Effect of Collagen Turnover on the Accumulation of Advanced Glycation End Products

Nicole Verzijl
Jeroen DeGroot
Suzanne R. Thorpe
Ruud A. Bank
J. Nikki Shaw

See next page for additional authors

Follow this and additional works at: https://scholarcommons.sc.edu/chem_facpub

Part of the Chemistry Commons

Publication Info
Published in Journal of Biological Chemistry, Volume 275, Issue 50, 2000, pages 39027-39031. This research was originally published in Journal of Biological Chemistry. Verzijl N, DeGroot J, Thorpe SR, Bank RA, Shaw JN, Lyons TJ, Bijlsma JWJ, Lafeber FPJG, Baynes JW, TeKoppele JM. Effect of Collagen Turnover on the Accumulation of Advanced Glycation End Products. Journal of Biological Chemistry. 2000; 275:39027-39031. © the American Society for Biochemistry and Molecular Biology. http://dx.doi.org/10.1074/jbc.M006700200

This Article is brought to you by the Chemistry and Biochemistry, Department of at Scholar Commons. It has been accepted for inclusion in Faculty Publications by an authorized administrator of Scholar Commons. For more information, please contact digres@mailbox.sc.edu.
PROTEIN SYNTHESIS
POST-TRANSLATION MODIFICATION
AND DEGRADATION:
Effect of Collagen Turnover on the
Accumulation of Advanced Glycation End
Products

Nicole Verzijl, Jeroen DeGroot, Suzanne R.
Thorpe, Ruud A. Bank, J. Nikki Shaw,
Timothy J. Lyons, Johannes W. J. Bijlsma,
Floris P. J. G. Lafeber, John W. Baynes and
Johan M. TeKoppele
J. Biol. Chem. 2000, 275:39027-39031.
doi: 10.1074/jbc.M006700200 originally published online September 6, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M006700200

Find articles, minireviews, Reflections and Classics on similar topics on the JBC Affinity Sites.

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 6 of which can be accessed free at http://www.jbc.org/content/275/50/39027.full.html#ref-list-1
Collagen molecules in articular cartilage have an exceptionally long lifetime, which makes them susceptible to the accumulation of advanced glycation end products (AGEs). In fact, in comparison to other collagen-rich tissues, articular cartilage contains relatively high amounts of the AGE pentosidine. To test the hypothesis that this higher AGE accumulation is primarily the result of the slow turnover of cartilage collagen, AGE levels in cartilage and skin collagen were compared with the degree of racemization of aspartic acid (%D-Asp, a measure of the residence time of a protein). AGE (N\textsuperscript{-}carboxymethyl)lysine, N\textsuperscript{-}carboxyethyl)lysine, and pentosidine) and %D-Asp concentrations increased linearly with age in both cartilage and skin collagen (p < 0.0001). The rate of increase in AGES was greater in cartilage collagen than in skin collagen (p < 0.0001). %D-Asp was also higher in cartilage collagen than in skin collagen (p < 0.0001), indicating that cartilage collagen has a longer residence time in the tissue, and thus a slower turnover, than skin collagen. In both types of collagen, AGE concentrations increased linearly with %D-Asp (p < 0.0005). Interestingly, the slopes of the curves of AGES versus %D-Asp, i.e. the rates of accumulation of AGES corrected for turnover, were identical for cartilage and skin collagen. The present study thus provides the first experimental evidence that protein turnover is a major determinant in AGE accumulation in different collagen types. From the age-related increases in %D-Asp the half-life of cartilage collagen was calculated to be 117 years and that of skin collagen 15 years, thereby providing the first reasonable estimates of the half-lives of these collagens.

