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Identification of N^{ϵ}-Carboxymethyllysine as a Degradation Product of Fructoselysine in Glycated Protein*

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The chemistry of Maillard or browning reactions of glycated proteins was studied using the model compound, N^{α}-formyl-N^{ϵ}-fructoselysine (fFL), an analog of glycated lysine residues in protein. Incubation of fFL (15 mM) at physiological pH and temperature in 0.2 M phosphate buffer resulted in formation of N^{ϵ}-carboxymethyllysine (CML) in about 40% yield after 15 days. CML was formed by oxidative cleavage of fFL between C-2 and C-3 of the carbohydrate chain and erythronic acid (EA) was identified as the split product formed in the reaction. Neither CML nor EA was formed from fFL under a nitrogen atmosphere. The rate of formation of CML was dependent on phosphate concentration in the incubation mixture and the reaction was shown to occur by a free radical mechanism. CML was also identified by amino acid analysis in hydrolysates of both poly-L-lysine and bovine pancreatic ribonuclease glycated in phosphate buffer under air. CML was also detected in human lens proteins and tissue collagens by HPLC and the identification was confirmed by gas chromatography/mass spectroscopy. The presence of both CML and EA in human urine suggests that they are formed by degradation of glycated proteins *in vivo*. The browning of fFL incubation mixtures proceeded to a greater extent under a nitrogen *versus* an air atmosphere, suggesting that oxidative degradation of Amadori adducts to form CML may limit the browning reactions of glycated proteins. Since the reaction products, CML and EA, are relatively inert, both chemically and metabolically, oxidative cleavage of Amadori adducts may have a role in limiting the consequences of protein glycation in the body.

One of the direct, chemical consequences of hyperglycemia in diabetes is an increase in levels of glycation (nonenzymatic glycosylation) of proteins throughout the body (1, 2). Glycation proceeds through formation of a Schiff base between glucose and amino groups on protein, followed by the Amadori rearrangement to yield relatively stable ketoamine adducts to protein. From studies *in vitro*, glycated proteins are known to undergo further reactions, collectively described as the Maillard reaction, leading to cross-linking and polymerization of protein and the formation of poorly characterized brown and fluorescent compounds (3, 4). There is evidence that these same reactions take place in the body following glycation of protein, based on 1) the increased browning and fluorescence of structural proteins during normal aging and in diabetes (5–7) and 2) the detection of Maillard products in hydrolysates of tissue proteins (8). Concomitant structural and functional alterations of tissue protein via the Maillard reaction are thought to contribute to the development of pathologies associated with both diabetes and aging, such as blindness, renal failure, neuropathy, and vascular disease (9, 10).

In order to gain better insight into mechanisms and products of the Maillard reaction *in vivo*, we have initiated studies on the chemistry of reactions between glucose and proteins under physiological conditions *in vitro* (11–14). In a recent report (13), we observed by ^{13}C NMR spectroscopy that the structures and conformational equilibria of Amadori adducts to proteins were identical with those of the model Amadori compound, N^{α}-formyl-N^{ϵ}-fructoselysine (fFL[1]). In this paper we describe studies on the reactions of fFL and show that the amino acid, N^{ϵ}-carboxymethyllysine (CML), is a major product formed on oxidative degradation of fFL. We also show that CML is formed in protein under conditions commonly used for glycation *in vitro* and that CML is detectable in tissue proteins. The relevance of this pathway to the progress of the Maillard reaction *in vivo* is also discussed.

EXPERIMENTAL PROCEDURES

Materials—Bovine pancreatic ribonuclease A (RNase, Type XII-A), poly-L-lysine (M_r = 15,000), iodoacetic acid, D-erythrose, and *N*-formylglycine were obtained from Sigma.

Synthesis of Model Compounds—fFL was synthesized from N^{α}-formyllysine (15) and glucose by the procedure of Finot and Mauron (16). CML was obtained in 30% yield by incubating N^{α}-formyllysine and iodoacetic acid (0.1 M each in 0.2 M phosphate buffer, pH 10) for 40 h at room temperature. Unreacted iodoacetate was discharged by the addition of 0.2 volume of concentrated NH_4OH, followed by continued incubation overnight. Phosphate and iodide were then removed by applying the reaction mixture to a column of Dowex 1-X8 acetate and eluting with 2 N acetic acid. The eluate was concentrated by rotary evaporation and the products were deformylated by hydrolysis in 2 N HCl for 0.5 h at 95 °C. CML was isolated by ion exchange chromatography on Dowex 50-X8, using the procedure of Chin and Wold (17). D-Erythronic acid (EA) was synthesized by oxidation of D-erythrose with molecular oxygen over a palladium-charcoal catalyst, according to Thompson *et al.* (18). Poly-L-lysine and RNase were each glycated by incubation with 1 M glucose in 0.2 M phosphate buffer, pH 7.4, for 7 days at 37 °C, as described previously (11). Amino acid analysis of the $NaBH_4$-reduced preparations (11) indicated 3 mol of Glc/mol of RNase and 0.2 mol of Glc/mol of Lys in poly-L-lysine.

