, N^5-carboxymethyl arginine; G-H3, imidazolinone, 5-(2-imino-5-oxo-1-imidazolidinyl)norvaline; EDTA, ethylenediaminetetraacetate; HD patients, patients undergoing hemodialysis; CRP, C-reactive protein; DTPA, diethylenetriamine pentaacetic acid; ESI, electron spray ionization; DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; LOD, limit of detection; LOQ, limit of quantitation; EI, electron ionization; SD, standard deviation; DIN, 5-(4,5-dihydroxy-2-imino-1-imidazolidinyl)norvaline

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REVIEW

Pathways of the Maillard reaction under physiological conditions

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Abstract Initially investigated as a color formation process in thermally treated foods, nowadays, the relevance of the Maillard reaction *in vivo* is generally accepted. Many chronic and age-related diseases such as diabetes, uremia, atherosclerosis, cataractogenesis and Alzheimer's disease are associated with Maillard derived advanced glycation endproducts (AGEs) and α-dicarbonyl compounds as their most important precursors in terms of reactivity and abundance. However, the situation *in vivo* is very challenging, because Maillard chemistry is paralleled by enzymatic reactions which can lead to both, increases and decreases in certain AGEs. In addition, mechanistic findings established under the harsh conditions of food processing might not be valid under physiological conditions. The present review critically discusses the relevant α-dicarbonyl compounds as central intermediates of AGE formation *in vivo* with a special focus on fragmentation pathways leading to formation of amide-AGEs.

Keywords Maillard reaction · Carbohydrate chemistry · Advanced glycation endproducts AGE · Fragmentation pathways · β-Dicarbonyl cleavage · Amide-AGEs · Oxidative stress

Introduction

The non-enzymatic chemistry taking place during thermal food processing was studied since Louis-Camille Maillard discovered in 1912 that mixtures of amino acids with sugars lead to intensively brown colored structures and decarboxylation upon heating [1]. Soon it became evident, that the development of color, aroma and taste are closely linked to the complex reaction pathways of the so-called Maillard reaction. The timeline of research in this field was recently summarized by Hellwig and Henle [2]. As the performance of the analytical equipment increased it was discovered that non-enzymatic browning reactions also lead to free and protein-bound Maillard products under physiological conditions. Today these processes, which lead to posttranslational modification are accepted as important pathogenic events in a number of chronic and age-related disorders such as diabetes [3, 4], uremia [5], atherosclerosis [6] and Alzheimer's disease [7].

On a molecular basis, the Maillard reaction results in a plethora of chemical compounds, which are more generally divided into reactive intermediates and stable endproducts (advanced glycation endproducts, AGEs).

α-Dicarbonyl compounds (α-DCs) have to be regarded as the most important central intermediates in the course of amino acid and protein modification. Therefore, knowledge of the *in vivo* concentrations in different matrices is required for the understanding of the mechanistic background of AGE formation and profile. However, the analytical approach to the complete α-DC spectrum is very challenging. Their high reactivity hampers direct analytical assessment and, thus, derivatization to more stable compounds is a prerequisite. Different trapping reagents like *o*-alkyl hydroxylamines, hydrazines, cysteamines, *o*-diaminobenzene derivatives and aminoguanidines are discussed in the literature [8–13]. However, derivatization of α-DCs with *o*-phenylenediamine (OPD) to yield the corresponding quinoxalines has evolved as the most accepted procedure [14]. Furthermore, sample collection and sample preparation were found to have a major impact on the results and have to be strictly controlled [12, 15–17].

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Initial phase

Glucose and ascorbic acid as the major carbohydrate players *in vivo* constitute the beginning of the Maillard reaction cascade. In a first step, glucose reacts to an 1,2-enaminol by nucleophilic attack of the ε-amino group of free or protein bound lysine. Rearrangement leads to 1-amino-1-deoxyfructose (fructoselysine, Amadori product) [18, 19]. Ascorbic acid is oxidized to dehydroascorbic acid (DHA) [20]. When the Maillard reaction proceeds, both structures lead to reactive α-DCs.

The initial state preserves the C6-carbon skeleton but includes carbonyl shifts along the entire carbohydrate backbone (Fig. 1). 3-Deoxyglucosone (3-DG) is formed non-oxidatively via 1,2-enolisation and dehydration of the Amadori product [21]. 2,3-Enolization leads to 1-deoxyglucosone (1-DG) [22] and Lederer's glucosone (N^6-(3,6-dideoxyhexos-2-ulos-6-yl)-L-lysine) originates from the 5,6-enediol [23, 24]. Oxidation of the Amadori product gives rise to glucosone [25, 26]. Ascorbic acid can be recycled by enzymatic pathways from dehydroascorbic acid [27–29]. In physiological solutions, however, part of the dehydroascorbic acid hydrolyzes irreversibly to 2,3-diketogulonic acid (2,3-DKG). These dicarbonyl compounds are direct precursors of certain AGEs, *e.g.* pyrraline and glucosepane, or undergo fragmentation reactions to yield further reactive α-DCs with a carbon backbone smaller than C6. Important breakdown cascades of the carbon backbone relevant for the formation of AGEs are presented in the following sections.

The modern view of the Maillard reaction *in vivo* considers also diverse other physiological sources for α-DCs, irrespective of Maillard chemistry, like metabolism and fat autoxidation. On the one hand, as an example most of the methylglyoxal formed *in vivo* stems from the enzymatic and spontaneous degradation of the triose phosphates glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, and the metabolism of ketone bodies [30], whereas large amounts of glyoxal are formed by lipid peroxidation [31]. On the other hand, both compounds are detoxified by the glyoxalase system [32].

