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Oxidation of Glycated Proteins: Age-Dependent Accumulation of N^{ϵ}-(Carboxymethyl)lysine in Lens Proteins†

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ABSTRACT: N^{ϵ}-(Carboxymethyl)lysine (CML) has been identified as a product of oxidation of fructoselysine (FL) in glycated (nonenzymatically glycosylated) proteins in vitro and has also been detected in human tissues and urine [Ahmed et al. (1986) *J. Biol. Chem. 261*, 4889-4894]. In this study, we compare the amounts of CML and FL in normal human lens proteins, aged 0-79 years, using specific and sensitive assays based on selected ion monitoring gas chromatography-mass spectrometry. Our results indicate that the lens content of FL increases significantly between infancy and about age 5 but that there is only a slight, statistically insignificant increase in FL between age 5 and 80 (mean ± SD = 1.4 ± 0.4 mmol of FL/mol of Lys). In contrast, the lens content of the oxidation product, CML, increased linearly with age, ranging from trace levels at infancy up to 8 mmol of CML/mol of lysine at age 79. The ratio of CML to FL also increased linearly from 0.5 to 5 mol of CML/mol of FL between age 1 and 79, respectively. These results indicate that CML, rather than FL, is the major product of glycation detectable in adult human lens protein. The age-dependent accumulation of CML in lens protein indicates that products of both glycation and oxidation accumulate in the lens with age, while the constant rate of accumulation of CML in lens with age argues against an age-dependent decline in free radical defense mechanisms in this tissue.

Glycation (nonenzymatic glycosylation) is a common posttranslational modification of proteins in vivo, resulting from reaction between glucose and amino groups on protein (Baynes et al., 1989). The adduct formed by glycation of lysine residues in protein in termed fructoselysine (FL)[1] (Figure 1), and levels of FL in hemoglobin, plasma proteins, collagen, hair, lens, and numerous other proteins in the body are known to increase in proportion to the degree of hyperglycemia in diabetes (Kennedy & Baynes, 1984). We recently described two products of oxidation of FL, N^{ϵ}-(carboxymethyl)lysine (CML) (Figure 1) and 3-(N^{ϵ}-lysino)lactic acid, and showed that these compounds were also detectable in human lens protein, collagen, and urine (Baynes et al., 1986; Ahmed et al., 1986, 1988). The present study was undertaken to compare the amounts of FL and its major oxidation product, CML, in proteins in vivo. Human lens proteins were chosen for initial studies because these proteins are among the longest lived, most slowly turned over proteins in the body (Harding & Dilley, 1976; Zigler & Goosey, 1981) and thus have the longest time to accumulate glucose adducts and their oxidation products. Because of the hypothesized role of both glycation (Cerami, 1985; Monnier, 1989) and oxidation (Cutler, 1984; Mehlhorn & Cole, 1985; Harman, 1987) in the aging of proteins in vivo, we also explored the relationship between age and the absolute and relative amounts of CML and FL in lens proteins. The results of these studies indicate that CML, rather than FL, is the major product of glycation present in adult lens proteins and also provide insight into the role of glycation and oxidation in the aging of lens proteins in vivo.

Experimental Procedures

Materials. Unless otherwise indicated, reagents were of the highest quality obtainable from Sigma or Aldrich Chemical Co. CML was prepared from reaction of glyoxalic acid with N^{α}-acetyllysine in the presence of sodium cyanoborohydride, followed by ion-exchange purification as described previously (Ahmed et al., 1986, 1988). N^{α}-Formyl-N^{ϵ}-fructoselysine (fFL) was synthesized from N^{α}-formyllysine and glucose, as described by Finot and Mauron (1969). The reaction mixture was concentrated by rotary evaporation, reconstituted in 0.2 M ammonium acetate, pH 9, and applied to a column of phenylboronic acid affinity resin (Amicon Matrex-Gel PBA-60). The fFL was eluted with 0.3 M acetic acid, concentrated by rotary evaporation, reconstituted in deionized water, and stored frozen at −70 °C.

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[1] Abbreviations: CML, N^{ϵ}-(carboxymethyl)lysine; GC/MS, gas chromatography-mass spectrometry; fFL, N^{α}-formyl-N^{ϵ}-fructoselysine; FL, fructoselysine; SIM, selected ion monitoring; TFAME, trifluoroacetyl methyl ester.