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[1] The abbreviations used are: fFL, N^{α}-formyl-N^{ϵ}-fructoselysine; CML, N^{ϵ}-carboxymethyllysine; EA, erythronic acid; FL, N^{ϵ}-fructoselysine; GC, gas chromatography; MS, mass spectrometry; HPLC, high pressure liquid chromatography; RNase, bovine pancreatic ribonuclease A; TAPSO, 3-[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid.

Analytical Procedures—Amino acid analyses were performed on a Waters high pressure liquid chromatography (HPLC) system by cation exchange chromatography using the sodium citrate-NaCl gradient system described by the manufacturer (Buffer A: 0.2 M sodium citrate, pH 3.2; Buffer B: 0.2 M sodium citrate, 1 M NaCl, pH 7.0). Amino acids and derivatives were detected by fluorescence using *o*-phthaldialdehyde as a post-column reagent.

Gas chromatography/mass spectroscopy (GC/MS) was performed on a Finnigan model 4201 instrument, using a 25-m fused silica capillary column with a 7% cyanopropyl-, 7% phenylmethyl-silicone bonded phase (Scientific Glass Engineering, Inc.: 25QC3/BP10-0.5, equivalent to OV-1701). The column was programmed from 50–270 °C at 15 °C/min and maintained at 270 °C for 20 min. For GC analyses, products were esterified by treatment with 1 N methanolic HCl for 0.5 h at 65 °C. After evaporation to dryness under a stream of nitrogen, *N*- and *O*-acetylation was performed by treatment with acetone/trimethylamine/acetic anhydride (5:2:1) for 5 min at 65 °C (19). The sample was evaporated under nitrogen and redissolved in ethyl acetate for GC/MS analysis.

Reactions of fFL—Standard reaction mixtures contained 15 mM fFL in 0.2 M phosphate buffer, pH 7.4. Solutions were sterilized by ultrafiltration and incubated in the dark at 37 °C in Teflon-lined screw-cap test tubes. *N*-Formylglycine (15 mM) was included as an internal standard. For reactions under nitrogen, the incubation mixtures were gassed for 5 min with a stream of nitrogen gas. For analysis by HPLC, aliquots of incubation mixtures were diluted into 10 volumes of 2 N HCl and deformylated as above, concentrated by rotary evaporation, and redissolved in HPLC Buffer A. For GC and GC/MS analyses, samples were desalted on Dowex 1-X8 as above (100-μl aliquot/1-ml column). The column was eluted with 5 volumes of 2 N acetic acid and products were deformylated and derivatized as described above.

RESULTS

In order to facilitate characterization of products formed during later stages of the Maillard reaction of glycated proteins, we studied reactions of the model compound, fFL, an analog of glycated lysine residues in protein. Standard incubations of fFL were carried out at physiological pH (7.4) and temperature (37 °C) under an air atmosphere, but in 0.2 M phosphate buffer to maintain good control of pH. Analysis of the starting reaction mixture on an HPLC amino acid analyzer (Fig. 1*A*) showed only fructoselysine (FL) and the inter-

