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Publication Info

Published in *Journal of Biological Chemistry*, Volume 263, Issue 18, 1988, pages 8816-8821.
This research was originally published in the Journal of Biological Chemistry. Ahmed MU, Dunn JA, Walla MD, Thorpe SR, Baynes JW. Oxidative Degradation of Glucose Adducts to Protein: Formation of 3-(Ne-Lysino)-Lactic Acid from Model Compounds and Glycated Proteins. *Journal of Biological Chemistry*. 1988; 263:8816-8821. © the American Society for Biochemistry and Molecular Biology.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 263, No. 18, Issue of June 25, pp. 8816-8821, 1988
Printed in U.S.A.
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Oxidative Degradation of Glucose Adducts to Protein

FORMATION OF 3-(N^ϵ-LYSINO)-LACTIC ACID FROM MODEL COMPOUNDS AND GLYCATED PROTEINS*

(Received for publication, February 8, 1988)

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The chemistry of Maillard or browning reactions of glycated proteins is being studied in model systems *in vitro* in order to characterize potential reaction pathways and products in biological systems. In previous work with the Amadori rearrangement product N^α-formyl-N^ϵ-fructoselysine (fFL), an analog of glycated lysine residues in proteins, we showed that fFL was oxidatively cleaved between C-2 and C-3 of the carbohydrate chain to yield N^ϵ-carboxymethyllysine (CML) and D-erythronic acid. We then detected CML in proteins glycated *in vitro*, as well as in human lens proteins and collagen *in vivo* (Ahmed, M. U., Thorpe, S. R., and Baynes, J. W. (1986) *J. Biol. Chem.* 261, 4889–4894). This work provided an explanation for the origin of CML in human urine and evidence for non-browning pathways of the Maillard reaction *in vivo*. In this report we describe the identification of a second set of products resulting from oxidative cleavage of fFL between C-3 and C-4 of the sugar chain, *i.e.* 3-(N^ϵ-lysino)-lactic acid (LL) and D-glyceric acid. The formation of LL from fFL was increased at slightly acid pH, representing about 30% of the yield of CML at pH 6.4, compared with 4% at pH 7.4 in phosphate buffer. By gas chromatography-mass spectroscopy, LL was detected in proteins glycated *in vitro* and then identified as a natural product in human lens proteins and urine. Our results indicate that oxidative degradation of Amadori adducts to proteins occurs *in vivo*, leading to formation and excretion of CML and LL. These non-browning pathways for reaction of Amadori compounds may be physiologically relevant mechanisms for averting potentially damaging consequences of the Maillard reaction.

The Maillard or browning reaction is a complex series of reactions which occur between reducing sugars and amines, leading to the formation of poorly characterized, polymeric, brown, and fluorescent compounds (1–4). Maillard reactions between sugars and proteins under physiological conditions *in vitro* (pH 7.4, 37 °C) may also lead to denaturation, cross-linking, and precipitation of the protein, as well as the formation of protein-bound pigments with a characteristic brown color and fluorescence. The first step in this reaction is the formation of a Schiff base between a reducing sugar and an amino group on the protein, followed by an Amadori rearrangement to yield a relatively stable ketoamine adduct. The early stages of the Maillard reaction, resulting in the formation of the Amadori adduct, are described as glycation or nonenzymatic glycosylation of proteins and are known to occur between glucose and proteins *in vivo* (5–9). The level of glycation of numerous body proteins, such as hemoglobin, albumin, and collagen, also corresponds closely with the mean circulating blood glucose concentration, and glycation of protein is increased in proportion to the degree of hyperglycemia in diabetes. It is widely believed that subsequent browning reactions of glycated proteins *in vivo* may contribute to the denaturation, cross-linking, and insolubilization of tissue proteins and the gradual development of the chronic pathophysiology of diabetes (5–9). In addition, because of the decline in glucose tolerance and decreased rates of protein turnover with age, there is reason to believe that Maillard reactions may also be one mechanism involved in the age-dependent chemical modification of proteins (9).

One of the goals of research in our laboratory has been to study the intermediates and mechanisms of protein browning and cross-linking during the later stages of the Maillard reaction. We recently showed that the compound N^ϵ-carboxymethyllysine (CML)[1] is formed on oxidation of the Amadori compound N^α-formyl-N^ϵ-fructoselysine (fFL), an analog of glycated lysine residues in proteins (Fig. 1, *top*) (10, 11). We also observed that CML was formed following glycation of proteins *in vitro* and was detectable in human lens protein and collagen. Our observations suggested that CML, which had been detected previously in human urine (12), is derived from the oxidative degradation of glycated proteins. Both CML and the split product erythronic acid are inactive in the Maillard reaction, since the carboxylic acid functional groups of CML and erythronic acid are relatively inert compared to the carbonyl functionality in the Amadori adduct. We have now identified the hitherto unknown compound 3-(N^ϵ-lysino)-lactic acid (LL) as a second product of oxidation of fFL *in vitro* (Fig. 1, *bottom*). In this report we describe the characterization of LL and some factors affecting its formation *in vitro*, and we also report the presence of this compound in human lens proteins and as the free amino acid in human urine.