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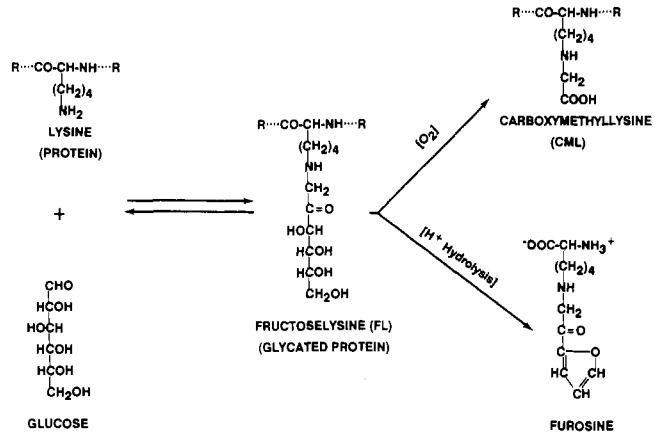

FIGURE 1: Reaction scheme for glycation of protein and formation of FL and CML. A Schiff base intermediate is formed initially between glucose and protein (R···R) and then undergoes the Amadori rearrangement to form FL. A fraction of the protein-bound FL is oxidized to CML in vivo. During acid hydrolysis to release CML and FL from protein, the FL is converted to furosine in about 40% yield.

Lens Preparations. Human lenses were obtained from the Medical College of Georgia Eye Bank, Augusta, GA, and the South Carolina Lions Eye Bank, Columbia, SC. Whole lenses (ages 0–79 years) were weighed (110–160 mg wet weight) and then finely homogenized in 0.5 mL of deionized water at 4 °C using a Reacti-Ware glass homogenizer (Pierce Chemicals). Samples were diluted to 100 mg/mL (wet weight) with water and then dialyzed overnight against deionized water at 4 °C in Spectrapor membrane tubing (6000–8000 MW cutoff; Fisher Scientific Co.). Protein concentration was measured by the Biuret method (Layne, 1957) using bovine serum albumin as standard.

Analytical Methods. Amino acid analyses were performed on a Waters cation-exchange amino acid analysis system using citrate buffers and a sodium chloride gradient, as described previously (Ahmed et al., 1986). The eluent was monitored for amino acids by postcolumn reaction with *o*-phthaldialdehyde and fluorometric detection. Gas chromatography–mass spectrometry (GC/MS) was performed on a Hewlett Packard Model 5890 gas chromatograph equipped with a Model 7673A autosampler and a Model 5970 mass selective detector, using a 30-m DB-5 capillary column (J & W Scientific). The temperature program was as follows: 2 min at 70 °C, ramp to 245 °C at 5 °C/min, then to 280 °C at 15 °C/min, hold for 4 min at 280 °C.

Sample Preparation for GC/MS. Samples of lens protein (3 mg in 3 mL of 7.8 N HCl) were hydrolyzed for 24 h at 110 °C under nitrogen in 13 × 100 mm screw-top test tubes (Teflon-lined caps). The hydrolysis conditions were selected to maximize the percent conversion of FL to furosine, as described by Erbersdobler (1986). Varying the protein concentration between 0.3 and 2 mg/mL did not affect the results of the analyses. The hydrolysates were dried in vacuo using a Savant Speed-Vac concentrator and converted to their *N*-trifluoroacetyl methyl ester (TFAME) derivatives for analysis. For preparation of the methyl esters, the hydrolysate was dissolved in 1.5 mL of 1 N methanolic HCl and heated for 0.5 h at 65 °C. Solvent was evaporated at room temperature under a stream of nitrogen and the product redissolved in 0.5 mL of dry methylene chloride. Trifluoroacetic anhydride (1 mL) was then added and the mixture incubated at room temperature for 1 h to obtain the trifluoroacetyl amides. After removal of the solvent under a stream of nitrogen, the sample was dissolved in 0.3 mL of methylene chloride, and 2 μL of this solution was injected for GC/MS analysis.

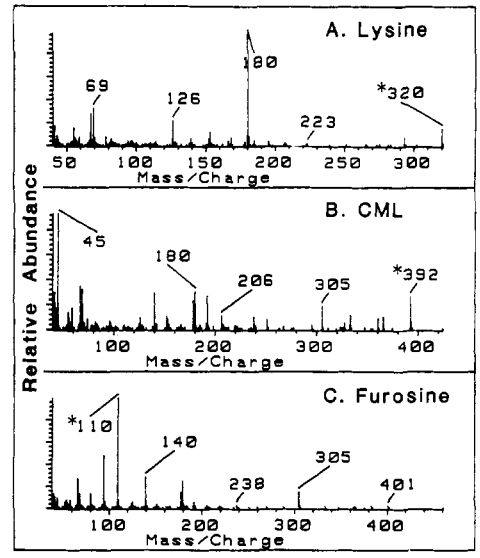

FIGURE 2: Full-scan mass spectra of the TFAME derivatives of (A) lysine, (B) CML, and (C) furosine. The ions used for quantitation of these compunds by SIM-GC/MS are indicated by asterisks, i.e., the m/z = 320, 392, and 110 ions for lysine, CML, and furosine, respectively.