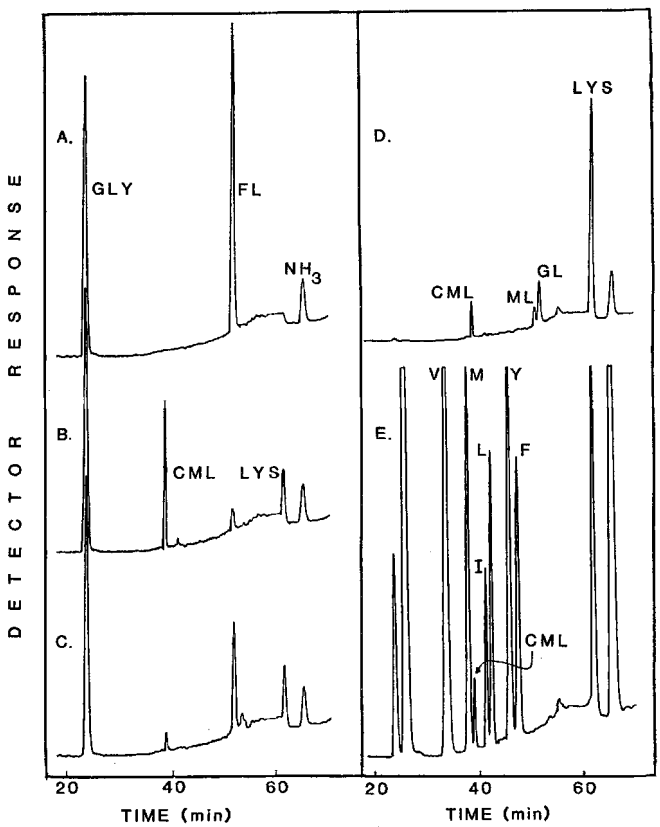

FIG. 1. **HPLC chromatograms of fFL incubation mixtures and of glycated macromolecules.** *A*, starting fFL incubation mixture, as described under "Experimental Procedures," showing peaks from internal standard, glycine (*Gly*), and FL. *B*, identical reaction mixture as in *A*, after incubation for 15 days at 37 °C under an air atmosphere. *C*, reaction mixture as in *A*, except after a 15-day incubation under nitrogen atmosphere. *D*, amino acid analysis of $NaBH_4$-reduced, glycated poly-L-lysine. *ML*, N^{ϵ}-mannitollysine; *GL*, N^{ϵ}-glucitollysine. *E*, segment of amino acid chromatogram of glycated RNase. The amino acids in RNase adjacent to CML are identified by conventional symbols.

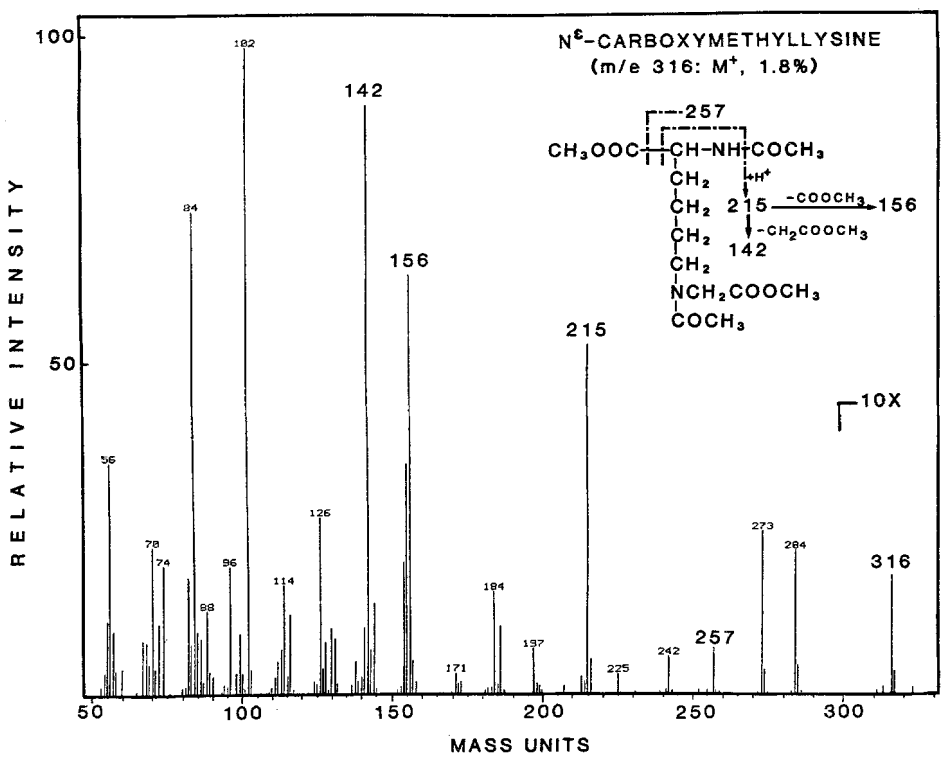

FIG. 2. **Electron impact mass spectrum of CML, derivatized as the N^{α},N^{ϵ}-diacetyl-N^{ϵ}-carboxymethyllysine methyl ester.**

nal standard, glycine. Visible browning of the reaction mixture occurred gradually within the first week of incubation at 37 °C, but only lysine and a second major product were detected on the amino acid analyzer. This product eluted in the neutral amino acid region and was apparently an oxidation product of fFL since it was formed in air (Fig. 1*B*), but not under a nitrogen atmosphere (Fig. 1*C*). It was also observed in hydrolysates of glycated poly-L-lysine and RNase (Fig. 1, *D* and *E*), indicating that it is formed during either the preparation or storage of glycated peptides and proteins *in vitro*.

The unknown compound was pooled from a series of semi-preparative HPLC runs of fFL incubation mixtures, desalted on Dowex 50, and derivatized and analyzed by GC/MS. The electron impact fragmentation pattern (Fig. 2) showed a molecular ion at *m/e* 316 and a base peak at *m/e* 102. The fragmentation pattern permitted a tentative identification of the unknown as CML and the identification was confirmed by synthesis. The unknown compound and synthetic CML were identical in their behavior on HPLC and GC and yielded identical mass spectra.