Nonenzymatic glycation is a post-translational modification of proteins in vivo, which is initiated by the spontaneous reaction of sugars with lysine residues in proteins and eventually results in the formation of advanced glycation end products (AGEs),\textsuperscript{1} such as N\textsuperscript{-}carboxymethyl)lysine (CML), N\textsuperscript{-}carboxyethyl)lysine (CEL), and pentosidine (1–3). Because AGES are irreversible chemical modifications of protein, they accumulate with age in long-lived proteins such as lens crystallins and tissue collagens (1, 3–9). Because collagen molecules in articular cartilage have an exceptionally long lifetime (>100 years) (10, 11), they are highly susceptible to the accumulation of AGES. Indeed, in comparison to other collagen-rich tissues (such as skin), articular cartilage contains relatively high amounts of pentosidine (3, 12). Although differences in AGE levels between different proteins have been attributed to differences in protein turnover rates (3, 12–14), no quantitative evidence to support this assumption is available.

To compare protein turnover rates, information on the residence time of a protein in tissue can be obtained from the racemization of aspartic acid. Amino acids are incorporated into peptides and proteins as the L-enantiomers. During aging, racemization slowly converts the l-form into a racemic mixture of d- and l-forms. Aspartic acid is one of the fastest racemizing amino acids (15, 16), so that d-aspartic acid can be detected in proteins that turn over slowly. There is a close relationship between chronological age and d-aspartic acid accumulation in the white matter of the brain (17), the eye lens (16), dentin (18), and bone (19, 20). Furthermore, several studies have shown the age-related accumulation of d-aspartic acid in cartilaginous tissues such as the intervertebral disc (21), rib cartilage (22), and articular cartilage (11, 23). Measurement of aspartic acid racemization has proven to be a valuable tool to derive quantitative information on protein residence time, i.e. protein turnover (11, 23).

The present study was designed to establish the effect of protein turnover rate on the kinetics of accumulation of CML, CEL, and pentosidine in cartilage and skin collagen. Differences in the rate of accumulation of these AGES were then corrected for differences in residence times of these collagens in the tissue, estimated from the degree of racemization of aspartic acid. The results indicate that differences in levels of AGES in cartilage and skin collagens may be almost solely attributed to differences in the rate of turnover of these proteins.

**EXPERIMENTAL PROCEDURES**

**Tissue Samples**—Macroscopically normal human articular cartilage was obtained post mortem from femoral condyles within 18 h after

---

\textsuperscript{1} The abbreviations used are: AGEs, advanced glycation end products; CEL, N\textsuperscript{-}carboxyethyl)lysine; CML, N\textsuperscript{-}carboxymethyl)lysine; ANCOVA, analysis of covariance.
Effect of Collagen Turnover on AGE Accumulation

FIG. 1. AGES accumulate linearly with chronological age in both cartilage and skin collagen, but the accumulation rate is higher in cartilage collagen. A–C show the age-dependent increase in the concentration of CML (A), CEL (B), and pentosidine (C) in human articular cartilage collagen (●) and skin collagen (○). Normal full depth femoral condylar cartilage of 20 subjects in the age range 3–81 years and normal skin from the medial aspect of the buttock of 26 subjects in the age range 19–91 years were analyzed. Linear regression analysis and ANCOVA were performed on cartilage (solid line) and skin (dashed line) collagen data ≥18 years.

dead from patients who had no clinical history of joint disorders or diabetes (n = 23). Skin samples were obtained from the medial aspect of the buttock of nondiabetic donors (n = 27; healthy volunteers or at autopsy within 6 h after death). In a few additional cases, paired cartilage and (knee) skin samples (n = 6; age 76.4 ± 4.4 years; mean ± S.D.) were obtained at autopsy within 18 h after death. Bovine cartilage and skin were obtained from the metacarpophalangeal joint of a 6-month-old calf. All samples were stored at −20 °C until analyzed.

**Purification of Collagen**—Articular cartilage collagen was isolated by depleting the tissue of all proteoglycans and other non-collagenous proteins using sequential enzymatic treatment with chondroitinase ABC (Sigma), trypsin (Roche Molecular Biochemicals), and Streptomyces hyaluronidase (Sigma) for 20 h at 37 °C as described by Schmidt et al. (24). This procedure resulted in the removal of more than 97% of the glycosaminoglycans with a minimal loss of collagen (<1%, measured as hydroxyproline). Skin collagen was isolated by sequential extraction for 24 h at 4 °C with 1 M NaCl, chloroform/methanol (2:1), and 0.5 M acetic acid as described previously (5). All collagen samples were lyophilized following purification.