GC/MS Analyses. The lysine, CML, and FL content of lens proteins was determined by selected ion monitoring GC/MS (SIM-GC/MS). Lysine and CML were measured directly as their TFAME derivatives, and FL as the TFAME derivative of furosine which is formed in about 40% yield from FL during acid hydrolysis (Erbersdobler, 1986). The mass spectra of the TFAME derivatives of these compounds are shown in Figure 2. The m/z 320, 392, and 110 ions were used for detection and quantitation of lysine, CML, and furosine, respectively, by SIM-GC/MS. These ions were chosen because they provided good sensitivity and reproducibility, and were well resolved from ions of other components in the mixture. The areas of the CML and furosine peaks were normalized to the lysine area for each sample to correct for variations in protein concentration, recovery, and derivatization efficiency. Absolute amounts of CML and FL in lenses were quantitated by standard addition, adding known amounts of CML and fFL standards (0–12 nmol/3 mg of protein) to a lens protein pool prior to hydrolysis (Kennedy, 1984). The initial amounts of CML and FL in the pool were determined by extrapolation of the standard addition curve to the x axis (zero added standard). Using the lysine concentration obtained by amino acid analysis and the extrapolated concentration of CML and FL, we calculated calibration factors for conversion of the relative ion areas (CML/lysine and furosine/lysine) to the corresponding molar ratios of CML and FL to lysine in individual lens samples. Each lens sample was hydrolyzed, derivatized, and analyzed as above, with an aliquot of the hydrolysate removed to quantitate lysine content by amino acid analysis. All samples were assayed twice (on 2 separate days), and average results are reported. A lens protein pool was included in each set of analyses (maximum of 12 samples per set) for quality control. Day-to-day coefficients of variation in the CML/lysine and furosine/lysine ratios in the pool were 10.5 and 10.7% for CML and FL, respectively (n = 19).

RESULTS

Measurement of CML and FL in Lens Proteins. Figure 3 shows typical ion chromatograms obtained on analysis of young and old human lens proteins by SIM-GC/MS. The identification of the lysine, CML, and furosine peaks was confirmed

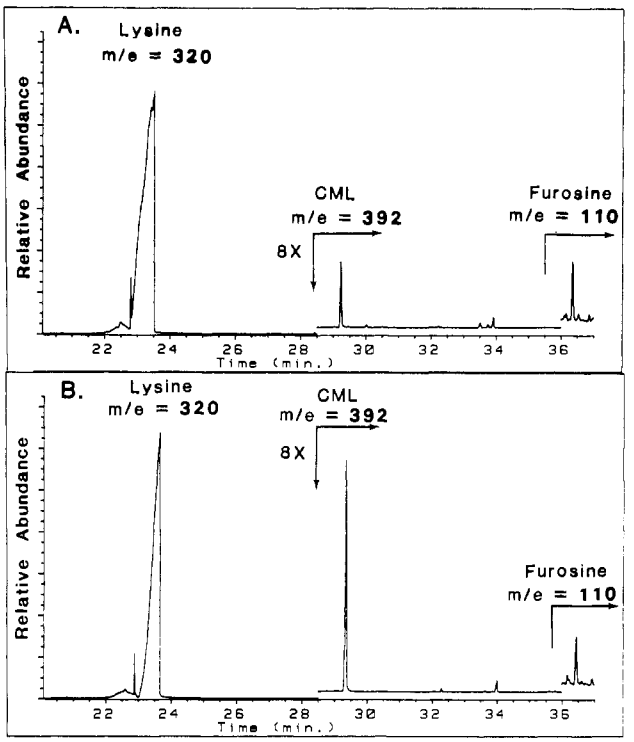

FIGURE 3: Typical SIM-GC/MS chromatograms of lens samples. Hydrolysis and derivatization conditions were as described under Experimental Procedures. (A) Donor age: 17 years. (B) Donor age: 68 years. The peaks for lysine (m/z = 320), CML (m/z = 392), and furosine (m/z = 110) are at 23, 29, and 36 min, respectively. The scale is expanded 8×, starting at 28.5 min.