Cyclization

Especially important in view of age-related alterations of long lived proteins are structures that crosslink two amino acid side chains. A major bivalent lysine-arginine crosslink is

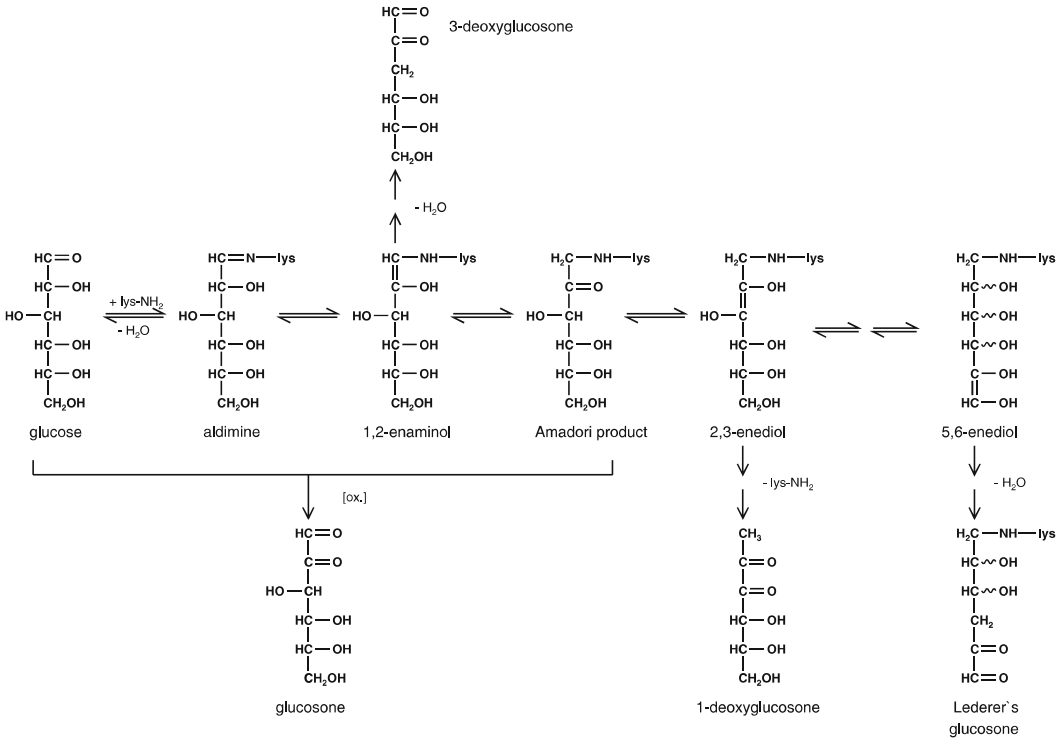

Fig. 1 Initial state stages of amine induced glucose degradation retaining the intact C6-carbon backbone

glucosepane which stems from Lederer's glucosone (Fig. 2). Ring closure and dehydration lead to an intramolecular aldimine which then reacts with the guanidinium group of arginine [33, 34]. As a similar but quantitatively minor crosslink, pentosidine can be explained via the same mechanism with the 4,5-enediol of a pentose as the precursor [33]. Alternatively, fragmentation of C6 α-DCs also yields the required C5 precursor for pentosidine formation. Vesperlysine (LM-1), the analogous bivalent lysine-lysine structure to pentosidine, is supposed to form via totally different pathways with ascorbic acid as the starting compound although the detailed mechanism is still unknown [35–37]. Pyrraline represents a monovalent lysine modification originating from 3-deoxyglucosone. The formation proceeds via nucleophilic attack of the ε-amino group of lysine at the C2-carbonyl moiety, followed by dehydration, cyclization and aromatization (Fig. 3) [38–41].

Isomerization

First discovered by Baynes' group in 1986 [42], N^6-carboxymethyl lysine (CML) is the most important monovalent lysine modification *in vivo*. They proposed the oxidative fragmentation of the Amadori product of glucose to yield CML and erythronic acid. However, a comprehensive examination of the underlying mechanism is still lacking. An alternative route proceeds with lysine and glyoxal as precursors via glyoxal-imine to the hydrate and subsequent isomerization (Fig. 4) [43]. Furthermore, glycolic acid lysine amide (GALA), glyoxal lysine amide (GOLA) and glyoxal lysine dimer (GOLD) can arise from the very same CML reaction cascade [44]. If the nucleophilic addition of water takes place at the imine group, isomerization yields the amide-AGE GALA. A nucleophilic attack of the ε-amino group of a second lysine molecule at the carbonyl group of glyoxal-imine leads to the bivalent lysine-lysine crosslink GOLA. Reaction of the intermediate state with a second molecule of glyoxal, ring closure and water elimination gives the imidazolium crosslink GOLD [45]. Analogous imidazolium salts are methylglyoxal lysine dimer (MOLD) [46, 47] and 3-deoxyglucosone lysine dimer (DOLD) [48] with the corresponding imine structures as precursors. The CML-glyoxal reaction cascade has been successfully translated to methylglyoxal leading to N^6-carboxyethyl lysine (CEL) and N^6-lactoyl lysine. However, here in contrast to CMLs additional Amadori product route, the formation via isomerization is exclusive.