**CML, CEL, and Pentosidine Analyses**—For analysis of CML and CEL, 2 mg of dry collagen was reduced overnight at 4 °C in 500 μL of 0.1 m NaBH₄ (Sigma) in 0.1 m sodium borate buffer (pH 9.0). To remove excess NaBH₄, the samples were washed 3 times with 5 mL of deionized water and then hydrolyzed in 1 mL of 6 M HCl at 100 °C for 4 h. After hydrolysis, an aliquot of these samples (30%) was reserved for pentosidine analysis. CML, CEL, and lysine content of the collagen hydrolysates were simultaneously measured as their N-acetyl-N-(t-butoxycarbonyl)-L-cysteine (Sigma) in a MIDAS autosampler (Spark Holland, Emmen, The Netherlands). Derivatized D- and L-Asp were separated on a C18 column (TSKgel ODS-80TM, 150 × 4.6 mm, 5-μm particle size, Tosoh-Haas, Stuttgart, Germany) at a flow rate of 0.8 mL/min. Solvent A was 50 mM sodium acetate (pH 5.9). Solvent B was 80% (v/v) methanol (Rathburn, Walkerburn, UK) and 20% (v/v) solvent A. Elution of D- and L-Asp was achieved in two steps as follows: isocratic elution with 9% (v/v) solvent B for 5 min and then a linear increase in the content of solvent B to 100% over a period of 5 min. The column was washed for 10 min with 100% solvent B and equilibrated for 10 min in 9% (v/v) solvent B before injecting the next sample. Fluorescence was monitored at 340/440 nm. Standard solutions of either D- or L-Asp were calibrated using nonchiral FMOC (9-fluorenylmethyl chloroformate) derivatization as described previously (29) and mixed into standards of known % D-Asp.

All of the % D-Asp data were corrected for the amount of D- and L-Asp present in papain (5–10% of the aspartic acid in the samples) and for racemization during the hydrolysis step. The latter was performed either by subtracting the intercept of the graph for % D-Asp versus donor age (for the cartilage and skin collagen age ranges) or by subtracting the % D-Asp measured in young bovine cartilage and skin collagen (for the paired cartilage and skin samples of n = 6 aged donors), with both methods resulting in comparable corrections.

**Measurement of in Vitro Rate of Aspartic Acid Racemization in Different Collagen Types**—In order to estimate the rate of aspartic acid racemization in cartilage collagen (type II) and in skin collagen (type I), a heating experiment on young bovine cartilage and skin collagen was performed as described by Ohtani (30). Dry collagen samples (2–7 mg) were placed in glass test tubes and heated at 120, 140, or 160 °C for 15–120 min. Samples were digested in papain (5 units/mL in papain buffer, 100 μL per mg of collagen), and the racemization of aspartic acid (i.e. the D/L-Asp ratio) was determined after hydrolysis, as described above.

Amino acid racemization follows first-order kinetics described by Equation 1 (31, 32),

\[
\ln(1 + D/L)/(1 - D/L)) = 2k_{rpm} \cdot t + \ln((1 + D/L)/(1 - D/L))_{t=0} \quad \text{(Eq. 1)}
\]

in which D/L is the ratio of D- to L-Asp; t is any given time during racemization; k_{rpm} is the rate constant of aspartic acid racemization, and the logarithmic term at t = 0 describes the amount of D-Asp measured in unheated collagen. The Arrhenius equation was used to determine the activation energy for the racemization of aspartic acid residues in each type of collagen. This equation describes the quantitative relationship between temperature and reaction rate (33) as shown in Equation 2,
The intrinsic rate constant for racemization of aspartic acid is identical in cartilage and skin collagen. Arrhenius plot showing the identical relation between the rate constant for racemization of aspartic acid (k_{Asp})(h)) and the reciprocal of the absolute temperature (1/T) for bovine cartilage (○) and skin (△) collagen, determined in a heating experiment as described under “Experimental Procedures.” Data are mean ± S.D. The Arrhenius equations for cartilage (solid line) and skin (dashed line) were ln(k_{Asp}(h)) = −11.472 (1/T) + 14.77 and ln(k_{Asp}(h)) = −11.472 (1/T) + 15.15, respectively.