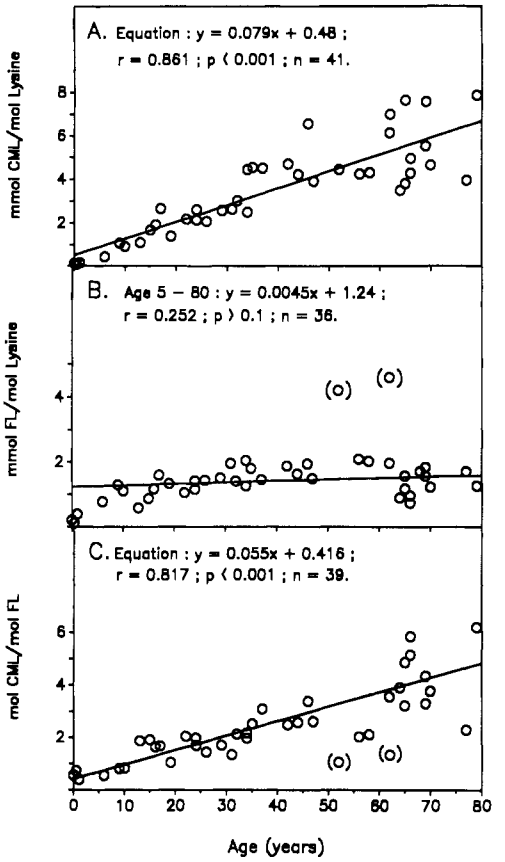

FIGURE 4: Levels of CML and FL in lens protein versus age. (A) Age-dependent accumulation of CML in lens proteins. The line drawn is a least-squares fit to the data. (B) Relationship between lens content of FL and lens age. The line drawn on the graph is a least-squares fit to the data for lenses aged 5–80, excluding the two outlier points (in parentheses). The average extent of glycation of protein was 1.4 ± 0.4 mmol of FL/mol of lysine (n = 39). (C) Age-dependent increase in the ratio of CML to FL in lens proteins. The two outlier points [in parentheses and identified in (B)] were excluded from the least-squares fit.

by retention time and by measuring the relative heights of other ions in the peaks (m/z = 180 and 305), compared to the ion ratios in the authentic compounds (Figure 2). While the lysine peak (Figure 3) was generally skewed because of sample load (equivalent to 20 μg of lens protein injected per analysis), the response (peak area) for the m/z 320 ion was linear within the range of 0.2–2-μL sample injection. The relative peak areas (CML/lysine and furosine/lysine) measured on duplicate samples analyzed on consecutive days had mean relative deviations of 5.1 ± 3.6 and 6.1 ± 4.5%, respectively.

Age-Dependent Changes in CML and FL Content of Lens Proteins. Since lysine is the parent amino acid from which CML and FL are derived, analytical data were normalized to the lysine content of the protein; however, we and others (Dilley & Harding, 1975; Coghlan & Augusteyn, 1977) observed that the lysine content of lens protein did not change significantly with age. Figure 4A shows that there was a strong correlation ($p < 0.001$) between lens age and the CML content of lens proteins. A least-squares fit to the data yielded a slope of 0.08 mmol of CML/mol lysine per year through the range of 0–79 years, with CML representing from 0.02 to 0.8% of the total lysine residues in lens protein. Figure 4B shows that, in contrast to the trend with CML, there was little evidence of a relationship between lens age and levels of FL in lens protein ($p > 0.1$). The ratio of FL/lysine remained relatively constant between age 5 and 79, yielding an average value of 1.4 ± 0.4 mmol of FL/mol of lysine. Only trace levels of FL were detectable in infant lenses; however, there was a rapid increase in FL between birth and age 5. The adult level of FL attained at about age 5 may reflect the time at which rates of lens protein synthesis and turnover decline to adult levels. Although all samples were obtained from apparently normal donors, two samples (in parentheses in Figure 4B) showed significantly higher levels of FL than the remainder of the population and may represent lenses from donors with undiagnosed, maturity-onset diabetes.

