

6-15-1993

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### Publication Info

Published in *Journal of Biological Chemistry*, Volume 268, Issue 17, 1993, pages 12348-12352.

This research was originally published in the *Journal of Biological Chemistry*. Wells-Knecht MC, Huggins TG, Dyer DG, Thorpe SR, Baynes JW. Oxidized Amino Acids in Lens Protein with Age: Measurement of o-Tyrosine and Dityrosine in the Aging Human Lens. *Journal of Biological Chemistry*. 1993; 268:12348-12352. © the American Society for Biochemistry and Molecular Biology.

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## Oxidized Amino Acids in Lens Protein with Age

### MEASUREMENT OF *o*-TYROSINE AND DITYROSINE IN THE AGING HUMAN LENS\*

(Received for publication, December 22, 1992, and in revised form, February 26, 1993)

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The concentrations of *ortho*-tyrosine (*o*-Tyr) and dityrosine (DT) were measured in noncataractous human lenses in order to assess the role of protein oxidation reactions in the aging of lens proteins. The measurements were conducted by selected ion monitoring-gas chromatography/mass spectrometry using deuterium-labeled internal standards, which provided both high sensitivity and specificity for the quantitation of *o*-Tyr and DT. Between ages 1 and 78 years, the *o*-Tyr concentration in lens proteins varied from 0.3 to 0.9 mmol of *o*-Tyr/mol of Phe ( $n = 19$ ), while DT ranged from 1 to 3  $\mu$ mol of DT/mol of Tyr ( $n = 30$ ). There were no significant changes in levels of *o*-Tyr with lens age. There was a statistically significant, but only slight, increase in DT in lens proteins with age (~33% increases between ages 1 and 78,  $r = 0.5$ ,  $p < 0.01$ ). At the same time, total protein fluorescence, measured at DT wavelengths ( $E_x = 317$  nm,  $E_m = 407$  nm), increased 11-fold between ages 1 and 78 and correlated strongly with age ( $r = 0.82$ ,  $p < 0.0001$ ). Although the fluorescence maxima of lens proteins were similar to those of DT, DT accounted for less than 1% of the DT-like fluorescence in lens protein at all ages. These observations indicate that oxidation of Phe and Tyr plays a limited role in the normal aging of lens proteins *in vivo*.

Lens proteins are among the longest-lived proteins in the body and, as such, provide an excellent model system for studying the long-term effects of aging and oxidative stress on the chemical composition of proteins. Numerous studies (reviewed in Refs. 1 and 2) have shown that with age the proteins of the human lens undergo a broad range of molecular and macromolecular changes, including racemization, deamidation and oxidation of amino acids, accumulation of covalently attached fluorescent compounds, and aggregation and insolubilization. The age-related increase in lens fluorescence (3) and the association of fluorescence both with high molecular weight protein aggregates and the water-insoluble fraction of lens protein (3, 4) suggest that fluorescent compounds may be involved in the cross-linking and insolubilization of lens protein, both during normal lens aging and in cataractogenesis.

\* This work was supported by United States Public Health Service Grants DK19971 and DK25373. 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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The age-related, visible wavelength fluorescence in lens protein, often described as non-tryptophan fluorescence, is a characteristic alkaline blue fluorescence with broad excitation and emission maxima at 315–340 and 415–440 nm, respectively (3, 4). Blue fluorescent compounds of known structure which have been isolated from the lens include dityrosine (DT)<sup>1</sup> (5–7), anthranilic acid (6), and  $\beta$ -carboline (8). All three of these compounds are oxidation products of aromatic amino acids (either Tyr or Trp), and DT and  $\beta$ -carboline would contribute to the cross-linking of proteins. Although DT could be a major contributor to the non-tryptophan fluorescence that accumulates in human lens proteins with age, there is no information available concerning changes in amounts of DT or other oxidation products of aromatic amino acids in the noncataractous, aging human lens.