Statistical Analysis—Linear regression analysis, analysis of covariance (ANCOVA), and paired Student’s t tests were performed with SPSS version 8.0 for Windows (SPSS, Chicago); p < 0.05 was considered to represent statistically significant differences.

RESULTS AND DISCUSSION

In the present study, AGE levels in cartilage and skin collagen were compared with the degree of racemization of aspartic acid, a measure of the residence time of a protein (11, 15, 23, 34, 35), to provide quantitative data on the relationship between turnover rates and AGE accumulation.

AGE Levels in Human Cartilage Collagen and Skin Collagen—After maturity has been reached (> 18 years), CML levels increase linearly with age in both human articular cartilage collagen (r = 0.89, p < 0.0001; Fig. 1A) and skin collagen (r = 0.80, p < 0.0001; Fig. 1A). The rate of CML accumulation with age is significantly higher in cartilage collagen than in skin collagen (ANCOVA with age as covariant; p < 0.0001). Consistent with the results for CML, the levels of CEL and pentosidine in cartilage and skin collagen also increase linearly with age (p < 0.0001 in all cases; Fig. 1B and C), with the rate of accumulation being higher in cartilage than in skin collagen (p < 0.0001 for both; Fig. 1B and C). Taken together, our data on three different AGEs consistently show that AGE levels in cartilage collagen increase more rapidly with age and eventually reach higher levels than in skin collagen. In the late 1980s, Sell and Monnier (3) already reported higher levels of pentosidine in cartilage compared with skin from elderly subjects and suggested that this difference may be due to a higher turnover of skin. Up to this time, no quantitative data on protein turnover have been related to AGE levels to substantiate this suggestion.

Rate Constant of Racemization of Aspartic Acid in Cartilage and Skin Collagen—Using % D-Asp levels in cartilage and skin collagen to compare the residence time of collagen in these two tissues assumes that the intrinsic rate constant of aspartic acid racemization at body temperature is identical in both collagens. Because differences in the rate of aspartic acid racemization have been shown to exist between collagens from other sources, e.g. bone and dentin (30, 36), a comparison of these rates in cartilage and skin collagen was necessary. Therefore, the relation between the rate constant of racemization of L-Asp into D-Asp and temperature was determined for both cartilage and skin collagen in an in vitro heating experiment. The Arrhenius equations, describing the quantitative relation between the rate constant of racemization of aspartic acid (k_{Asp}) and the reciprocal of the absolute heating temperature (1/T), are shown in Fig. 2. This relation is identical for cartilage and skin collagen (ANCOVA with 1/T as covariant; p = 0.58). The temperatures of human knee cartilage and skin are lower than 37 °C but comparable; the intra-articular temperature in the knee joint is 31.7–33.9 °C (37, 38), and the mean body skin temperature is 32.6 °C (39). Extrapolating the Arrhenius equations to 33 °C shows that the respective rate constants for aspartic acid racemization in cartilage and skin collagen in the human body are 1.78 × 10^{-6} and 1.71 × 10^{-6} per year, respectively. Consequently, the % D-Asp levels in cartilage and skin collagen can be used validly as a measure of residence time of the collagen in the tissue.

Racemization of Aspartic Acid in Human Cartilage Collagen and Skin Collagen—Inasmuch as the intrinsic rate constant of aspartic acid racemization at body temperature is identical in human articular cartilage collagen and skin collagen, rates of turnover of these collagens were compared by measuring the degree of racemization of aspartic acid. In both cartilage collagen and in skin collagen, the racemization of aspartic acid (% D-Asp) increases linearly with age (r = 0.95, p < 0.0001, and...
The goal of the present study was to establish the effect of protein turnover rate on the accumulation of AGEs in cartilage and skin collagen. As such, the rate of protein turnover is an important determinant in AGE accumulation.