Figure 4C shows the relationship between the CML and FL content of lens proteins. The data indicate that while the level of FL reaches a steady state early in life (Figure 4B), its oxidation product, CML, continues to accumulate with age. The molar ratio of CML to FL increased linearly with age, from a ratio of ~0.5 in infancy to ~5 at 80 years of age, i.e., approximately a 10-fold increase during a normal lifespan. After about 10 years of age, CML was in fact the major product of protein glycation identifiable in the adult lens. The slope of the least-squares fit in Figure 4C indicates that FL is converted to CML at a rate of about 7% per year. The total extent of modification of lysine as CML and FL increases from ~0.1% at age 5 to ~1% at age 80.

Distribution of CML and FL among Lens Protein Fractions. In some experiments (n = 6), the CML and FL content of the soluble, insoluble, and capsular protein fractions of lenses was examined in detail for two reasons: first, it has been reported that dityrosine, a product of oxidation of tyrosine residues in lens proteins, is located exclusively in capsular proteins (McNamara & Augusteyn, 1980); thus, CML, the product of oxidation of FL, might also be found primarily in the capsular protein; second, since insoluble proteins are found largely in the older, nuclear fraction of lens proteins, the ratio of CML to FL might be significantly higher in the insoluble protein fraction. These analyses (data not shown) indicated

that neither FL nor CML was enriched in the capsular protein fraction and that, since the capsule accounted for only 1–2% of total lens protein, the majority (>97%) of the CML and FL in lens protein was in the crystallin protein fraction. There was also no consistent enrichment of CML or FL in the insoluble (sedimented by centrifugation for 20 min at 16000*g*), compared to the soluble, proteins in the lens homogenate.

Discussion

Relationship between Age and Glycation of Human Lens Proteins. Because glycation is a slow reaction under biological conditions, it is most readily measured in long-lived proteins, such as hemoglobin, collagens, and lens crystallins. While the extent of glycation of these proteins is known to increase with hyperglycemia in diabetes (Kennedy & Baynes, 1984), several studies have also suggested that the glycation of proteins increases with age in the bovine, human, and rat lens (Chiou et al., 1981; Garlick et al., 1984; Swamy et al., 1987). Our observation that glycation of human lens protein is essentially constant after about age 5 stands in contrast to the results of these earlier studies. Because of the specificity of the GC/MS assay and the number and age range of samples evaluated in this work, the conclusions of the present work are judged to be more reliable. They are also supported by a more limited study from our laboratory (Patrick et al., 1989) using an assay based on reduction of lens proteins with NaB^3H_4 and isolation of glycated amino acids by affinity chromatography on phenylboronate resin. This method measures total glycated amino acids, including glycated amino-terminal amino acids and fructose adducts to lysine residues in lens protein (Walton & McPherson, 1987). Using this assay, we also failed to detect any change in the total glycation of proteins in the adult lens.

Earlier studies arguing for an age-dependent increase in glycation of bovine lens proteins (Chiou et al., 1981) were based largely on changes in total incorporation of radioactivity from NaB^3H_4 into lens proteins. Only limited data were provided regarding increases in FL, measured as [^{3}H]hexitollysine, so that a statistical analysis of the significance of the age-related increase in glycation of bovine lens proteins is not possible. The conclusions of these studies and those on human lenses were also based on analysis of a smaller number of samples than the present work, and in the case of the human lenses, there was a significant overlap in the estimate of hexitollysine content of young and old normal lenses (Figure 4; Garlick et al., 1984). Studies on rat lens protein (Swamy et al., 1987) show clearer evidence for increased glycation with age; however, the greatest changes occur within the first 4 months of life. Analysis of the data on both water- and urea-soluble proteins between 4 and 28 months of age suggests that further age-related increases in glycation of total lens proteins may not be statistically significant. Thus, the studies in rats are consistent with our own data which show a large increase in glycation of human lens proteins between infancy and age 5, but a relatively constant value thereafter. We conclude that there is not a statistically significant increase in glycation in the adult lens and that an age-dependent increase in glycation, per se, is not a likely cause of lens pathology. It should be emphasized, however, that even a steady-state level of glycation may contribute to subsequent, cumulative chemical modifications of lens proteins, which, in the long term, may be a factor in the development of both senile and diabetic cataracts.

CML Is a Major End Product of Glycation of Adult Lens Protein. The original goal of these studies was to determine the quantitative significance of oxidation of glucose adducts to protein in vivo. The results of this study indicate that in the adult lens the concentration of the oxidation product, CML, actually exceeds that of its precursor, FL. Thus, to date, CML may be described as the major, chemically identified end product of glycation of protein in the adult lens. These data indicate that measurement of FL alone is not an adequate indicator of the overall extent of modification of long-lived proteins by glucose.

