Biochem. J. (1981) **197**, 759–762
Printed in Great Britain

Collagen cross-linking compounds in human urine

Zeenat GUNJA-SMITH* and Robert J. BOUCEK
Department of Medicine (R-127), University of Miami School of Medicine, P.O. Box 016960, Miami, FL 33101, U.S.A.

(*Received 10 April 1981/Accepted 21 May 1981*)

We report the isolation and chemical characterization of collagen cross-linking compounds, 3-hydroxypyridinium and dihydroxylysinonorleucine, from human urine.

Collagen cross-linking compounds are biochemical markers of polymeric collagen, and their quantification in human urine would provide an unambiguous and clinically useful measure of degradation of extracellular collagen. In their initial forms, collagen cross-linking compounds are chemically unstable Schiff-base compounds; however, dehydrodihydroxylysinonorleucine and 3-hydroxypyridinium, a proposed cross-link, are sufficiently stable to withstand chemical and enzymic degradation of collagen under laboratory conditions (Robins & Bailey, 1973; Fujimoto & Moriguchi, 1978; Eyre & Oguchi, 1980), and therefore might be present in human urine.

We have described techniques for isolating urinary desmosines, the chemically stable lysyl-derived intermolecular cross-links of elastin (Gunja-Smith & Boucek, 1981). Similar methods were used for the isolation and chemical characterization of 3-hydroxypyridinium and dihydroxylysinonorleucine from human urine.

Materials and methods

Materials

NaB^3H_4 (10 Ci/mmol) was purchased from ICN, Irvine, CA, U.S.A. Achilles-tendon collagen was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. A sample containing 3-hydroxypyridinium was kindly provided by Dr. David Eyre, Children's Hospital, Boston, MA, U.S.A. Urine, collected from patients with radiographic and clinical-chemistry features of Paget's disease, was provided by Dr. Roy Altman, Professor of Medicine, Division of Arthritis, University of Miami, Miami, FL, U.S.A.

Isolation of 3-hydroxypyridinium from bovine Achilles tendon

3-Hydroxypyridinium was obtained from an acid hydrolysate of bovine Achilles tendon by the method of Fujimoto & Moriguchi (1978), and is henceforth referred to as 3-hydroxypyridinium standard. Fluorescent fractions (2.5 ml each), with fluorescence and u.v.-absorption spectra similar to those reported for 3-hydroxypyridinium by Fujimoto *et al.* (1977), were collected from a phosphocellulose (H^+ form) column (15 cm × 1.5 cm). The standard was co-eluted on the Beckman Amino Acid Analyzer with a sample of 3-hydroxypyridinium, provided by Dr. David Eyre, with a specially programmed buffer system containing 0.35 M-citrate buffer, pH 4.42 (see the legend to Fig. 1).

Isolation of 3-hydroxypyridinium from human urine

Volunteers were placed on a collagen-free diet for 24 h before and during urine collection. A ninhydrin-positive peak (Y), that was co-eluted with the 3-hydroxypyridinium standard (Fig. 1) on the Beckman Amino Acid Analyzer, was isolated from human urine by using a multi-step procedure involving precipitation with acetone (80%, v/v), separation on Sephadex G-15, reduction with $NaBH_4$, hydrolysis (6 M-HCl) and paper chromatography and Amino Acid Analyzer separation as reported by Gunja-Smith & Boucek (1981) for the isolation of urinary desmosines.

Fluorescence spectra

Activation and emission spectra of the 3-hydroxypyridinium standard and peak Y from the urine in 0.1 M-HCl and 20 mM-potassium phosphate buffer, pH 7.4, were measured with an Amicon–Bowman spectrofluorophotometer as reported by Fujimoto *et al.* (1977).

Isolation of dihydroxylysinonorleucine from human urine

Urine collection from patients with Paget's disease of the bone was filtered successively through PM-30, PM-10, UM-2 and UM-0.5 membranes in an Amicon ultrafiltration cell. Each retained fraction

* To whom requests for reprints should be addressed.