EXPERIMENTAL PROCEDURES

General Methods—Diethylenetriaminepentaacetic acid (DTPA), Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid), poly-L-lysine-HBr

* This work was supported by National Institutes of Health Research Grant AM-19971 and Research Career Development Award AM-00931 (to J. W. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CML, N^ϵ-carboxymethyllysine; GC/MS, gas chromatography/mass spectrometry; FL, 1-deoxy-1-(N^ϵ-lysino)-D-fructose (fructoselysine); fFL, N^α-formyl-N^ϵ-fructoselysine; LL, 3-(N^ϵ-lysino)-lactic acid; HPLC, high performance liquid chromatography; DTPA, diethylenetriaminepentaacetic acid.

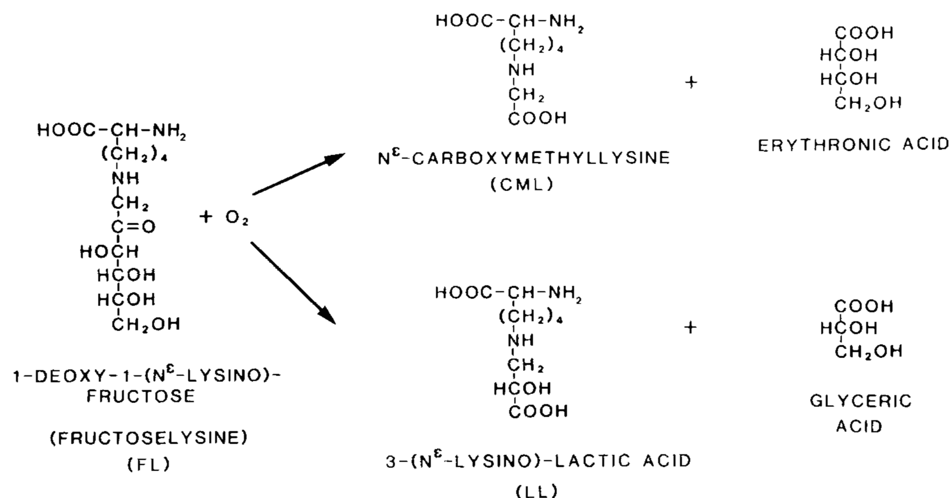

FIG. 1. **Pathways for oxidative degradation of fructoselysine in glycated proteins.** The upper pathway, leading to formation of CML, was characterized in Ref. 11. Evidence for the lower pathway, leading to formation of LL, is presented in this paper.

(M_r = 15,000), and mannitol were purchased from Sigma. N^α-Formyl-N^ϵ-fructoselysine (fFL) was prepared from glucose and N^α-formyllysine, as described previously (11, 13). Incubations of fFL were carried out at 37 °C in 0.2 M sodium phosphate buffer (6). Following hydrolysis in 2 N HCl for 30 min at 95 °C to remove the N^α-formyl blocking group, products were detected by cation-exchange chromatography on an HPLC amino acid analyzer (Waters), using *o*-phthaldialdehyde as post-column reactant (11, 14). For quantitative analyses, the color factors for FL, CML, and LL were assumed to be identical. The HPLC system was also used, without post-column reaction, for semi-preparative isolation of reaction products. Compounds were desalted on Dowex 50-X8, using 0.5 N NH_4OH as eluent, and then dried on a rotary evaporator.

Derivatization for Gas Chromatography/Mass Spectrometry (GC/MS)—Direct probe and GC/MS analyses of acetylated methyl ester and trimethylsilyl derivatives were performed on a Finnigan Model 4521C GC/MS system equipped with a 0.2-mm × 25-m Hewlett-Packard HP-5 column (cross-linked and bonded 95% dimethyl, 5% diphenylpolysiloxane, equivalent to SE-54). *N,O*-Trifluoroacetyl methyl ester derivatives were analyzed using a similar column (J & W Scientific DB-5) on a Hewlett-Packard Model 5890A GC interfaced to a Model 5970 mass selective detector. Injection port temperature was 250 °C, oven temperature was 70 °C for 2 min, and then a ramp to 300 °C at 5 °C/min. Reaction products were esterified by treatment with 1 N methanolic HCl for 30 min at 65 °C. After drying under a stream of nitrogen, acetylation was performed with a mixture of acetone:triethylamine:acetic anhydride (5:2:1) (15) for 5 min at 65 °C. Trimethylsilylation was carried out with a mixture of bis(trimethylsilyl)-trifluoroacetamide:pyridine:trimethylchorosilane (200:50:2), heated for 1.5 h at 95 °C. Trifluoroacetyl methyl esters were prepared by methyl esterification, followed by treatment with neat trifluoroacetic anhydride for 1 h at room temperature. Poorer recoveries of the LL derivative were obtained when derivatization was carried out at higher temperatures.