In addition to the ε-amino group of lysine the guanidinium group of arginine is prone to yield various Maillard modifications. Here, only α-DCs are known precursors for monovalent modifications. Our workgroup investigated the mechanistic pathway of the reaction with glyoxal [49]. The intermediate 2-imino-4,5-dihydroxyimidazolidine is slowly converted to N^7-carboxymethyl arginine (CMA) through ring opening of the imidazolidine to the open-chained hemiaminal and intramolecular disproportionation, *i.e.* isomerization. An alternative route, only present under strong acidic conditions, leads to glyoxal imidazolinone 3 (G-H3). Thus, formation of G-H3 is not possible under physiological conditions but occurs as an artifact of sample cleanup. CMA has to be regarded as the only stable arginine modification with glyoxal. Methylglyoxal as a precursor gives a different picture. Here, as illustrated in Fig. 5, the homologous structure N^7-carboxyethyl arginine (CEA) [50, 51] is a minor intermediate of the equilibrium between a kinetically controlled (MG-H3) and a thermodynamically controlled imidazolinone (MG-H1) [52]. In the methylglyoxal reactions the various cyclic imidazolinone and pyrimidine derivatives are

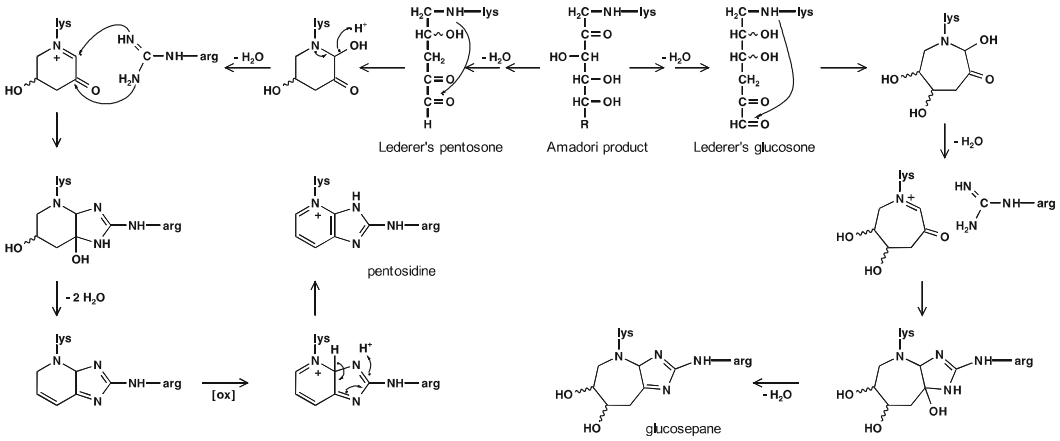

Fig. 2 Cyclisation of Lederer's glucosone (R = −CH_2OH) and Lederer's pentosone (R = −H) lead to bivalent lysine-arginine crosslinks glucosepane and pentosidine, respectively