Because of the prior evidence for the presence of DT in lens and the increase in blue fluorescence in lens proteins with age, we set out to measure the concentration of DT in noncataractous human lens and to determine whether DT also increased with age in this tissue. Since DT formation requires the reaction between neighboring Tyr residues and its yield may be affected by protein structure, the content of a second oxidized amino acid, *ortho*-tyrosine (*o*-Tyr), was also measured in lens protein as a function of age. *o*-Tyr is formed on oxidation of Phe residues in protein and, in the experiments described in the accompanying paper (9), was recovered in greater yield than DT during oxidation of model proteins *in vitro*. In the present studies, we have applied sensitive and specific assays for quantifying *o*-Tyr and DT in lens proteins using selected ion monitoring-gas chromatography/mass spectrometry (SIM-GC/MS). In addition, the total alkaline blue fluorescence and the DT contribution to this fluorescence were also measured, in order to determine the fraction of total fluorescence derived from DT in the aging lens. The results described below indicate that *o*-Tyr and DT are present in only trace concentrations in lens proteins, that *o*-Tyr content does not increase with age, and that DT content increases only slightly with age. However, alkaline blue fluorescence increases linearly with age in lens proteins, although DT itself accounts for <2% of the total fluorescence at any age.

#### EXPERIMENTAL PROCEDURES

**Materials**—Unless otherwise indicated, reagents were of the highest quality obtainable from either Sigma or Aldrich. Deuterium-labeled *o*-Tyr ( $d_4$ -*o*-Tyr, deuterated in four positions on the phenyl ring) was prepared by  $\text{Cu}^{2+}/\text{H}_2\text{O}_2$  oxidation of  $d_5$ -Phe, and DT was prepared by peroxidase/ $\text{H}_2\text{O}_2$  treatment of L-Tyr, as described in

<sup>1</sup> The abbreviations used are: DT, dityrosine; CML,  $N^{\epsilon}$ -(carboxymethyl)lysine; HFBA, heptafluorobutyric acid; MCO, metal-catalyzed oxidation; *o*-Tyr, *ortho*-tyrosine; SIM-GC/MS, selected ion monitoring-gas chromatography/mass spectrometry.

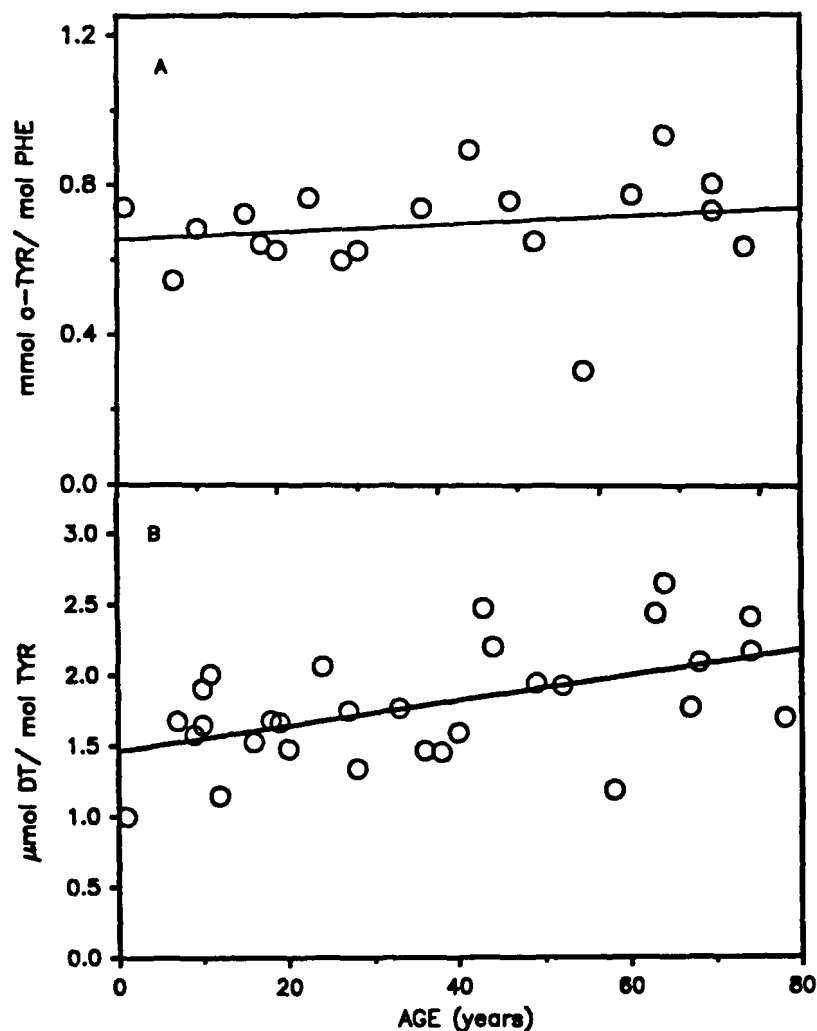


FIG. 1. Concentrations of o-Tyr and DT in lens protein versus age.