The cartilage and skin collagen samples for the age range (shown in Fig. 1 and 3) were obtained from two separate groups of donors. To confirm that within a single donor AGE levels and collagen residence time are also higher in cartilage collagen than in skin collagen, pentosidine and %D-Asp levels were measured in an additional set of paired samples of cartilage and skin collagen of aged donors (n = 6). Again, both the pentosidine content and %D-Asp were significantly higher in cartilage collagen than in skin collagen (paired Student’s t test; p < 0.0001 and p < 0.005, respectively; data not shown).

Relation between Collagen AGE Levels and Residence Time—The goal of the present study was to establish the effect of protein turnover rate on the accumulation of AGEs in cartilage and skin collagen. As such, the relation between AGE and %D-Asp levels represents the rate of AGE accumulation in proteins. Incubation of collagen in high glucose under aerobic conditions (40) did not induce aspartic acid racemization,2 which confirms that %D-Asp levels provide a measure of protein residence time that is independent of glycation. These results allowed us to examine the direct relation between AGE concentrations and %D-Asp in human articular cartilage collagen (●) and skin collagen (△). Skin samples are those described in the legend to Fig. 1. Cartilage samples are those with both AGE concentrations and %D-Asp ≤125% of the maximum skin collagen values (resulting in 12, 15, and 11 subjects in the age range 3–55 years in A–C, respectively). Linear regression analysis and ANCOVA were performed on all cartilage (solid line) and skin (dotted line) collagen data.

Since the rate constants of aspartic acid racemization in cartilage and skin collagen are identical, %D-Asp values could accurately based on a large number of samples (n = 23) covering a broad age range (3–81 years). Based on the model described by Maroudas et al. (11), it was calculated that the observed increase in %D-Asp in cartilage collagen of 0.0258 ± 0.0015% per year (mean ± S.D.; Fig. 3, ages 3–81 years) corresponds to a half-life of 117 years (95% confidence interval, 62–480 years).

The cartilage and skin collagen samples for the age range (shown in Fig. 1 and 3) were obtained from two separate groups of donors. To confirm that within a single donor AGE levels and collagen residence time are also higher in cartilage collagen than in skin collagen, pentosidine and %D-Asp levels were measured in an additional set of paired samples of cartilage and skin collagen of aged donors (n = 6). Again, both the pentosidine content and %D-Asp were significantly higher in cartilage collagen than in skin collagen (paired Student’s t test; p < 0.0001 and p < 0.005, respectively; data not shown).

Calculation of Half-lives of Human Cartilage and Skin Collagen—Although a slow turnover of collagen in articular cartilage has often been indicated (10, 11, 41–43), little experimental evidence exists for such a statement. Based on aspartic acid racemization data of only three cartilage collagen samples, Maroudas et al. (11) showed that the half-life of cartilage collagen is between 100 and 400 years. In the present study, we determined the half-life of articular cartilage collagen more accurately based on a large number of samples (n = 23) covering a broad age range (3–81 years). Based on the model described by Maroudas et al. (11), it was calculated that the observed increase in %D-Asp in cartilage collagen of 0.0258 ± 0.0015% per year (mean ± S.D.; Fig. 3, ages 3–81 years) corresponds to a half-life of 117 years (95% confidence interval, 62–480 years).

Since the rate constants of aspartic acid racemization in cartilage and skin collagen are identical, %D-Asp values could accurately based on a large number of samples (n = 23) covering a broad age range (3–81 years). Based on the model described by Maroudas et al. (11), it was calculated that the observed increase in %D-Asp in cartilage collagen of 0.0258 ± 0.0015% per year (mean ± S.D.; Fig. 3, ages 3–81 years) corresponds to a half-life of 117 years (95% confidence interval, 62–480 years).

Since the rate constants of aspartic acid racemization in cartilage and skin collagen are identical, %D-Asp values could also be used to calculate the rate of collagen turnover in skin based on this model. For skin collagen, the 0.0115 ± 0.0018% increase in %D-Asp per year (mean ± S.D.; Fig. 3, ages 19–91 years) indicates a half-life of 14.8 years (95% confidence interval: 9.4–22.3 years). Although no data on the half-life of human skin collagen are available in the literature, our data provide a reasonable first estimate.