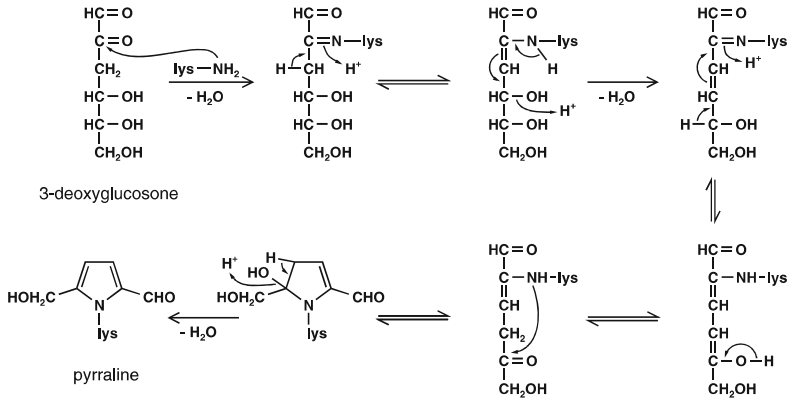

Fig. 3 Reaction pathway for the formation of pyrraline

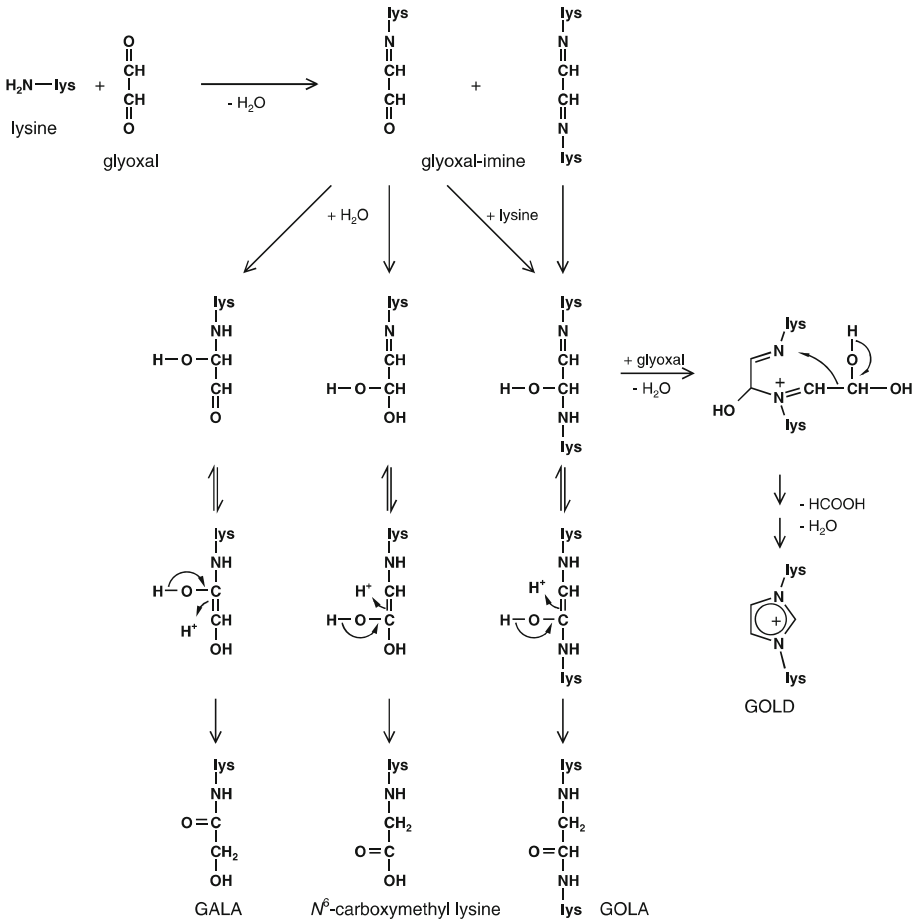

Fig. 4 The N^{ε}-carboxymethyl lysine (CML) reaction cascade explains the formation of glycolic acid lysine amide (GALA), glyoxal lysine amide (GOLA) and glyoxal lysine dimer (GOLD) via isomerization of the hydrated glyoxal-imine

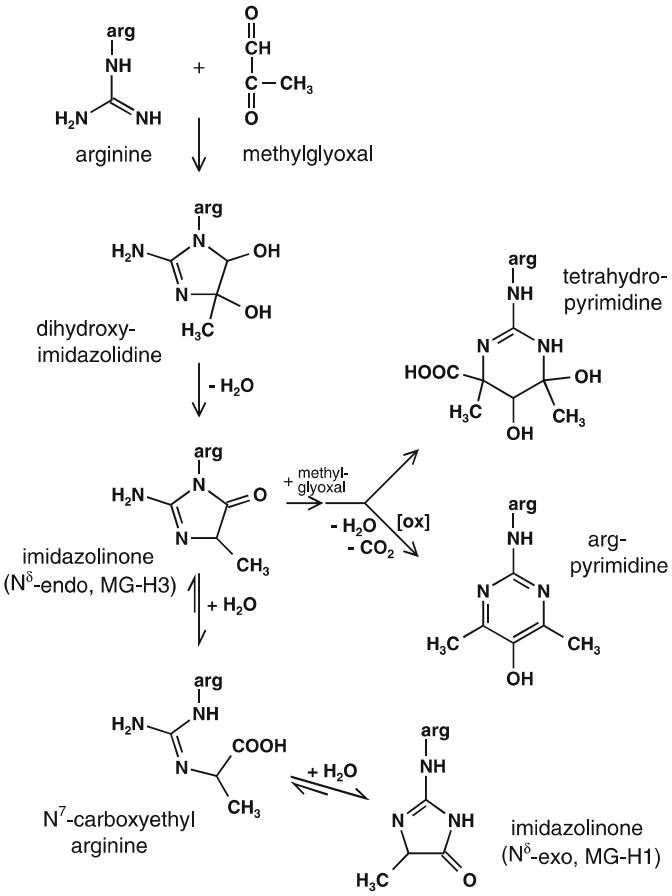

Fig. 5 Formation pathways of methylglyoxal-arginine modifications

of much higher quantitative importance. In general, three constitutional isomers of the imidazolinones originating from glyoxal, methylglyoxal and 3-DG can be formulated. The isomer type 1 refers to the imidazolinone with an exocyclic δ-amino group whereas isomer type 2 and type 3 possess an endocyclic δ-nitrogen atom [53, 54]. In addition, tautomerization of the imino group of MG-H1 or racemization of the chiral center of the imidazolinone ring system of MG-H2 and MG-H3 lead to a multitude of further isomeric structures [52]. A stable and fluorescent endproduct of the reaction of arginine with methylglyoxal, argpyrimidine (AP), was identified by Shipanova *et al.* [55]. The formation proceeds via the same precursor compound as the reaction to CEA with incorporation of a second molecule of methylglyoxal. An alternative route yields a tetrahydropyrimidine (THP) [56].

While the described isomerization reactions maintain the carbon backbone, the following sections discuss possible fragmentation pathways (Fig. 6). Basically, three different mechanisms are described in the literature [57, 58]: retro-aldol cleavage, α-dicarbonyl cleavage and β-dicarbonyl cleavage. This concept has to be re-evaluated in the light of new findings. First, differentiation into a hydrolytic [59] and an oxidative α-dicarbonyl cleavage [60] has to be made. Second, both the hydrolytic β-dicarbonyl and the oxidative α-dicarbonyl cleavage have to be complemented by an amine-catalyzed mechanism [61].

Retro-aldol cleavage

β-Hydroxy carbonyl compounds (aldols) can undergo retro-aldolization by cleavage of the carbon-carbon bond between Cα and Cβ next to the carbonyl group. This mechanism is still the most accepted fragmentation pathway in Maillard reactions for sugars, Amadori and Heyns rearrangement products and α-DCs used to explain formation of shorter-chain hydroxyl