0306-3275/81/090759-04$01.50/1

was freeze-dried, resuspended in 0.1 M-pyridine/acetate buffer, pH 5.5, and, after centrifugation, the supernatant solution was passed through a calibrated Sephadex G-15 column. Fractions containing excluded material of high molecular weight and fractions containing retarded peptides were reduced separately with a mixture of NaB^3H_4 (10 Ci/mmol) and $NaBH_4$ (20 mg) for 135 min (Gunja-Smith & Boucek, 1981). The reduced fractions were desalted and hydrolysed in 3 M-toluene-*p*-sulphonic acid (105°C, 18 h), under vacuum, after being flushed with N_2. The hydrolysates were then fractionated on an MR-201 resin (Mark Instruments, Villanova, PA, U.S.A.) cation-exchange column (60 cm × 0.9 cm) by using a complex nine-chamber gradient of sodium citrate buffers (0.25 M-sodium citrate, pH 2.93, and 0.4 M-sodium citrate) (Boucek *et al.*, 1979). Radioactive fractions corresponding to the regions of authentic dihydroxylysinonorleucine were pooled and further fractionated on an MR-208 resin (Mark Instruments) cation-exchange column (30 cm × 0.9 cm) to obtain purified dihydroxylysinonorleucine as described previously (Boucek *et al.*, 1979). The radioactive dihydroxylysinonorleucine was desalted on Dowex 50 (H^+ form).

Smith degradation

Periodate degradation of isolated urinary [^{3}H]-dihydroxylysinonorleucine was performed as described by Robins & Bailey (1975). The mixture (1 ml), containing cross-link and 10 mM-$NaIO_4$ in 0.1 M-sodium citrate buffer, pH 5.3, was kept for 7 min at 20°C in the dark. After addition of 2 M-NaOH, the reaction products were reduced with $NaBH_4$ (5–7 mg, 30 min) and the mixture was desalted on Dowex 50 (H^+ form). The reaction products were then analysed for [^{3}H]proline and [^{3}H]hydroxynorvaline on a Beckman Amino Acid Analyzer.

Results and discussion

A fluorescent and stable (non-reducible) 3-hydroxypyridinium cross-link has been reported in abundance in bone, cartilage and tendon collagens, and is a substituted ring compound, probably derived by the condensation of hydroxylysyl aldehydes and hydroxylysyl residues (Fujimoto *et al.*, 1979; Eyre & Oguchi, 1980).

By employing the procedures for isolating urinary desmosines, the urinary fraction remaining at the origin after development of the paper-chromatographic step was eluted and a portion was applied to the Amino Acid Analyzer. A ninhydrin-positive peak (Y) was eluted after phenylalanine (Fig. 1) in the region of the 3-hydroxypyridinium standard and the 3-hydroxypyridinium provided by Dr. David Eyre. Peak Y material was collected, without reaction with ninhydrin, from the Amino Acid Analyzer, desalted and found to have absorbance spectra and fluorescence excitation and emission spectra identical with those of a 3-hydroxypyridinium standard (Fig. 2) and similar to those

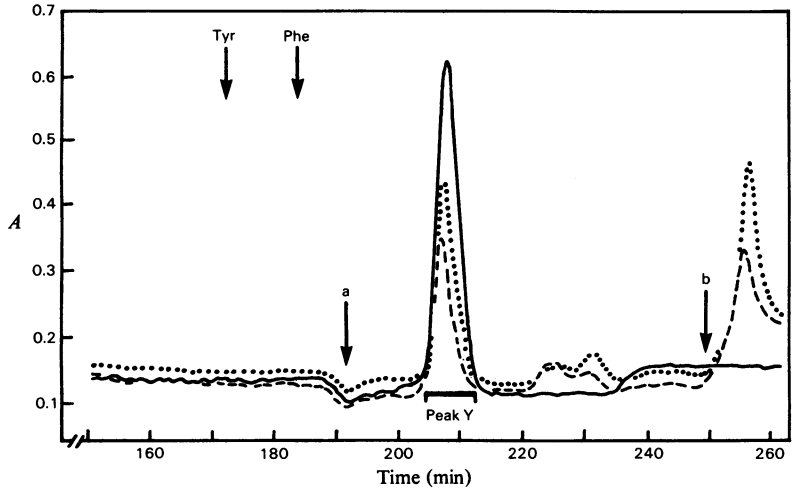

Fig. 1. *Elution patterns of bovine Achilles-tendon and urinary 3-hydroxypyridinium*
——, 3-Hydroxypyridinium from bovine Achilles-tendon collagen; – – – –, urinary peak Y (acetone precipitation method); ·····, urinary peak Y (retained fraction of UM-2 membrane). a, Buffer change from 0.2 M-citrate buffer, pH 4.25, to 0.35 M-citrate buffer, pH 4.42; b, buffer change to 0.35 M-citrate buffer, pH 5.26. For further experimental details see the text.