Besides human articular cartilage, aspartic acid racemiza-
Aspartic Acid Racemization in Collagen from Cartilage, Skin, and Dentin—In Table I, the in vivo rates of d-Asp accumulation in cartilage and skin collagen (based on Equation 1 and the data presented in Fig. 3) are compared with rates that had previously been determined in purified collagen (11, 44). Only collagens in which the activation energy for aspartic acid racemization is comparable (present study and Ref. 36), i.e., have similar intrinsic rates of racemization, are included. The rate of d-Asp accumulation in skin collagen is clearly the slowest and that in dentin collagen the fastest, whereas d-Asp accumulation in cartilage collagen has an intermediate rate. The rate of d-Asp accumulation in cartilage collagen as determined in the present study is close to the rate in cartilage collagen as determined by Maroudas et al. (11). Because Table I compares collagens with similar intrinsic aspartic acid racemization rates, these in vivo rate constants of d-Asp accumulation probably reflect the rate of turnover of the protein. Therefore, we conclude that the rate of collagen turnover is higher in skin than in cartilage (see above), whereas dentin collagen has the slowest rate of turnover.

In summary, we have shown that in both cartilage and skin collagen, AGE levels (CEL, CML, and pentosidine) are linearly related to levels of aspartic acid racemization, a direct measure of protein residence time, implying that protein turnover is an important factor in the accumulation of AGEs. Furthermore, consistent with a slower turnover of cartilage collagen compared with skin collagen, both % d-Asp levels and AGE levels are higher in cartilage collagen than in skin collagen. As a result, the rate of accumulation of AGEs corrected for turnover (based on % d-Asp) is similar for cartilage and skin collagen, which provides direct experimental evidence that protein turnover is a major determinant of AGE accumulation.

Acknowledgments—We thank Mark Sochaski (University of South Carolina, Columbia, SC) and Tom VanDenBroek and Esther Oldenhinkel (TNO Prevention and Health, Leiden, The Netherlands) for excellent technical assistance.