The formation of CML from glycated lysine was presumed to occur by oxidative cleavage of fFL between C-2 and C-3 of the glucose residue (Fig. 3). In this case, erythronic acid (EA) should be recoverable as the split product, but would not be seen on the amino acid analyzer. The formation of EA was confirmed by GC/MS analysis of the total incubation mixture described in Fig. 1*B*. The gas chromatogram of this sample is shown in Fig. 4*A*. A comparable chromatogram of the starting incubation mixture was blank in the region after the internal standard, glycine, since the acetylated methyl ester of fructoselysine did not elute from the GC column. The chemical ionization mass spectrum of the peak identified as EA is shown in Fig. 5. This identification was also confirmed by GC/MS comparison to the synthesized compound. The peak at 1760 s in the chromatogram in Fig. 4*A* was identified as CML by its mass spectrum, as was the peak at the same retention time in glycated RNase (Fig. 4*B*), confirming the identification of CML in the HPLC chromatogram in Fig. 1*E*. Overall, these data support the scheme shown in Fig. 3 and indicate that CML and EA are products of oxidative cleavage of both fFL and glycated lysine residues in protein.

The chromatograms in Figs. 1, *B* and *C* and 4*A* also indicate that lysine is a second major product formed during incubation of fFL, presumably by reversal of the Amadori rearrangement. The formation of mannose and glucose by the reverse reaction was also suggested by the cluster of peaks at about 1000 s in Fig. 4*A* (labeled *HEXOSE*), which had retention times and mass spectra expected of a mixture of anomers and conformers of acetylated methylhexosides. To confirm this identification, an aliquot of the fFL incubation mixture was reduced with $NaBH_4$ prior to GC/MS analysis and, as shown

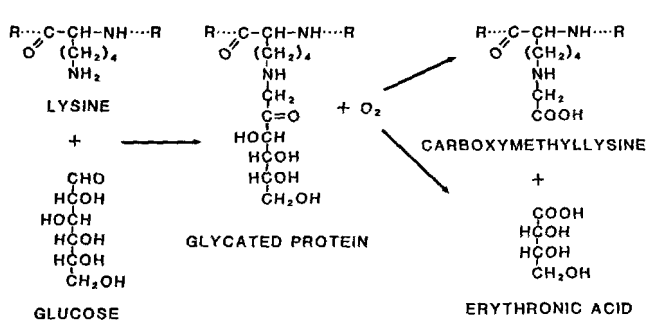

FIG. 3. **Reaction scheme for formation of CML and EA from glycated lysine residues.**

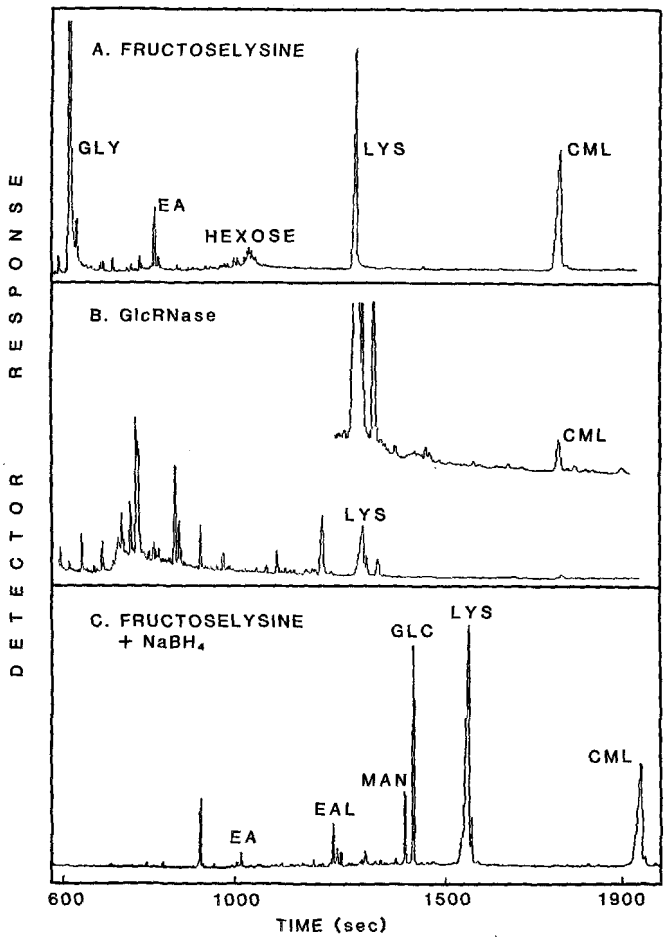

FIG. 4. **Gas chromatographic comparison of products formed from fFL and glycated RNase.** *A*, chromatogram of fFL incubation mixture described in Fig. 1*B*. *B*, chromatogram of glycated RNase (*inset* = 10× scale expansion), showing presence of CML. *C*, same sample as in *A* above, except that the sample was reduced with $NaBH_4$ to convert hexoses to hexitols prior to derivitization. *EAL*, erythronic acid lactone; *Man*, mannitol hexaacetate; *Glc*, glucitol hexaacetate.

in Fig. 4*C*, both mannitol and glucitol were identified as major reaction products. Thus, the formation of lysine from fFL appears to occur by reversal of the Amadori rearrangement.