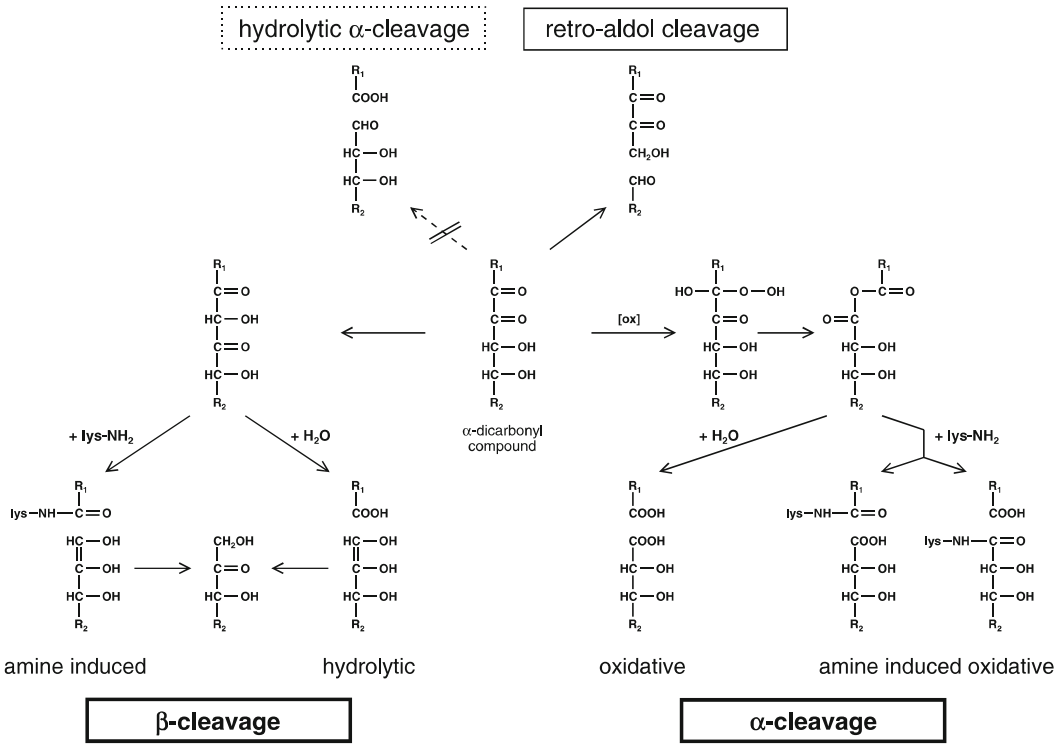

Fig. 6 Fragmentation pathways of α-dicarbonyl compounds. The relevant mechanisms *in vivo* are set in bold fonds

ketones, hydroxyl aldehydes and dicarbonyl compounds [57, 62–64]. However, as stated in a recent review, there is no convincing experimental proof for retro-aldolization in α-DC fragmentation [65]. The relevance of retro-aldol fragmentation under physiological conditions is more than questionable.

Hydrolytic α-cleavage

A second, frequently reported concept of α-DC fragmentation is the hydrolytic α-cleavage [59, 66–68]. Based on an intramolecular disproportionation, the carbon-carbon bond fission reaction takes place between both carbonyl moieties yielding a carboxylic acid and an aldehyde. However, experiments of Davidek *et al.* and others clearly disproved this mechanism and proposed an oxidative α-dicarbonyl cleavage mechanism instead [69].

Oxidative α-cleavage

The oxidative α-cleavage proposed by Davidek *et al.* [69] was further verified by *in vitro* experiments with 1-deoxyglucosone [60]. As expected, acetic acid and the counterpart erythronic acid were detected. More recently, we performed a mechanistic in-depth investigation of the degradation pathways of ascorbic acid [70]. Based on the incorporation of activated molecular oxygen generated by *e.g.* photooxidation processes or hydroperoxide species the mechanism starts with an attack at the C2 or C3 carbonyl moiety of 2,3-DKG (Fig. 7). After rearrangement hydrolysis of the intermediate acid anhydride yields a mixture of oxalic acid and threonic acid. Acylation is an amine driven alternative route where the ε-amino group of lysine initiates a nucleophilic attack on the anhydride to directly yield the amide-AGEs N^6-oxalyl lysine and N^6-threonyl lysine.

Hydrolytic β-cleavage

Hydrolytic β-dicarbonyl cleavage of sugars was already mentioned in 1961 as an alternative fragmentation pathway to retro-aldol reactions [71]. Again, Davidek *et al.* were the first to perform detailed mechanistic studies [72, 73]. The results were confirmed by investigations of our workgroup [74, 75]. We studied the degradation of 1-DG which is isomerized into its 2,4-tautomer. Addition of water leads to a hydrate which

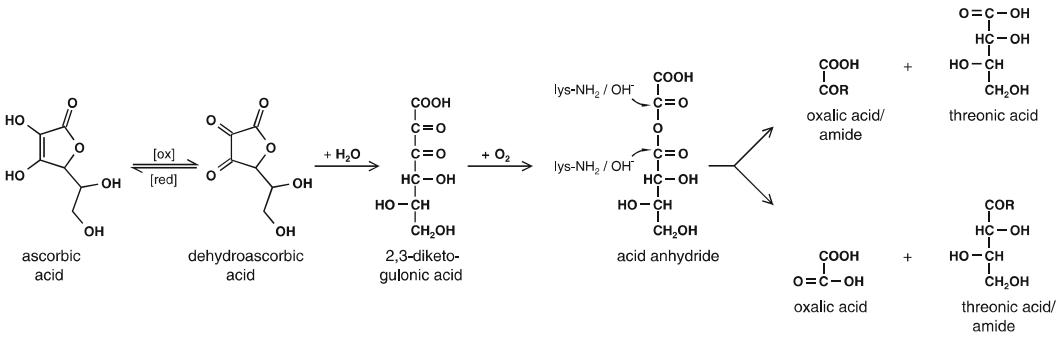

Fig. 7 Oxidative α-dicarbonyl cleavage reaction of 2,3-diketogulonic acid which is the product of ascorbic acid oxidation and hydrolytic ring opening. Hydrolytic attack leads to oxalic acid and threonic acid (R = −OH) whereas lysine induced fragmentation (R = −NH-lys) yields the amide structure and the corresponding acid

induces scission to acetic acid and a C4-enediol intermediate. Isomerization eventually yields tetruloses and tetrosones (Fig. 8). Similarly, 2,3-DKG can fragment to oxalic acid and the corresponding C4-enediol based on the same pathway. Hydration at the C2 carbonyl moiety without prior tautomerization results in decarboxylation as an alternative reaction pathway, which indeed follows the same mechanistic principle. The C5-enediol intermediate can dehydrate to form xylosone or isomerize to yield xylonic acid and lyxonic acid, respectively (Fig. 9) [70].