Preparation of 3-(N^ϵ-Lysino)-lactic acid—Bromopyruvic acid (Aldrich) (20 mM) was reacted with N^α-formyllysine (20 mM) in 0.2 M phosphate buffer, pH 7.5, at room temperature under N_2. An equal volume of a solution of 0.2 M $NaBH_4$ in 0.01 M NaOH was added at 3 h, followed by incubation for an additional 2 h. After acidification to discharge excess $NaBH_4$, the solution was diluted 5-fold, adjusted to pH 7.0, and applied to a 20-ml column of Dowex 1-X8 in the acetate form. The column was washed with water to remove reactants and the product eluted with 2 N acetic acid. The product, formyl-LL, was recovered by rotary evaporation and deformylated. The LL was not rigorously purified but accounted for more than 95% of total *o*-phthaldialdehyde-reactive material detected on the amino acid analyzer and was obtained in approximately 12% yield. The direct probe mass spectrum of the acetylated methyl ester of synthetic LL was identical to that of LL identified in human lens and urine samples, as described below in Fig. 3.

Analysis of Biological Samples—Normal, fasting human urine was obtained from laboratory volunteers. Normal human lenses were the gifts of the South Carolina Lions Eye Bank, Columbia, SC, and the Medical College of Georgia Eye Bank, Augusta, GA. For detection of CML and LL, lenses were homogenized in 1 ml H_2O, dialyzed overnight against H_2O at 4 °C, hydrolyzed in 6 N HCl at 1 mg of protein/ml HCl for 18 h at 95 °C, and then derivatized as the trifluoroacetyl methyl esters, as described above. Urinary creatinine was measured using a creatinine test kit (Sigma No. 555A). Free CML and LL were concentrated by sequential anion- and cation-exchange chromatography prior to derivatization. The urine sample (10 ml) was first adjusted to pH 9 with 1 N NaOH and then applied to a Dowex 1-X8 OH^- column (1 ml of resin/ml urine). The column was washed with 10 column volumes of 5 mM NH_4OH (pH ≈ 9) and then eluted with 5 volumes of 2 N acetic acid. The eluate (containing CML and LL) was concentrated by rotary evaporation, diluted to the original urine volume, adjusted to pH 7 with 1 N NaOH, and then applied to a similar column of Dowex 50-X8 H^+. After washing with 4 volumes of 5 mM acetic acid, the column was eluted with 4 volumes of 1 N NH_4OH and the eluate concentrated and derivatized as described above. Quantitative analyses of lens and urine were performed by selected ion monitoring GC/MS, using the *m/e* 392 and 518 ions for the trifluoroacetyl methyl esters of CML and LL, respectively. Diaminopimelic acid was used as an internal standard for measurement of CML and LL in urine samples.

RESULTS

Identification of 3-(N^ϵ-Lysino)-lactic acid—In previous work we showed that oxidative degradation of Amadori compounds occurs spontaneously in phosphate buffer under air in the presence of trace amounts of iron salts (11). CML was identified as the major product of oxidation of fFL at pH 7.4 (11). However, we also observed a minor product which eluted at 42 min on the amino acid analyzer, about 3 min later than CML (Fig. 2*A*). The yield of this compound was about 5% of the yield of CML at pH 7.4 (Fig. 2*A*) but increased to >20% of CML at pH 6.4 (Fig. 2*B*). As with CML, this product was formed in only trace amounts in the absence of air (see Table II, discussed below), and was also formed during glycation of poly-L-lysine (Fig. 2, *C* and *D*). Its elution time and yield were not affected by reduction with $NaBH_4$ (Fig. 2, *C versus D*), nor could it be labeled by reduction with $[^3H]NaBH_4$ (data not shown). The formation of this product during glycation of proteins *in vitro* could not be confirmed by HPLC amino acid analysis because of its coelution with leucine or isoleucine in several gradient systems. The compound was isolated by pooling peak fractions from several semi-preparative runs of fFL reaction mixtures on the amino acid analyzer. Following derivatization by methyl esterification and acetylation, it was tentatively identified as LL, based on its mass spectrum obtained by direct probe analysis (Fig. 3). This mass spectrum was identical to that of authentic LL prepared by reaction of bromopyruvic acid with N^α-formyllysine, followed by reduction *in situ* with $NaBH_4$, as described under "Experimental Procedures."