The kinetics of formation of CML and lysine from fFL under air and nitrogen atmospheres are illustrated in Fig. 6. Oxidative degradation of fFL to CML proceeds only in the presence of oxygen, while the formation of lysine by reversal of the Amadori rearrangement occurs at similar rates under air and nitrogen. Under the reaction conditions used, the formation of CML and lysine also proceed at similar rates under air and account for about 75% of the original fFL in the reaction mixture. The experiments presented in Fig. 7 show the temperature and pH dependence of CML formation. Initial rates increase with both temperature and pH in the ranges studied, but the eventual yield of CML at higher temperature is limited, undoubtedly because of competing reactions. Qualitatively, these competing reactions are readily apparent because of the increased browning, *i.e.* formation of melanoidins, observed in the higher temperature incubations. As shown in Fig. 8, the overall rate of loss of fFL through the several reactions leading to formation of CML, lysine, melanoidins, and possibly other products appears to be primarily a first order process, with a half-life of approximately 3.5 and 8.8 days under air and nitrogen, respectively, under the con-

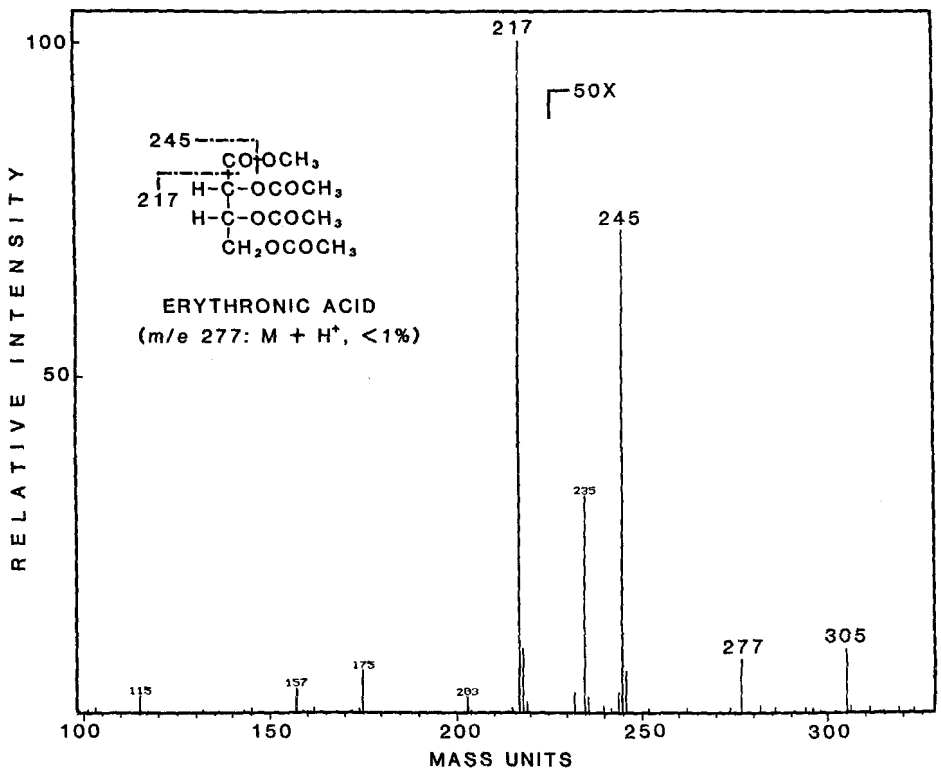

FIG. 5. **Methane chemical ionization mass spectrum of tri-O-acetylerythronic acid methyl ester.** The mass spectrum of the product identified as EA in Fig. 4A is shown. The M^+ ion is observed at *m/e* 277 and $M + C_2H_5$ at *m/e* 305.

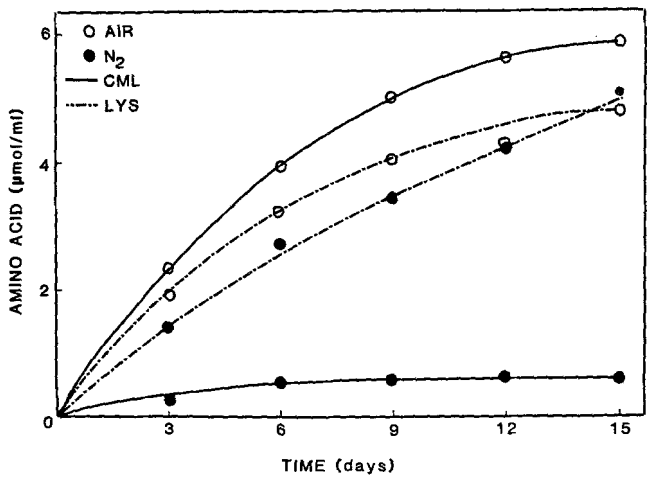

FIG. 6. **Comparative kinetics of formation of lysine (– — –) and CML (——) under air (○) and nitrogen (●) atmospheres.** Reactions were conducted as described under "Experimental Procedures" and aliquots were analyzed by HPLC.