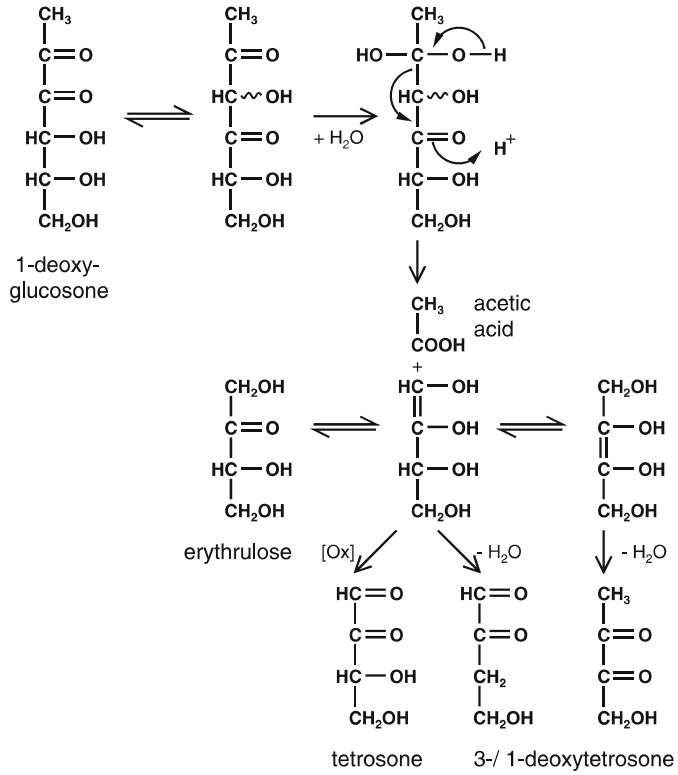

Fig. 8 Hydrolytic β-dicarbonyl cleavage reaction of 1-deoxyglucosone

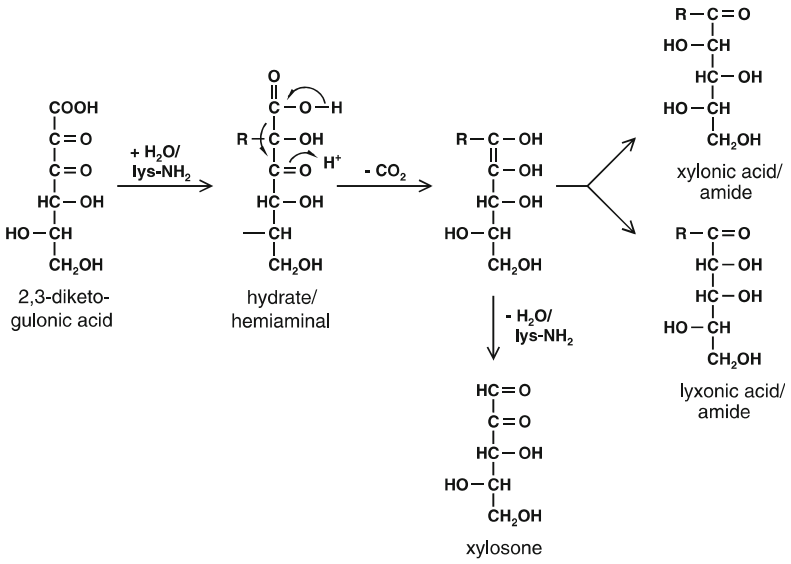

Fig. 9 Hydration (R = −OH) or amination (R = −NH-lys) and subsequent decarboxylation of 2,3-diketogulonic acid leads to the respective C5-enediol or enaminol

Amine induced β-cleavage

Recently, a novel class of AGEs (amide-AGEs) was discovered during the degradation of 1-DG in aqueous solutions under physiological conditions [61] and also *in vivo* [76, 77]. The formation of amide-AGEs was explained via an amine induced β-cleavage mechanism which parallels the above hydrolytic pathways (Fig. 10). Indeed, incubations of α-DCs in the presence of lysine yielded the enediol in form of the respective ketose, the matching carboxylic acid and the corresponding amide structure (*e.g.* N^6-acetyl lysine with 2, 4-deoxyglucosone as the precursor or N^6-oxalyl lysine with 2, 4-diketogulonic acid as the precursor) [61, 78].

Relevance *in vivo*

As already discussed above, the basis for the understanding of the complex Maillard processes *in vivo* is the knowledge of the complete spectrum of α-DCs. However, analytical investigations were often limited to few selected compounds like glyoxal, methylglyoxal and 3-DG [5, 12, 79–85]. A more comprehensive approach including important intermediates from a mechanistic point of view is needed, *e.g.* 1-DG and 2,3-DKG. Initial investigations for a complete set of α-DCs in human plasma and lens were recently published and should be continuously extended [15, 86].