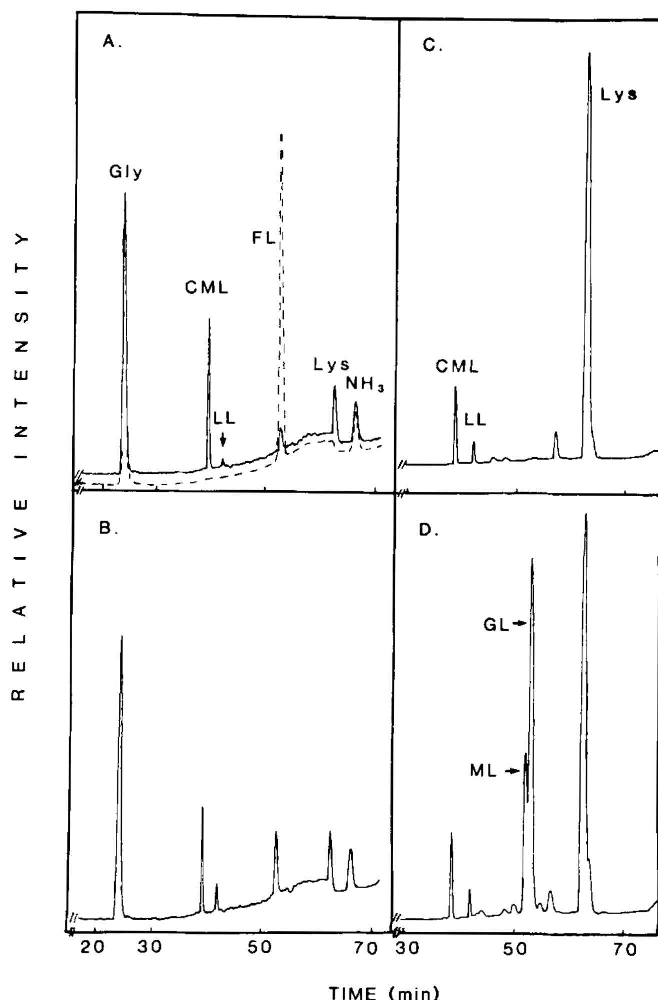

FIG. 2. **Detection of LL among Maillard reaction products.** *A*, a standard reaction mixture containing 15 mM fFL in 0.2 M phosphate buffer, pH 7.4, was incubated for 15 days at 37 °C under air, deformylated by mild acid hydrolysis, and then analyzed by cation-exchange HPLC as described under "Experimental Procedures;" Gly was internal standard. The *dotted line* is the chromatogram of the incubation mixture at day 0. The *solid line*, obtained after 15 days incubation, indicates that about 10% of the original fFL remains unreacted. Products are identified as CML, LL, and lysine, the latter formed by reversal of the Amadori rearrangement. *B*, an identical reaction mixture incubated at pH 6.4, showing increased yield of LL at slightly acid pH. *C*, poly-L-lysine (20 mg/ml) incubated with glucose (0.5 M) in 0.2 M phosphate buffer, pH 6.4, for 14 days at 37 °C. After dialysis to remove glucose, the sample was hydrolyzed in 6 N HCl for 18 h at 95 °C. FL in the glycated polymer is destroyed during the hydrolysis. *D*, the sample in *C* was reduced with $NaBH_4$ prior to hydrolysis. The total recovery of mannitollysine (*ML*) and glucitollysine (*GL*) indicates about 15% glycation of lysine residues in this preparation.

Although LL could be characterized as its acetylated methyl ester derivative using direct probe MS after its purification from a reaction mixture, it was not detectable in reaction mixtures by GC/MS, either because of its poor volatility or adsorption to the GC column. As an alternative approach to derivatization, the fFL reaction mixture was reduced with $NaBH_4$ (converting fFL to the hexitollysines, glucitol- and mannitollysine), desalted on Dowex 1-X8 acetate, deformylated, and derivatized directly by trimethylsilylation. The total ion chromatogram (Fig. 4) and mass spectra revealed the presence of both LL and its putative split product, glyceric acid (Fig. 1), and also of CML and erythronic acid. In addition, mannose and glucose (as mannitol and glucitol) and lysine, which are formed on reversal of the Amadori rearrangement, were among the major products detected in the reaction mixture. The hexitollysines, glucitol- and mannitollysine, formed on reduction of the remaining fFL, were also detected in the chromatogram. In other studies (data not shown), the reaction mixture was dried by rotary evaporation and then trimethylsilylated directly without prior reduction, desalting, and deformylation. Although the trimethylsilyl derivative of fFL was not detectable in the ion chromatograms, erythronic and glyceric acids were observed in amounts comparable to those obtained following the reduction and deformylation procedure. There were also prominent peaks at retention times and ion intensities relative to lysine and with mass spectra expected for the trimethylsilyl derivative of N^{α}-formyl-CML and N^{α}-formyl-LL. Thus, CML and LL appear to be formed in the reaction mixture *in situ*, rather than by degradation of labile intermediates during the work-up for analysis.