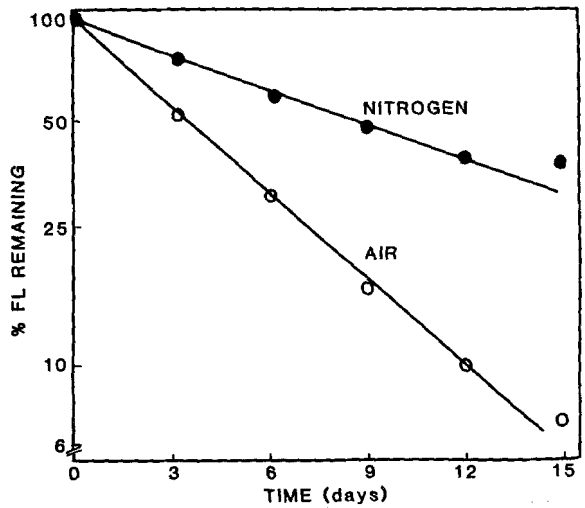

FIG. 8. **Kinetics of disappearance of fFL under air (○) and nitrogen (●) atmospheres.** Reaction mixtures were incubated at 37 °C and analyzed by HPLC.

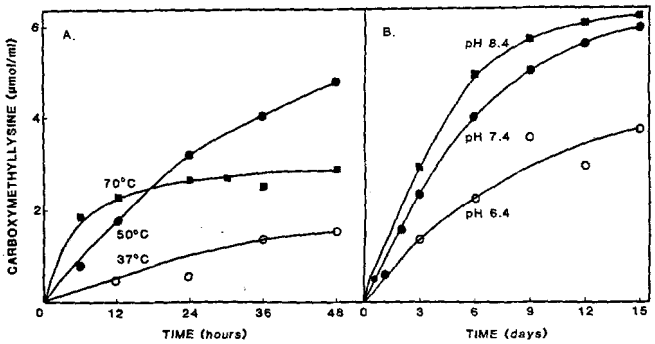

FIG. 7. **Effects of temperature (*A*) and pH (*B*) on kinetics of formation and yield of CML.** All reactions were conducted as described under "Experimental Procedures" except for variation in temperature or pH of phosphate buffer. Aliquots were analyzed by HPLC.

ditions described. The difference between the rate of fFL degradation under air *versus* nitrogen can be attributed largely to the formation of CML in the oxygen-containing incubations. Under nitrogen, the concentration of fFL also remains higher with time and this may explain the increased formation of browning products in the nitrogen incubations (Fig. 9). Thus, oxidative degradation of fFL appears to limit the browning reaction under air by converting fFL to the relatively nonreactive compounds, CML and EA. It is also possible that the decreased browning under air could result from bleaching of browning products by oxygen. This seems less likely, however, since the brown products, once formed, appear to be stable during prolonged incubation in air (Fig. 9, air incubation, 7th–15th day).

Oxidative degradation of sugars is known to proceed by free radical mechanisms (20, 21) which involve the participation

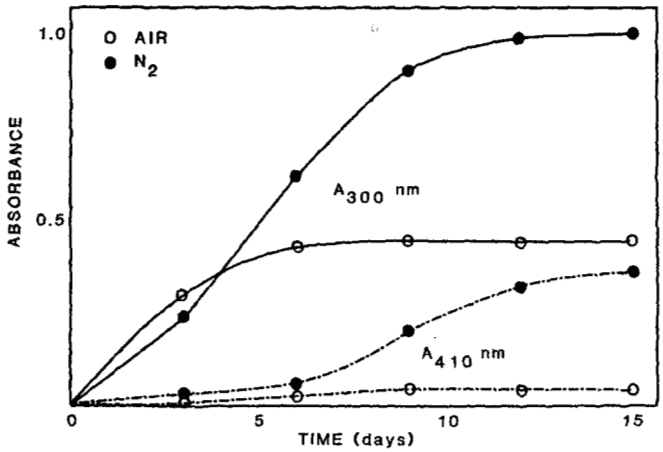

FIG. 9. **Kinetics of browning of fFL incubation mixtures under air (○) and nitrogen (●) atmospheres.** The progress of the Maillard reaction was followed in both the ultraviolet (300 nm) and visible (410 nm) region of the spectrum.