A, for measurement of o-Tyr, lenses ( $n = 19$ ) were decapsulated, hydrolyzed, and analyzed for o-Tyr content by SIM-GC/MS as described under "Experimental Procedures"; between ages 1 and 78 yr,  $r = 0.18$ ,  $p = 0.2$ . B, lenses ( $n = 30$ ) were processed for measurement of DT as described under "Experimental Procedures"; between ages 1 and 78,  $r = 0.52$  and  $p < 0.01$ .

detail previously (9). Deuterium-labeled DT ( $d_6$ -DT, three deuteriums on each phenyl ring), used as the internal standard for quantitation of DT in protein, was synthesized from  $d_4$ -L-Tyr (98% pure; Cambridge Isotope Laboratories) by the same procedure as DT.

**Extraction of Lens Protein and Protein Hydrolysis**—Human lenses were obtained from the South Carolina Lions Eye Bank, Columbia, SC and the Medical College of Georgia Eye Bank, Augusta, GA. Individual whole lenses were weighed and then decapsulated. The lens was placed in 1 ml of ice-cold deionized water; after 10–15 min, the sample was vortexed gently, and the capsule was removed with forceps. The lens was then homogenized in 1 ml of water in a Reacti-Ware glass homogenizer (Pierce). Lens homogenates were dialyzed for 18–24 h against several changes of 500 volumes of deionized water at 4 °C. Protein concentration was measured by the biuret method (10) using bovine serum albumin as standard. Proteins were hydrolyzed under vacuum in 6 N HCl (2 mg of protein/ml of acid, 5 ml total) for 24 h at 110 °C. The hydrolysates were dried *in vacuo* using a Savant Speed-Vac concentrator (Savant Instruments, Farmingdale, NY) to remove acid and then redissolved in 1 ml of H<sub>2</sub>O.

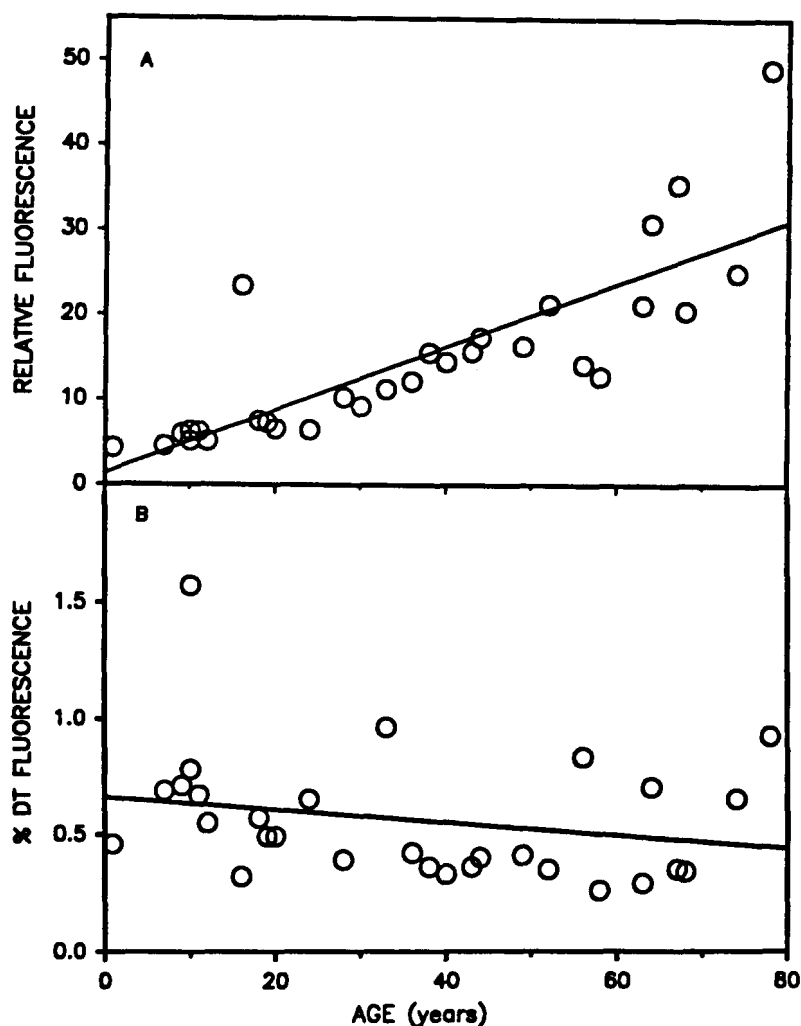
**Measurement of o-Tyr**—A 2-mg aliquot of protein hydrolysate prepared as described above was removed, and the deuterium-labeled internal standards  $d_4$ -o-Tyr (156 ng) and  $d_5$ -Phe (100 μg) were added to the sample. The internal standards were added after hydrolysis of the protein because of partial hydrogen-deuterium exchange during hydrolysis. Amino acids were derivatized as their *N,O*-acetyl isopropyl esters and analyzed by SIM-GC/MS, as described previously (9). The  $m/z = 265$  and 269 ions were used for measurement of o-Tyr and  $d_4$ -o-Tyr, and the  $m/z = 190$  and 195 ions were used for Phe and  $d_5$ -Phe, respectively. Essentially identical results were obtained using the  $m/z = 146$  and 150 ions which were measured simultaneously for o-Tyr and  $d_4$ -o-Tyr, and the  $m/z = 249$  and 254 ions used for Phe and  $d_5$ -Phe, respectively. Quantification was based on an external calibration curve using o-Tyr and Phe as standards and  $d_4$ -o-Tyr and  $d_5$ -Phe as