Characteristics of the Reaction—Having established that LL is a product of oxidative cleavage of fFL, we compared the kinetics of formation of LL and CML and the effects of various reaction conditions on their yield. As shown in Fig. 5, the kinetics of formation of LL closely paralleled those for formation of CML. The data in Table I indicate that, within the range of pH 5.4–8.4, formation of LL, relative to CML, was favored at more acidic pH, while the absolute yield of LL was maximal at pH 6.4. The experiments described in Table II indicate that LL, like CML, is formed by a mechanism involving oxygen free radicals. These reactions require both oxygen and traces of redox-active metal ions, such as copper or iron, and are inhibited by strong chelators and oxygen radical scavengers (16, 17). Thus, only traces of CML or LL were formed under nitrogen or in the presence of the chelator DTPA. Partial inhibition was observed with cyanide, probably as a result of precipitation or formation of complexes with metal ions. Good inhibition was also obtained with mannitol, a hydroxyl radical scavenger, and with Tiron which may act as either a chelator (18) or a superoxide radical scavenger (19, 20). The differential effect of Tiron on formation of CML and LL was consistently observed but is not fully understood. Overall, however, these data clearly support a free radical mechanism for the formation of both CML and LL.

Detection of LL in Biological Samples—Having detected LL in model reaction systems *in vitro* we next tested for the presence of this compound in biological samples. The trimethylsilyl derivatization procedure proved unsatisfactory for this purpose because of incomplete and variable efficiency of derivatization of amino acids in protein hydrolysates and concentrated urine samples. However, preparation of trifluoroacetyl methyl esters yielded reproducible results with lysine, CML, and LL, although FL and the hexitollysines were not detectable by this procedure. The total ion chromatogram of a typical fFL reaction mixture, prepared by trifluoroacetyl methyl ester derivatization, is shown in Fig. 6*A*, along with the mass spectrum of the compounds identified as CML and LL (Fig. 6, *B* and *C*, respectively). Using the trifluoroacetyl methyl ester derivatives, both CML and LL were also detected in hydrolysates of ribonuclease and lysozyme glycated under air *in vitro* (data not shown) but were not detectable in the native proteins. As shown in the selected ion chromatograms in Fig. 7, CML and LL were also detected in hydrolysates of human lens proteins and as the free amino acids in human urine. The peaks identified as CML and LL co-eluted with authentic standards in mixing experiments and had total ion chromatograms characteristic of CML and LL, respectively. The possibility that these compounds were formed from en-