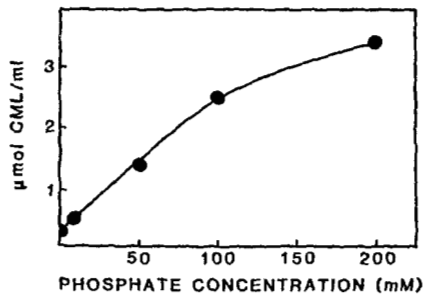

FIG. 10. **Effect of phosphate concentration on the formation of CML.** Incubations were conducted for 7 days at pH 7.4 and 37 °C, then analyzed by HPLC. Phosphate concentration was increased at a constant, 0.2 M TAPSO concentration.

TABLE I

Factors affecting the formation of CML

Addition to reaction mixture[a]	% inhibition	Remarks
None	0[b]	Control
Tiron[c] (1 mM)	100	$O_2^{\cdot-}$ scavenger
Mannitol (50 mM)	30	$OH^{\cdot}$ scavenger
Catalase (1000 units/ml)	88	H_2O_2 scavenger
DTPA[d] (1 mM)	100	Iron and copper chelator

[a] Starting reaction mixtures contained 15 mM fFL in 0.2 M phosphate buffer, pH 7.4. Incubation was carried out at 37 °C and aliquots were withdrawn at 5 days for analysis by HPLC.

[b] Conversion of fFL to CML was 42% in the control experiments.

[c] 4,5-Dihydroxy-1,3-benzenedisulfonic acid.

[d] Diethylenetriaminepentaacetic acid.

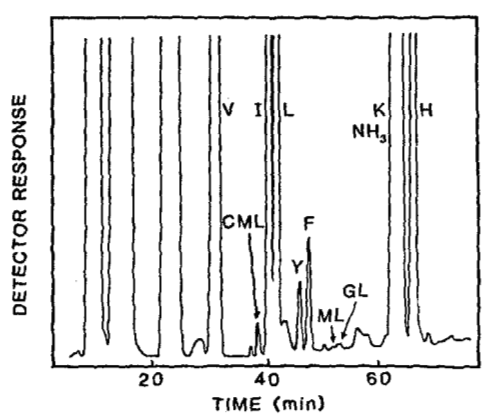

FIG. 11. **Detection of CML in lens protein by HPLC amino acid analysis.** The lens was obtained from a 56-year-old adult onset diabetic with unknown duration of the disease. The total lens was homogenized in 2.5 ml of distilled water, and an aliquot was reduced with $NaBH_4$ in phosphate buffer at pH 8.5 (14), dialyzed against distilled water to remove salts, and then hydrolyzed in 6 N HCl for 18 h at 95 °C (14). The amino acid analyzer is overloaded to enhance the signal from CML, which is approximately 1% of the intensity of the lysine peak. The intensity of the peak identified as CML was similar to that from a separate aliquot which was not reduced with $NaBH_4$. *ML* and *GL* mark the position of elution of mannitol- and glucitollysine, respectively.

of H_2O_2 and superoxide and hydroxyl radicals. Since these reactions are catalyzed by metal ions and phosphate (22, 23), we investigated the effects of phosphate concentration, chelators, and radical scavengers on the formation of CML. As shown in Fig. 10, the formation of CML from fFL was significantly affected by phosphate concentration, although some CML was formed in TAPSO buffer alone. Chelation of metal ions with diethylenetriaminepentaacetic acid, even in the presence of phosphate (Table I), completely inhibited the formation of CML. Inclusion of catalase and superoxide and hydroxyl radical scavengers in the incubation mixtures (Table I) also inhibited the reaction, confirming the involvement of H_2O_2 and oxygen radicals as mediators of the oxidative degradation of fFL. Similar effects of phosphate, metal ions, and radical scavengers on the formation of CML were also observed during glycation of protein *in vitro*.[2]

Although phosphate concentration *in vivo* is significantly less than 0.2 M and numerous enzymatic and chemical mechanisms operate to quench oxygen radical reactions, it seemed possible that oxidative degradation of Amadori adducts to protein might nevertheless proceed at slow rates in the body. As shown in Fig. 11, we observed that CML was readily detectable in hydrolysates of lens proteins by HPLC amino acid analysis. The identification of CML was confirmed by Selective Ion Monitoring GC/MS, as shown in Fig. 12. Interestingly, the level of hexitollysines in this sample of total lens protein (*ML* and *GL* in Fig. 11) was at the limit of sensitivity of the amino acid analyzer, indicating that there are higher levels of CML than of reducible Amadori adducts in lens protein. It is possible, however, that because of the structure and insolubility of some lens proteins, not all Amadori adducts were reducible under the conditions used. In addition to lens proteins, CML has also been detected, albeit at lower levels, in hydrolysates of tendon collagen, but was not detectable in hemoglobin or plasma proteins.