internal standards. Standard curves were constructed using 0–200 ng of o-Tyr and 0–200 μg of Phe. The o-Tyr measured in lens proteins was normalized to its precursor Phe. Based on amino acid analysis (9) of the hydrolysates, the ratio of Phe/Val showed no statistical change with age, indicating that the Phe content of lens protein was also unchanged with age. Val was chosen for this analysis because post-translational modifications of Val are not known or expected in lens protein with age, and because Val is well resolved from other amino acids in the mixture. A lens protein pool was analyzed for quality control yielding a day-to-day coefficient of variation of 14.8% ( $n = 4$ ).

**Measurement of DT**—A 5-mg aliquot of protein hydrolysate prepared as described above, with 10 ng of  $d_6$ -DT added as internal standard, was mixed with an equal volume of 4% heptafluorobutyric acid (HFBA). The mixture was applied to a 1-ml Supelclean LC-18 solid phase extraction (SPE) tube (Supelco Inc., Bellefonte, PA) equilibrated in 2% HFBA. The column was washed with 1 ml of 2% HFBA, then eluted with 3 ml of 2% HFBA in 10% acetonitrile. The eluate, which contained >90% of the applied DT but <10% of the other amino acids, was concentrated to dryness under vacuum, and the amino acids were derivatized as their *N,O*-pentafluoropropyl isopropyl esters. After esterification of carboxyl groups with 1 N isopropanolic HCl (9), samples were evaporated to dryness at room temperature under a stream of nitrogen, and the product was redissolved in 0.8 ml of acetonitrile. Pentafluoropropionic acid anhydride (0.2 ml) was added, and the mixture was incubated at room temperature for 0.5 h to obtain the *N,O*-pentafluoropropyl isopropyl ester derivatives. Solvent was removed under nitrogen, samples were redissolved in 100 μl of benzene, and 2 μl were injected for SIM-GC/MS analysis.

SIM-GC/MS analysis of DT was performed using a VG 70SQ Mass Spectrometer (electron impact mode) (VG Instruments, Manchester, UK), equipped with a Hewlett-Packard 5890 Gas Chromatograph

FIG. 2. Relationship between total lens fluorescence (A) and contribution of DT to total DT-like fluorescence (B) with age. A, aliquots (200  $\mu\text{g}$ ) of the lenses described in the legend to Fig. 1B were digested with chymotrypsin and analyzed for total protein fluorescence at  $E_x = 317$  nm,  $E_m = 407$  nm, as described under "Experimental Procedures"; Between ages 1 and 78,  $r = 0.82$  and  $p < 0.0001$ . B, the amount of DT measured by SIM-GC/MS for each sample in Fig. 1B was converted to relative fluorescence units, based on comparison to authentic DT (see text). Points represent the calculated fluorescence contribution for DT divided by the total fluorescence measured in each sample shown in A, multiplied by 100.