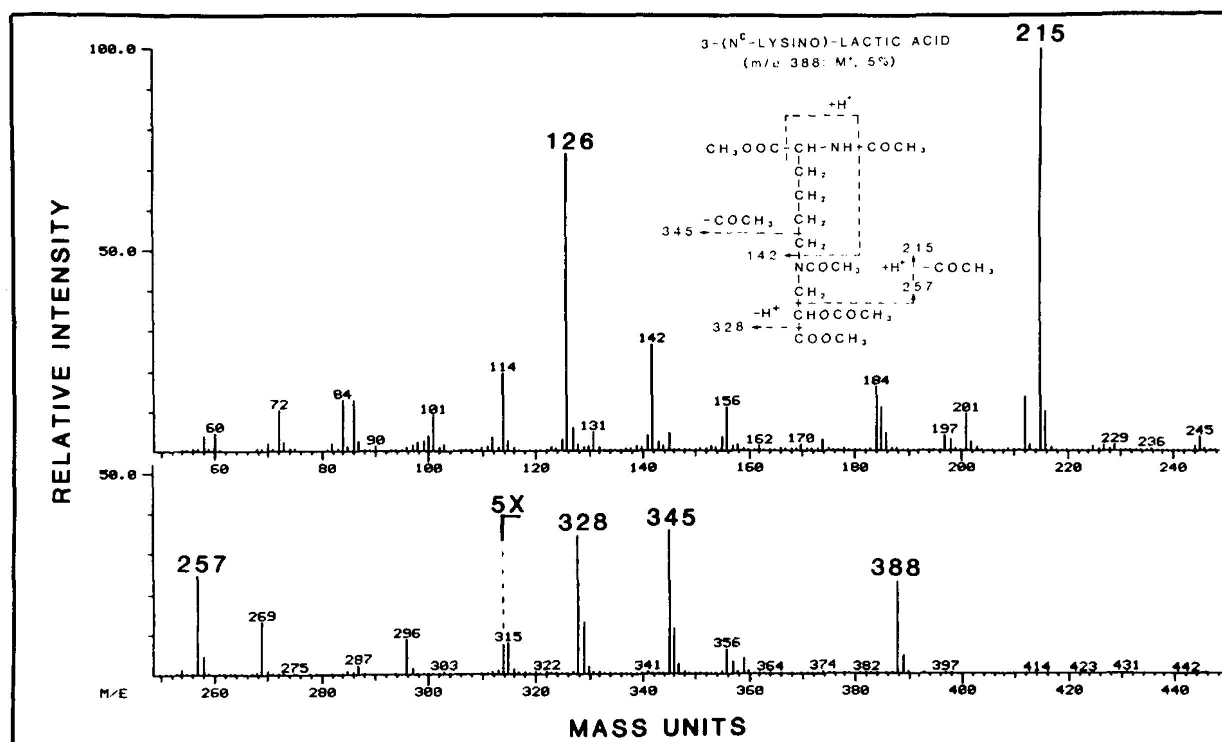

FIG. 3. **Direct probe electron impact mass spectrum of 3-(N^ϵ-lysino)-lactic acid.** LL was isolated by HPLC from an fFL reaction mixture incubated at pH 6.4 for 15 days at 37 °C, derivatized by methyl esterification and N- and O-acetylation, and analyzed on a Finnigan Model 4521C mass spectrometer.

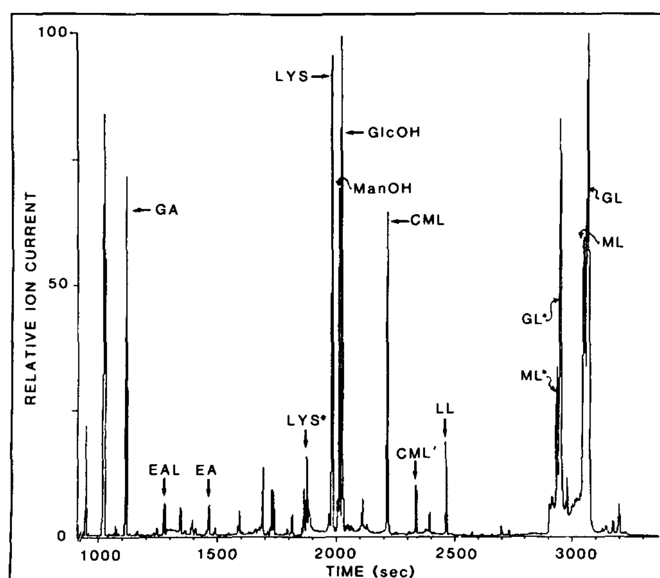

FIG. 4. **Total ion chromatogram of an fFL reaction mixture after incubation for 7 days at pH 6.4.** The products were reduced with $NaBH_4$, deformylated, and then derivatized by trimethylsilylation. *GA*, glyceric acid; *EA*, erythronic acid; *EAL*, erythronic acid lactone; *GlcOH* and *ManOH*, glucitol and mannitol; *ML* and *GL*, mannitol- and glucitol-lysine. *Peaks* marked with an *asterisk*, Lys*, ML*, and GL*, contain one less trimethylsilyl group than the major peak. CML′ contains 5 trimethylsilyl groups *versus* 4 in CML.

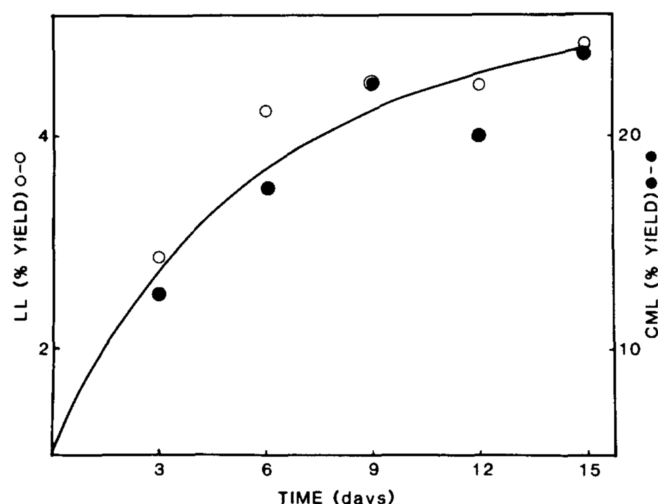

FIG. 5. **Kinetics of formation of CML and LL.** Incubations of fFL were conducted at pH 6.4, as described in the legend to Fig. 2*B*, and analyzed by HPLC. Percent yields are based on original content of fFL in the incubation mixture.

dogenous FL during the hydrolysis or derivatization procedures was excluded since similar results were obtained following prior reduction of the samples with $NaBH_4$. Overall, the trifluoroacetyl methyl ester derivatization procedure not only permitted the detection of LL in biological samples but also provided significantly higher sensitivity for detection of CML than was obtained previously using the N-acetyl methyl ester derivative (11). Preliminary experiments indicate that CML and LL are present at 3.5 ± 0.9 and 0.2 ± 0.6 mmol/mol lysine in adult human lens proteins (n = 4, donor age = 45–60) and 1.6 ± 0.8 and 0.35 ± 0.3 mg/g creatinine in fasting adult human urine (n = 5, age = 20–50).