DISCUSSION

The pathway described here for the formation of CML represents a new route for degradation of Amadori compounds. The pH dependence of the reaction, *i.e.* increased rate at higher pH (Fig. 7), and the sensitivity to phosphate, chelators, and radical scavengers (Fig. 10 and Table I) indicate that the process is analogous to the oxidative degradation of ketoses in alkali to yield glyconic and other organic acids (20, 21). For the ketoamine, in this case the Amadori product formed between glucose and lysine, the oxidation reaction yields CML and EA. Although CML was first characterized in the fFL model system, it had been observed previously in RNase glycated *in vitro* (peak U-79 in amino acid analysis of glycated RNase, Fig. 1A of Ref. 11). The appearance of CML during short-term glycation reactions of protein *in vitro* (Fig.

[2] M. U. Ahmed, S. R. Thorpe, and J. W. Baynes, manuscript in preparation.

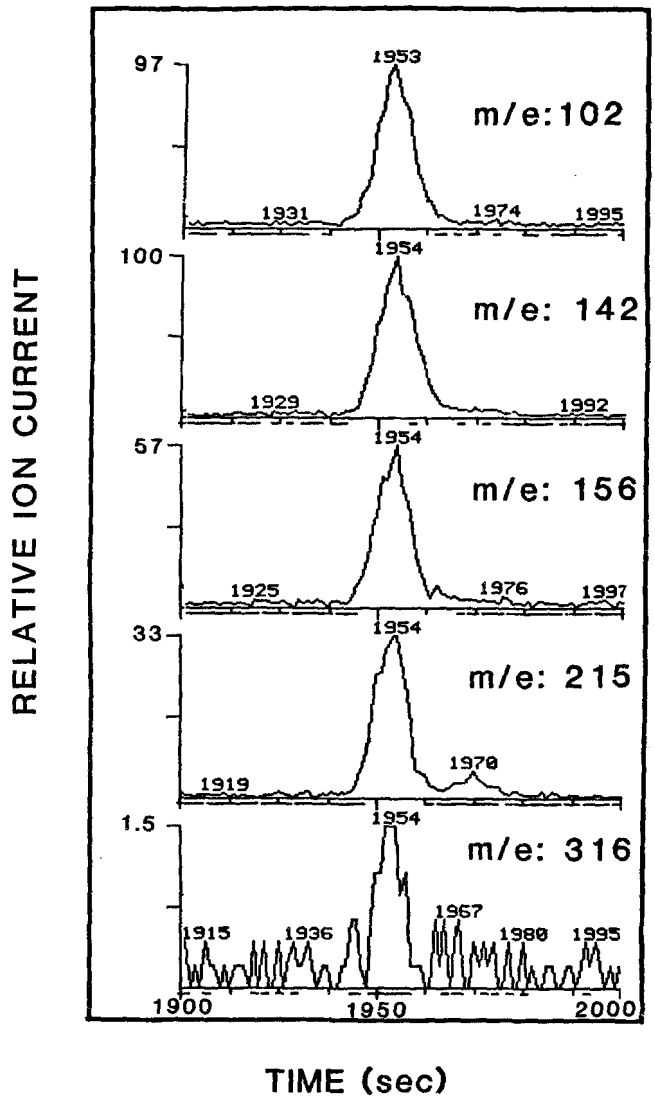

FIG. 12. **Identification of CML in lens protein by selective ion monitoring mass spectrometry.** The hydrolysate described in Fig. 11 was derivatized by *N*-acetylation and methyl esterification and subjected to analysis by GC/MS. Characteristic CML fragment ions were monitored (*cf.* Fig. 2) and are shown in separate frames.

1*E* and Ref. 11) suggested that the reaction might also occur at measurable rates *in vivo*. This hypothesis is supported by the detection of CML in lens proteins (Figs. 11 and 12) and tendon collagen. Further studies are in progress to determine if CML is present in other proteins and if it accumulates in proteins as a function of age and diabetes. The presence of CML in urine (24) and of EA in both urine (18, 25) and plasma (26) has been known for many years although their metabolic origin was unknown. The work described here suggests that they may both be derived from the oxidation of Amadori adducts to lysine residues in protein and that, as observed for fructoselysine (27), increased levels of CML and EA may also be observed in the urine of diabetics. CML may eventually prove to be a generally useful marker for assessing the age of extracellular proteins in the body or assessing the cumulative exposure of proteins to glucose *in vivo*. Based on studies with fFL, it seems possible that the formation of CML may be enhanced in regions of locally high phosphate concentration, *e.g.* in the connective tissue matrix, or in areas of inflammation where the concentration of radical species may be elevated.

The data in Fig. 9 indicate that the formation of browning products from fFL is decreased under air *versus* nitrogen. As noted earlier, this probably results from the fact that in air the concentration of the Amadori compound is more rapidly decreased by its conversion to CML and EA. Both of these compounds are relatively inert compared to reducing sugars and the various intermediates (3, 4) formed during the browning pathway of the Maillard reaction. Thus, oxidative cleavage of Amadori adducts, along with reversal of the Amadori rearrangement, may have a role in limiting the potential damage resulting from browning reactions *in vivo*.

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