(Hewlett-Packard). Chromatography was carried out on a 30-m DB-5 capillary column (J & W Scientific, Folsom, CA) using the following temperature program: 1 min at 70 °C, ramp to 260 °C at 5 °C/min, ramp to 290 °C at 15 °C/min, hold for 10 min at 290 °C. The full scan ( $m/z$  750–1050) mass spectra of DT revealed the following major ions:  $m/z$  1028 =  $M^+$ ;  $m/z$  1009 =  $M^+ - F$ ;  $m/z$  865 =  $M^+ - (F_5C_2CO_2)$ ;  $m/z$  853 =  $M^+ - (2(CO_2C_3H_7) + H)$ ;  $m/z$  805 =  $M^+ - (F_5C_2CO_2 + OC_3H_7 + H)$  (data not shown). Although the molecular ion was visible in the spectra, the more intense  $m/z = 865$  and 871 ions were chosen for quantitation of DT and  $d_6$ -DT, respectively. Essentially identical results were obtained using the  $m/z = 805$  and 811 ions, for DT and  $d_6$ -DT, respectively, which were measured simultaneously. Quantification was based on an external calibration curve using DT as standard and  $d_6$ -DT as internal standard; the standard curve was constructed using 0–2.5 ng of DT. The DT content of the protein was normalized to the content of the precursor amino acid Tyr, determined by amino acid analysis of the hydrolysate (9). As described above for Phe, there was no statistically significant change in the ratio of Tyr to Val with age, so that Tyr content was also considered to be constant with age. Repeated analysis of a lens protein pool yielded a day-to-day coefficient of variation of 18.8% ( $n = 6$ ).

**Measurement of Fluorescence in Lens Proteins**—Because of the significant increase in fluorescence of lens protein following acid hydrolysis, endogenous protein fluorescence ( $E_x = 317$  nm,  $E_m = 407$  nm) was measured in lens homogenates solubilized by chymotrypsin digestion (9). Essentially identical results were obtained for protein solubilized using urea (data not shown). Three-dimensional fluorescence plots were constructed as described previously (9).

**Statistical Analyses**—Data were analyzed for statistical significance using nonparametric statistics by the Spearman rank-correlation method.

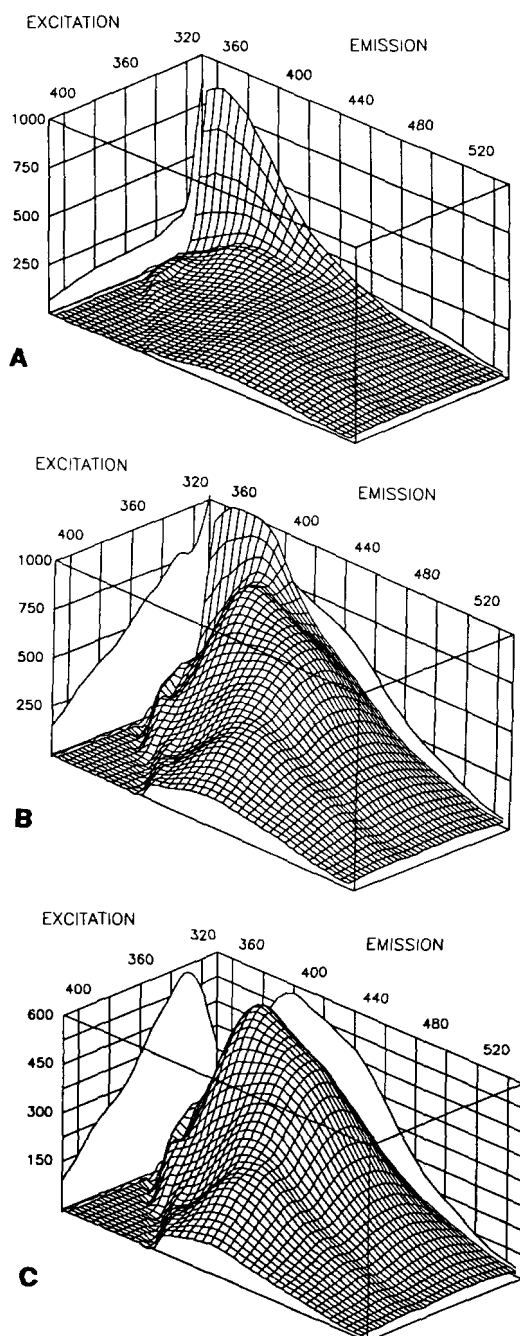
## RESULTS

**Measurements of o-Tyr and DT Concentration in Lens Proteins**—Results of SIM-GC/MS analyses for o-Tyr in lens

proteins from a group of donors aged 1–78 years are summarized in Fig. 1A. The concentration of o-Tyr was  $0.69 \pm 0.14$  mmol of o-Tyr/mol of Phe (mean  $\pm$  S.D.). Statistical analysis revealed no significant correlation between o-Tyr concentration and lens age.