DISCUSSION

The studies reported here extend our previous observations (10, 11) on the oxidative degradation of Amadori adducts to

TABLE I

Effect of pH on yield of CML and LL

Reaction mixtures containing 15 mM fFL in 0.2 M phosphate buffer were incubated for 15 d at 37 °C, then analyzed by HPLC. Yields are based on initial concentration of fFL (15 mM).

pH	Percent yield		Total yield	Ratio (LL/CML)
	CML	LL		
			%	
5.4	3.3	2	5.3	0.6
6.4	19	5.3	24	0.3
7.4	40	1.3	41	0.03
8.4	35	1.3	36	0.04

TABLE II

Effect of various conditions on yield of CML and LL

Numbers in parentheses represent actual percent yield of CML and LL in standard reaction mixture incubated for 7 days at pH 6.4.

Variation	Percent inhibition	
	CML	LL
Control	(13.6)	(5.5)
+ DTPA (1 mM)	>98	>98
N_2 atmosphere	>98	>98
+ KCN (1 mM)	32	35
+ Mannitol (50 mM)	28	21
+ Tiron (1 mM)	78	37

protein and re-emphasize that glycation of protein does not necessarily imply a commitment to the cross-linking and browning reactions characteristic of the later stages of the Maillard reaction. Our preliminary data indicate that CML and LL in adult human lens proteins account for less than 1% of lysine residues in lens proteins, but their total (3–4 mmol/mol lysine, equivalent to ~1.5 nmol/mg protein) is comparable to amounts of FL in lens protein, as reported from other laboratories, *i.e.* 1–2 nmol/mg lens protein (21, 22). Although LL has not been described previously, our estimate of the free CML concentration in human urine is 10–20-fold lower than that previously reported, *i.e.* 27 ± 17 mg/g creatinine (calculated from data in Table I of Wadman *et al.* (12)). The difference may result from differences in the populations under study, *i.e.* pediatric (12) *versus* adult donors, or from dietary sources of CML in the pediatric population (12). Our estimate of daily urinary excretion of free CML and LL (1–2 μmol/kg body weight/day) is about 10% of the estimated daily excretion of total (free plus peptide bound) glycated amino acids (2.7 μmol/kg/day, estimated from data of Brownlee *et al.* (23)). Although we have not yet obtained an estimate of total urinary CML and LL, the amounts detected are reasonable since only a fraction of glucose adducts would be oxidized, and CML and LL would accumulate primarily on longer-lived proteins which turn over more slowly. Even on these proteins, the rates of formation of CML and LL would be limited by the concentration of active oxygen and metal ion species in tissues and body fluids and inhibited by the activity of numerous endogenous chemical and enzymatic inhibitors of free radical reactions.

Since the products of oxidation of FL, such as CML, LL, erythronic and glyceric acid, are colorless, the reactions leading to their formation may be described as non-browning pathways of the Maillard reaction. These oxidation products are chemically inert compared with FL, and thus do not contribute to the further chemical modification and cross-linking of protein via the Maillard reaction. Indeed, the oxidation reactions appear to compete with the browning reactions since the yield of browning products from fFL incubation mixtures *in vitro* is significantly depressed under air