Age-Dependent Accumulation of CML in Lens Proteins. Because of the slow turnover of lens crystallins and alterations in their physical and chemical properties with age, the lens has been used frequently as a model for studies on the aging of protein in vivo (Zigler & Goosey, 1981). There is evidence for both nonoxidative and oxidative changes in lens crystallins with age and/or cataractogenesis, including racemization, deamidation, and oxidation of amino acids (Harding & Dilley, 1976; Zigler & Goosey, 1981; Harding & Crabbe, 1984); however, assessment of the extent of oxidative damage often yields contradictory results. Thus, while there is an age-dependent increase in oxidation of sulfhydryl groups in normal rat lenses (Swamy & Abraham, 1987; Ozaki et al., 1987), similar trends were not observed in normal human or guinea pig lenses (Yu et al., 1985). Oxidation of methionine residues in human lenses also appears to be associated with cataractogenesis, as opposed to normal aging (Zigler & Goosey, 1981; Brot & Weissbach, 1983). These studies on age-related oxidation of cysteine and methionine residues may be complicated because the oxidation of sulfur amino acids can be induced and reversed by enzymatic mechanisms (Brot & Weissbach, 1983). In contrast, CML appears to be an inert end product which is excreted largely in intact form in urine (Liardon et al., 1987), and measurement of its accumulation in proteins may prove useful as an indicator of age-dependent oxidative modification of proteins. While it is admittedly a derivative of another chemical modification of protein, FL, the level of FL in proteins remains relatively constant with age (Baynes et al., 1989). On the basis of the data in Figure 4A,B, the rate of oxidation of FL may be estimated as slightly less than 0.1 mol of CML (mol of FL)$^{-1}$ year^{-1} in lens proteins, assuming negligible turnover of these proteins. Studies are in progress to determine if similar ratios of CML to FL, i.e., rates of oxidation of FL, are observed in other tissues and how oxidation rates are affected by various dietary (caloric restriction), pathological (inflammatory), and genetic (progeric) conditions which may affect the rate of formation of oxygen radicals in tissues.

Relationship between Glycation, Oxidation, and Aging. Glycation is seen as the first step in a cascade of Maillard or Browning reactions between reducing sugars and amines (Maillard, 1912; Njoroge & Monnier, 1989). The long-term products of this reaction between glucose and protein or nucleic acids have been termed advanced glycosylation end products (AGE) (Cerami, 1985). According to the glycation theory of aging (Monnier, 1989), AGE products accumulate in and contribute to the aging of tissues by causing age-dependent chemical modification, cross-linking, insolubilization, and loss of function of proteins and DNA. Alternatively, according to the free radical theory, aging is interpreted as the result of cumulative oxidative, free radical damage to biomacromolecules (Cutler, 1984; Mehlhorn & Cole, 1985; Harman, 1987). The age-dependent accumulation of CML, a product of oxidation of glycated proteins, is consistent with both of these theories, and its accumulation suggests that other products of glycation and oxidation may also accumulate with age in lens proteins. The lens, however, is a unique physiological system with limited protein turnover, and it is not clear

that the chemical mechanisms involved in the aging of lens proteins are significant in other tissues where proteins are turned over more rapidly. Indeed, there is no evidence that, even in the lens, CML is detrimental to protein function, although it may be one factor in the age-dependent acidification of lens proteins (Zigler & Goosey, 1981). In any case, the accumulation of CML in proteins may prove useful as an indicator of protein exposure to oxidative stress and the extent to which oxidation is involved in the aging of proteins.

The linear relationship between lens age and CML content suggests that the rate of oxidation of FL and formation of CML is constant throughout adult life. Thus, our data provide no support for the hypothesis that free radical defense mechanism decline with age (Sohal, 1988) in the mammalian lens. Our observations on the lens are consistent with other work (submitted for publication) from our laboratory which reveal that the urinary concentration of CML and the ratio of CML to FL in human urine do not change with age. Thus, to the extent that urine is the mirror of whole-body oxidation and metabolism, these results also indicate that whole-body oxidative stress is constant with age. Our results are also in agreement with work by Ames and co-workers (Saul et al., 1987; Ames, 1988) which indicates that the urinary excretion of thymine glycol and thymidine glycol, products of oxidation of DNA, are constant with age. While cumulative damage to protein and DNA is limited by turnover and repair processes, our results support the argument that chronic oxidative stress to these molecules may lead to the accumulation of products which place limits on the lifespan of species.

Registry No. CML, 5746-04-3; FL, 21291-40-7.

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