In previous studies we described a reversed-phase HPLC assay for measurement of DT formed during radiolytic and MCO of the model proteins RNase and lysozyme (9). However, this assay proved to be of limited value for analysis of lens proteins because of the trace concentrations, 1–3  $\mu\text{mol}$  of DT/mol of Tyr, present in lens protein, compared to  $>100$   $\mu\text{mol}$  of DT/mol of Tyr in the oxidized model proteins. For this reason, a SIM-GC/MS assay was developed for measurement of DT in lens proteins, using  $d_6$ -DT as an internal standard. The results of analyses of a series of lens homogenates from persons aged 1–78 years are summarized in Fig. 1B. Statistical analysis indicated a weakly significant ( $r = 0.52$ ,  $p < 0.01$ ) 33% increase in the DT content of lens between ages 1 and 78. Finally, because of the limited amount of protein available, we were unable to detect DT in individual lens capsules. However, analyses of pools of young ( $n = 6$ ; age =  $15 \pm 5$  yr) and old ( $n = 5$ ; age =  $63 \pm 7$  yr) yielded comparable levels of DT, 6.2 and 5.4 pmol of DT/mg of protein, respectively.

**Age-dependent Changes in Lens Fluorescence**—While we observed only a slight increase in the DT content of lens proteins with age using the SIM-GC/MS assay, Satoh *et al.* (3) reported an age-dependent increase in alkaline blue fluo-



**FIG. 3. Comparison of three-dimensional fluorescence spectra of young and old lens protein.** Protein samples were solubilized with chymotrypsin, as described under "Experimental Procedures," and fluorescence spectra were recorded for samples at a protein concentration of 50  $\mu\text{g}/\text{ml}$  in 25 mM phosphate buffer, pH 11. Spectra shown are for lenses ages 1 year (A) and 67 years (B); the difference spectrum in C was obtained by subtraction of A from B. The spectra shown are representative of spectra obtained with other lenses evaluated in this study.

rescence ( $E_x = 340$  nm,  $E_m = 420$  nm) associated with urea-soluble proteins from normal lenses. Since DT could contribute significantly to fluorescence at these wavelengths, the relationship between age and total DT-like fluorescence in lenses was measured, using the fluorescence maxima for DT ( $E_x = 317$  nm,  $E_m = 407$  nm). The data in Fig. 2A illustrate that lens fluorescence at DT wavelengths increased over 10-fold between 1 and 78 years of age with a strong correlation between lens fluorescence and age ( $r = 0.82$ ,  $p < 0.0001$ ), in

agreement with the earlier work of Satoh *et al.* (3). As noted above, the low levels of DT in lens protein prevented accurate measurement of DT fluorescence following reversed-phase HPLC of protein hydrolysates. Therefore, to estimate the contribution of DT to total fluorescence at DT wavelengths, the amount of DT in each lens preparation, measured by SIM-GC/MS (Fig. 1B), was converted to relative fluorescence units, based on a standard curve made using authentic DT. As shown in Fig. 2B, however, when expressed as a fraction of total protein fluorescence, DT was a minor contributor to total lens fluorescence at DT wavelengths, accounting on average for <1% of the DT-like fluorescence in lens proteins, regardless of age.

Despite the limited contribution of DT to total lens alkaline blue fluorescence, the three-dimensional fluorescence plots in Fig. 3 illustrate that the excitation and emission maxima of fluorescence in older lenses, approximately 330 and 400 nm, respectively, are similar to those of DT at 317 and 407 nm. The fluorescence in the 1-year-old lens (Fig. 3A) is attributable to Trp, while the fluorescence in the 67-year-old lens (Fig. 3B) shows a broad and complex envelope in the visible wavelength range. The difference spectrum between the old and young lens samples (Fig. 3C) has fluorescence maxima at 330 and 400 nm, similar to the wavelengths obtained on radiolytic and MCO of model proteins (9).