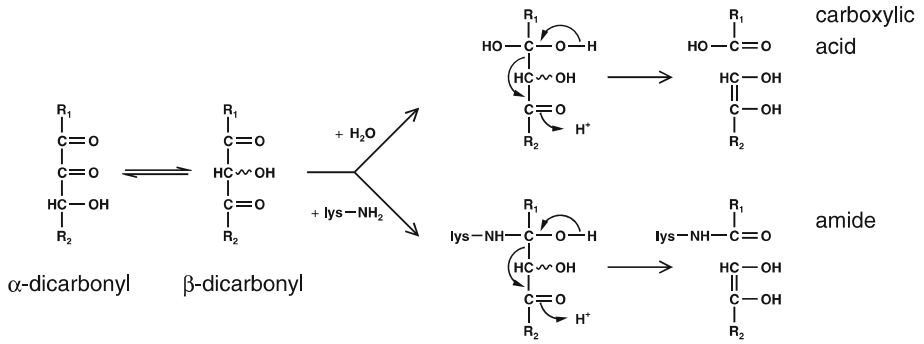

Fig. 10 Hydrolytic β-dicarbonyl cleavage leads to an enediol structure and a carboxylic acid as the counterpart. The analogous amine induced β-dicarbonyl cleavage yields the amide structure as the counterpart of the enediol

The analytical assessment of AGE free adducts (AGE-modified amino acids) in different biological matrices is a difficult task. Even more challenging is the quantitation of protein bound AGEs because hydrolysis of the peptide bonds is a necessity. Total hydrolysis is achieved by strong acidic treatment at high temperatures. However, under these harsh conditions, many of the above described AGEs are not stable. To circumvent this problem to achieve a complete AGE spectrum different protocols of enzymatic protein hydrolyses were developed [44, 77, 87, 88]. It is important in this regard to determine the efficiency of the enzymatic digestion and to reassure the stability of *e.g.* above mentioned amidation under these conditions. Indeed, our multistep enzymatic approach including treatment with Pronase E, leucine aminopeptidase and carboxypeptidase Y showed no degradation of reference amide-AGEs.

Table 1 summarizes mono- and bivalent lysine derived AGEs, monovalent arginine derived AGEs and bivalent lysine-arginine crosslinks which are relevant *in vivo*. For each compound the known mechanism of formation and the analytical detection in different matrices are listed without subdivision into protein bound modifications and free adducts. Obviously, there is no claim of completeness.

CML and the crosslink pentosidine represent the most investigated AGEs. CML was the first AGE discovered [42] and is of significant quantitative importance. This fact and its acid stability allowed the quantitation in many different matrices [89]. Pentosidine represents an acid stable fluorophore and thus enabled very sensitive detection in a variety of human tissues even before the coupling of liquid chromatography with mass spectrometry became a common analytical tool [115–117].

A novel class of important glycation products are amide-AGEs. Their quantitative relevance in lens protein [44, 77] and human plasma [76] was already established. Investigations on the mechanistic reaction pathways *in vivo* often raise the question, if the observed AGEs stem mainly from glucose degradation or if ascorbic acid has to be considered as the dominant precursor. The discovery of ascorbic acid specific amide-AGEs N^6-threonyl lysine and N^6-xylonyl/lyxonyl lysine in *in vitro* studies [70] and their quantitation in human lens [77] were a first attempt to clarify this issue. Their low abundance raises questions about the role of ascorbic acid as the predominant precursor of AGEs in lens proteins.

Another interesting aspect which needs critical reflection is the chemical stability of AGEs *in vivo*. Common lysine modifications like CML, CEL, pyrraline, GOLD, MOLD etc. are thought to be stable under physiological conditions. However, *in vitro* experiments clearly showed that monovalent arginine modifications except CMA and argpyrimidine are only of limited chemical stability with half-lives from one to several weeks, strongly dependent on incubation conditions like pH value, the choosen buffer and buffer strenght [49, 52, 53, 107]. Consequently, they are not likely to show a time-dependent accumulation in long-lived proteins, *e.g.* lens protein, over a time scale of years. However, our findings in normal human lenses contradict this assumption [77]. All target AGEs like CML, CEL, CMA, CEA, MG-H1, MG-H3, THP, glucosepane and most of the amide-AGEs correlated with donor age. Obviously, the formation rate of arginine modifications with limited stability has to be higher than their degradation rate. On the other hand, for lysine-amide-AGEs exclusively N^6-acetyl lysine and the C4 lysine amides (N^6-threonyl and N^6-erythronyl lysine) showed no age-related accumulation. This did not reflect the *in vitro* situation, where all amide-AGEs proved to be stable under physiological conditions. Indeed, formation of N^6-acetyl lysine is known to be paralleled by non-Maillard reactions [118] but alternative mechanisms for C4 lysine amide synthesis/degradation *in vivo* are still unclear.

Markers for protein oxidation

It is a matter of common knowledge, that AGE formation *in vivo* is promoted by a general increase in oxidative stress associated with many chronic diseases. This is not surprising, considering that important degradation pathways in Maillard chemistry like the oxidative α-dicarbonyl cleavage or the formation of dehydroascorbic acid require oxygen. Consequently, to evaluate the stress status *in vivo*, one promising approach is the quantitation of specific advanced protein oxidation products. To use AGEs like CML which originate from oxidative reaction pathways as analytical probes is therefore an obvious idea. However, most AGEs or precursors thereof can stem from various sources. Therefore, direct oxidation products of protein side chains independent of Maillard chemistry are often discussed as markers for oxidative stress. Methionine sulfoxide is the oxidation product of methionine residues [111, 119, 120]. *o*-Tyrosine, the isomer of the natural occurring *p*-tyrosine, is formed via attack of hydroxyl radicals at phenylalanine [121]. 3-Nitrotyrosine [122] and *o*,*o*'-dityrosine [121] are the oxidation products of tyrosine residues. In this concept we introduced a new marker based on the redox-equilibrium formed by protein bound glycolaldehyde and glyoxal [123]. Glyoxal represents the oxidized form of glycolaldehyde. The respective imines were stabilized as N^6-hydroxyethyl lysine derivatives by reduction with sodium borodeuteride. Recently, the potential of this approach was proven in a clinical trial with uremic patients versus healthy controls. Uremia is characterized by a state of inflammatory stress resulting from increased oxidation of carbohydrates and lipids [124–131]. Indeed, a clear shift towards the factor of 17 of the ratio of glyoxal derived N^6-hydroxyethyl lysine to the glycolaldehyde derived derivative was observed [submitted to JBC]. In healthy human subjects glyoxal and glycolaldehyde are present in approximately equal amounts.