REFERENCES

1. Dunn, J. A., Patrick, J. S., Thorpe, S. R., and Baynes, J. W. (1989) Biochemistry 28, 9464–9468
2. Ahmed, M. U., Brinkmann Frye, E., Degenhardt, T. P., Thorpe, S. R., and Baynes, J. W. (1997) Biochem. J. 324, 565–570
3. Sell, D. R., and Munnier, V. M. (1988) J. Biol. Chem. 263, 21597–21602
4. Uchiyama, A., Oishi, T., Takahashi, M., Kushida, K., Inoue, T., Fujie, M., and Marumo, F. (1992) Biochem. J. 285, 9–16
5. Dunn, J. A., McCance, D. R., Thorpe, S. R., Lyons, T. J., and Baynes, J. W. (1991) Biochemistry 30, 1205–1210
6. Lavoie, T. J., Bailie, K. F., Dyer, D. G., Dunn, J. A., and Baynes, J. W. (1991) J. Clin. Invest. 87, 1910–1915
7. Bank, R. A., Baylis, M. T., Lafeber, F. P. J. G., Maroudas, A., and TeKoppele, J. M. (1998) Biochem. J. 330, 345–351
8. DeGroot, J., Verzijl, N., Bank, R. A., Lafeber, F. P. J. G., Bijlsma, J. W. J., and TeKoppele, J. M. (1999) Arthritis & Rheum. 42, 1003–1009
9. Verzijl, N., DeGroot, J., Oldenhinkel, E., Bank, R. A., Thorpe, S. R., Baynes, J. W., Baylis, M. T., Bijlsma, J. W. J., Lafeber, F. P. J. G., and TeKoppele, J. M. (2000) Biochem. J. 350, 381–387
10. Maroudas, A. (1980) in Studies in Joint Disease I (Maroudas, A., and Holibarem, E. J., eds) pp. 59–86, Pitman Medical, London
11. Maroudas, A., Palla, G., and Gilav, E. (1992) Connect. Tissue Res. 33, 151–158
12. Maroudas, A., Palla, G., and Gilav, E. (1992) Connect. Tissue Res. 33, 151–158
13. van Boekel, M. A. (1991) Annu. Rev. Med. 42, 525–552
14. Man, E. H., Sandhouse, M. E., Burg, J., and Fisher, G. H. (1983) Science 220, 1407–1408
15. Helfman, P. M., and Bada, J. L. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2891–2894
16. Masters, P. M., Bada, J. L., and Ziegler, J. S. J. (1977) Nature 268, 71–73
17. Man, E. H., Sandhouse, M. E., Burg, J., and Fisher, G. H. (1983) Science 220, 1407–1408
18. Helfman, P. M., and Bada, J. L. (1976) Nature 262, 279–281
19. Pfeiffer, H., Mornstad, H., and Teivens, A. (1995) Int. J. Leg. Med. 108, 19–23
20. Ohtani, S., Matsuishita, Y., Kobayashi, Y., and Kishi, K. (1998) J. Forensic Sci. 43, 949–953
21. Ritz, S., and Schutz, H. W. (1993) J. Forensic Sci. 38, 633–640
22. Pfeiffer, H., Mornstad, H., and Teivens, A. (1995) Int. J. Leg. Med. 108, 24–26
23. Maroudas, A., Bayliss, M. T., Uchitelkauhansky, N., Schneiderman, R., and Gilav, E. (1998) Arch. Biochem. Biophys. 356, 61–71
24. Schmidt, M. B., Mow, V. C., Chun, L. E., and Eyle, D. R. (1990) J. Orthop. Res. 8, 353–363
25. Bank, R. A., Beekman, E., Verzijl, N., de Roos, A., Saakke, A. N., and TeKoppele, J. M. (1997) J. Chromatogr. B. Biomed. Appl. 703, 37–44
26. Dyer, D. G., Blackledge, J. A., Thorpe, S. R., and Baynes, J. W. (1991) J. Biol. Chem. 266, 11054–11060
27. Aswad, D. W. (1984) Anal. Chem. 56, 405–409
28. Kim, Y. J., Sah, R. L., Doong, J. Y., and Grodzinsky, A. J. (1988) Anal. Chem. 60, 174–176
29. Bank, R. A., Jansen, E. J., Beeckman, B., and TeKoppele, J. M. (1996) Anal. Biochem. 240, 167–176
30. Ohtani, S. (1998) Am. J. Forensic Med. Pathol. 19, 284–287
31. Bada, J. L., and Protasch, R. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1331–1334
32. Smith, G. G., Williams, K. M., and Winnacott, D. M. (1978) J. Org. Chem. 43, 1–5
33. Arhennius, S. (1889) Z. Phys. Chem. 4, 226–248
34. Cloos, P. A., and Fleediusus, C. (2000) Biochem. J. 343, 473–480
35. Gineyts, E., Cloos, P. A., Borel, O., Grimaud, L., Delmas, P. D., and Garnero, P. (2000) Biochem. J. 345, 481–485
36. Ohtani, S., Kato, S., and Sugeno, H. (1996) Growth Dev. Aging 60, 1–6
37. Horvath, S. M., and Hollander, J. L. (1949) J. Clin. Invest. 28, 469–473
38. Haimovic, N. (1989) Prog. Clin. Biol. Res. 330, 1169–1180
39. Choi, J. K., Miki, K., Sagawa, S., and Shikami, K. (1997) Int. J. Biometabol. 41, 68–77
40. Weller Knecht, M. C., Lyons, T. J., McCance, D. R., Thorpe, S. R., and Baynes, J. W. (1997) J. Clin. Invest. 100, 839–846
41. Libby, W. F., Berger, R., Mead, J. F., Alexander, G. V., and Ross, J. P. (1964) Science 146, 1170–1172
42. Eyle, D. R., McDevitt, C. A., Billingham, M. E., and Muir, H. (1980) Biochem. J. 188, 823–837
43. Freeman, M. A. R. (1999) J. Orthop. Res. 17, 3–8
44. Ohtani, S., and Yamamoto, K. (1991) J. Forensic Sci. 36, 792–800