#### DISCUSSION

In previous work on the chemical modification of lens proteins, we identified two carbohydrate-derived adducts, *N*<sup>c</sup>-(carboxymethyl)lysine (CML) (11) and pentosidine (12, 13), which accumulated with age in lens proteins. Both of these compounds are derived from oxidation of carbohydrates or carbohydrate adducts to protein (14) and also increase with age in human skin collagen (15). Because CML and pentosidine are derived from both glycation and oxidation reactions, they have been termed glycooxidation products (14). We have interpreted the accumulation of these glycooxidation products in tissue proteins as evidence for cumulative, oxidative aging of proteins and biomarkers of oxidative stress *in vivo* (14). However, we recognized that this direct interpretation might be compromised by the fact that both CML and pentosidine are derived from a prior chemical modification of protein by glycation, *i.e.* that the accumulation of glycooxidation products depends on the exposure of proteins to both glycative and oxidative modifications. In addition, the exact precursors and routes of formation of CML and pentosidine in protein are uncertain (14), which also complicates a straightforward conclusion regarding the relationship between glycooxidation products and oxidative stress. We have, therefore, sought to identify amino acid oxidation products which accumulate with age in lens proteins in order to develop an independent index of oxidative damage and stress and to confirm our interpretation of the significance of glycooxidation products.

The compounds *o*-Tyr and DT seemed ideal candidates for assessing age-dependent oxidative damage to lens proteins because they were readily detected during oxidation of model proteins *in vitro* (9), they are stable to acid hydrolysis of protein, and the presence of DT in lens proteins has been reported (5–7). Despite these considerations, we observed that the level of DT in the normal lens was 1% or less than that previously reported for cataractous lenses (7), and that there was, at most, only a modest and weakly significant increase with age. Further, although higher concentrations of DT in cataractous lens capsules, compared to decapsulated lenses, were also reported (7), we found no evidence for unique localization of DT in the capsules of normal lenses, nor

evidence of an increase in DT in lens capsules with age. The differences between our and the earlier studies might relate specifically to differences between normal and cataractous lenses; higher levels of protein cross-links and oxidative damage might be expected in cataractous lenses. The greater sensitivity and specificity of SIM-GC/MS to detect DT in the complex mixture from lens hydrolysates could account for quantitative differences in our results and those of earlier work.

In comparison to DT, *o*-Tyr proved, in model studies (9), to be a much more sensitive indicator of MCO damage to proteins. Although there are no prior reports on levels of *o*-Tyr in lens or other tissue proteins, we did observe that *o*-Tyr was present at over 100-fold the concentration of DT in lens proteins, and that, like DT, it did not accumulate appreciably with age. These results suggest that the trace levels of DT and *o*-Tyr being detected in lens proteins probably reflect true oxidative damage, but do not exclude the possibility that the small amounts might have been formed during storage and processing of the samples. In any case, the levels of these oxidation products are not likely to be of any physiological or pathological significance.

The changes in the fluorescence spectrum of lens proteins with age (Fig. 3) are consistent with the accumulation of oxidative damage, since similar fluorescence spectra were obtained on both radiolytic and metal-catalyzed oxidation of model proteins, such as RNase (9, 16) and lysozyme (9). DT is also considered to contribute to the increase in non-tryptophan, alkaline blue-green fluorescence in lens with age (3, 4). However, despite significant increases in DT-like fluorescence in lens proteins with age (Fig. 2A), DT was a minor (<1%) contributor to the total fluorescence at DT wavelengths at any age, and there was not a comparable or corresponding increase in DT itself.

The present studies indicate that, despite the usefulness of *o*-Tyr and DT as indicators of oxidative damage to model proteins (9, 16) and the evidence for age-dependent oxidative changes in the lens, these compounds are not useful as indicators for assessing oxidative damage to lens proteins with age. It is possible that much of the oxidative damage to aromatic amino acids in lens proteins may be tryptophan-derived and photolytic in origin (1, 2), and that *o*-Tyr and DT may not be formed in high yields under these circumstances (17). We conclude at this point that glycooxidation products, which are derived from reducing sugars and sugar adducts to protein and are more readily oxidized than Phe and Tyr, are more sensitive indicators of oxidative damage to proteins. We are continuing to search for other amino acid oxidation products which may accumulate with age in lens proteins.

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