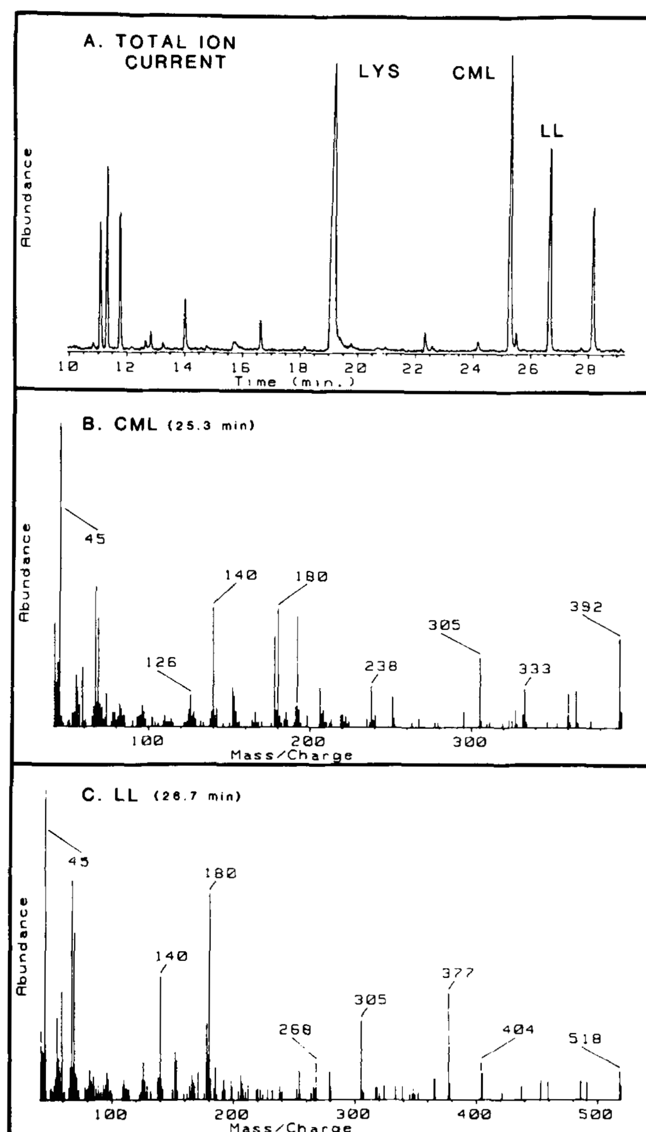

FIG. 6. **Detection of CML and LL in fFL reaction mixtures by GC/MS.** The reaction mixture was incubated for 7 days at pH 6.4 under air. The data were obtained on the Hewlett-Packard GC/MS system. *A*, total ion chromatogram of fFL reaction mixture after incubation for 7 days at pH 6.4 under air. Products were deformylated and then derivatized as trifluoroacetyl methyl esters. *B* and *C*, mass spectra of peaks identified as CML and LL, respectively, in *A*. The peaks identified as CML and LL co-eluted with authentic standards in mixing experiments and had total ion chromatograms identical to the standards. CML assignments: 392 = M − CH_3OH; 365 = M − $COOCH_3$; 333 = M − (CH_3OH + $COOCH_3$); 305 = M − (2 × $COOCH_3$ + H); 180 = M − ($CF_3CONCH_2COOCH_3$ + $COOCH_3$ + H). LL assignments: 518 = M − CH_3OH; 404 = M − (CF_3COO + CH_3OH + H); 377 = M − (CF_3COO + $COOCH_3$ + H); 305 = M − ($CF_3COOCHCOOCH_3$ + $COOCH_3$ + H); 180 = M − ($CF_3CONCH_2CH(OCOCF_3)COOCH_3$ + $COOCH_3$ + H).

compared with nitrogen (11). The fact that the non-browning reactions occur *in vivo* suggests that they may function as a physiological mechanism for limiting extended damage to long-lived glycated proteins via the Maillard reaction. Thus, if Maillard reactions of glycated proteins have any role in the development of pathophysiology in diabetes and aging (5–9), differences in the relative rates of browning *versus* non-browning pathways among individuals may be important in determining their relative susceptibility to tissue damage from these reactions.

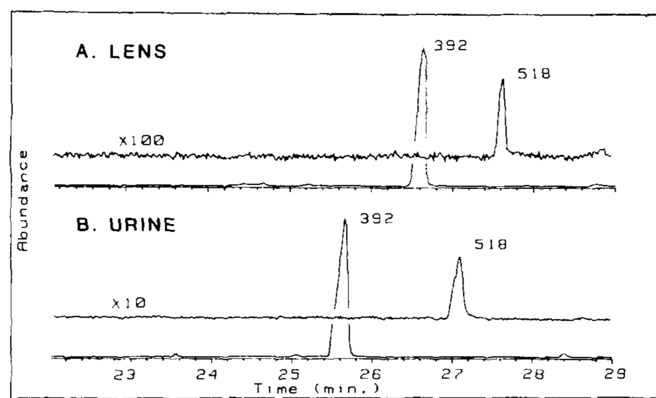

FIG. 7. **Simultaneous detection of CML and LL in human lens and urine samples by GC/MS using selected ion monitoring.** Chromatographic conditions were as described in Fig. 6*A*, except for focus on the M-30 ions, m/z = 392 and 518 for CML and LL, respectively. *A*, analysis of hydrolysate of human lens protein. Samples were prepared as described under "Experimental Procedures." The amount of sample injected in 1 μl of methylene chloride corresponded to approximately 80 μg of lens protein, estimated by the Biuret assay prior to hydrolysis using a standard of bovine serum albumin. *B*, analysis of human urine. The urine sample was enriched for amino acids by sequential anion- and cation-exchange chromatography, as described under "Experimental Procedures." The quantity of sample injected in 1 μl of methylene chloride corresponded to approximately 25 μl of the original urine sample. Differences in retention times between chromatograms result from differences in sample load.

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