Table 1 Advanced glycation endproducts (AGEs) relevant *in vivo*

AGE compound	Mechanism of formation	Quantitation *in vivo*
Monovalent lysine modifications		
N^6-carboxymethyl lysine (CML) [a]	isomerization, unknown ox. fragmentation	various tissues, plasma, blood cells, urine, faecis, lens, reviewed in [89, 90]
N^6-carboxyethyl lysine (CEL) [a]	isomerization	plasma [76, 91–93]; plasma, blood cells, urine [54, 94]; lens [77, 95]
glycolic acid lysine amide (GALA) [b]	isomerization	lens [77]
N^6-formyl lysine [b]	amine induced β-cleavage	plasma [76], lens [77]
N^6-acetyl lysine [b]	amine induced β-cleavage	plasma [76], lens [77]
N^6-oxalyl lysine [b]	ox. α-cleavage, amine induced β-cleavage	lens [77]
N^6-lactoyl lysine [b]	amine induced β-cleavage	plasma [76]; lens [77]
N^6-glycerinyl lysine [b]	amine induced β-cleavage	plasma [76]
N^6-threonyl lysine [b]	ox. α-cleavage	lens [77]
N^6-xylonyl lysine [b]	amine induced β-cleavage	lens [77]
N^6-lyxonyl lysine [b]	amine induced β-cleavage	lens [77]
pyrraline [b]	cyclisation	plasma [96, 97], serum [98]; urine [99–101]; lens [77, 102] various tissue [97]
Bivalent lysine modifications		
glyoxal lysine amide (GOLA) [b]	isomerization	lens [44, 77]
glyoxal lysine dimer (GOLD) [a]	isomerization	serum [98]; lens [103]; serum, lens [104]
methylglyoxal lysine dimer (MOLD) [a]	isomerization	serum [47, 98]; plasma, blood cells, urine [54, 94]; lens [103] ; serum, lens [104]
vesperlysine (LM-1) [a]	unknown	lens [37]
Monovalent arginine modifications		
N^7-carboxymethyl arginine [b]	isomerization	serum [105], lens [77]
N^7-carboxyethyl arginine [b]	isomerization	lens [77]
methylglyoxal imidazolinone (MG-H1) [b]	isomerization	plasma, blood cells, urine [54, 94]; lens [77, 106]
methylglyoxal imidazolinone (MG-H3) [b]	isomerization	lens [77]
3-deoxyglucosone imidazolinone (3DG-H) [b]	isomerization	plasma, blood cells, urine [54, 94];
argpyrimidine (AP) [a]	isomerization	plasma, blood cells, urine [54, 94]; lens [77, 106]
tetrahydropyrimidine (THP) [b]	isomerization	plasma [91]; lens [77]
Bivalent lysine-arginine modifications		
glucosepane [b]	cyclisation	lens [77]; serum, lens [107]; collagen [108]
pentosidine [a]	cyclisation	plasma [92, 109]; serum [98]; urine [99, 100]; urine, serum [110]; plasma, blood cells, urine [54, 94]; collagen [111–113]; lens [106, 114]

[a] acid stable; [b] total degradation under strong acidic conditions

Summary

First, it has to be stated, that the conventional retro-aldol fragmentation pathway is not a significant route for carbohydrate fragmentation in Maillard chemistry under physiological conditions. Second, there is no convincing mechanistic basis for the often suggested hydrolytic α-cleavage.

In contrast, the β-dicarbonyl cleavage route must be considered as the major carbohydrate fragmentation pathway in general (Fig. 6). The hydrolytic pathway is paralleled in the presence of amino acids or proteins by an analogous amine catalyzed cleavage mechanism. Indeed, acylation is a well-known regulatory concept *in vivo* [118]. The posttranslational acylation of amino acids in the polypeptide chain neutralizes the positive charge and, thus, changes protein function in various ways [132, 133]. Non-enzymatic acylation as described above parallels enzymatic regulatory mechanisms by affecting the same targets. However, the Maillard derived protein modifications might be irreversible and lead to accumulation and adverse effects on protein characteristics.

A further fragmentation route *in vivo*, which is of great mechanistic importance is based on oxidative α-dicarbonyl scission to yield carboxylic acids and related amine acylation. The quantitative aspects of this route still have to be evaluated.

Abbreviations

AGE advanced glycation endproduct, *α-DC* α-dicarbonyl compound, *OPD* *o*-phenylenediamine, *DHA* dehydroascorbic acid, *3-DG* 3-deoxyglucosone, *1-DG* 1-deoxyglucosone, *2,3-DKG* 2,3-diketogulonic acid, *CML* N^6-carboxymethyl lysine, *GALA* glycolic acid lysine amide, *GOLA* glyoxal lysine amide, *GOLD* glyoxal lysine dimer, *MOLD* methylglyoxal lysine dimer, *DOLD* 3-deoxyglucosone lysine dimer, *CEL* N^6-carboxyethyl lysine, *CMA* N^7-carboxymethyl arginine, *G-H3* glyoxal imidazolinone 3, *CEA* N^7-carboxyethyl lysine, *MG-H3* methylglyoxal imidazolinone 3, *MH-H1* methylglyoxal imidazolinone 1, *AP* argpyrimidine, *THP* tetrahydropyrimidine.

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12 Declaration of originality

Eigenständigkeitserklärung

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