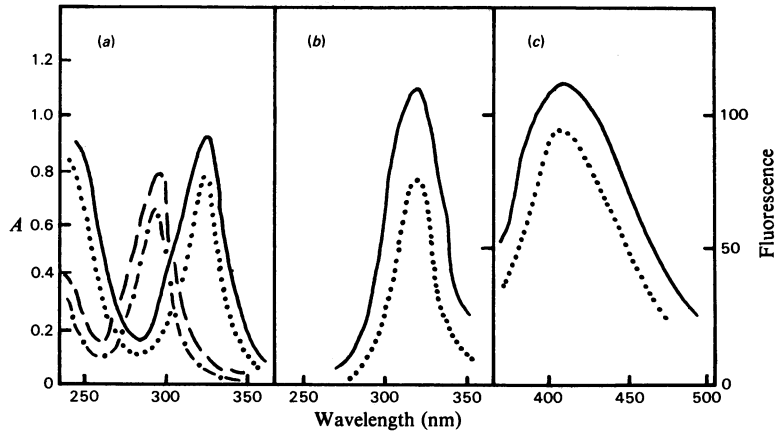

Fig. 2. *(a) U.v.-absorption spectra, (b) activation spectrum (fluorescence at 400 nm) and (c) emission spectrum (excitation at 320 nm) of 3-hydroxypyridinium and peak Y material*

——, 3-Hydroxypyridinium standard in 0.1 M-HCl; -·-·-, peak Y material in 0.1 M-HCl; ——, 3-hydroxypyridinium standard in 20 mM-potassium phosphate buffer, pH 7.4; ·····, peak Y material in 20 mM-potassium phosphate buffer, pH 7.4.

reported by Fujimoto *et al.* (1977) for 3-hydroxypyridinium. Furthermore, the u.v.-absorption spectra of both peak Y material and the 3-hydroxypyridinium standard (Fig. 2*a*) show a shift in absorption maximum from 295 nm in acid solution to 325 nm in neutral solution, and exhibit fluorescence excitation maximum at 295 nm (acid solution) and 320 nm (neutral solution, Fig. 2*b*) and emission maximum at 395 nm (acid solution) and 400–405 nm (neutral solution, Fig. 2*c*),

The standard 3-hydroxypyridinium remains at the origin when subjected to the paper-chromatographic procedure (butan-1-ol/acetic acid/water, 4:1:1, by vol.; 66 h). Therefore an eluate from the origin of the paper-chromatographic separation of a urine sample (see the Materials and methods section) was passed through a phosphocellulose column, and the fluorescent peak, when analysed on the Amino Acid Analyzer, produced a ninhydrin-positive peak in the region of peak Y and that was co-eluted with the 3-hydroxypyridinium standard.

These chemical characterizations strongly support the identity of peak Y material from human urine as 3-hydroxypyridinium.

To ensure that acetone precipitation did not produce or cause a loss of 3-hydroxypyridinium and to estimate the approximate molecular weight of peptidyl-3-hydroxypyridinium, half of the 24 h urine sample was successively passed through an Amicon ultrafiltration cell with PM-30, PM-10, UM-2 and UM-0.5 membranes, and the retained fractions were fractionated as described for desmosines by Gunja-Smith & Boucek (1981). The other half of the urine sample was processed by acetone precipitation. All of the urinary 3-hydroxypyridinium was found in the peptide-containing fraction of retained fractions of UM-2 membrane (~1000-mol.wt. cut-off), and the amount was 13% higher than that isolated by the acetone precipitation method. These findings suggest a peptidyl-3-hydroxypyridinium of molecular weight greater than 900 and that a small fraction of urinary 3-hydroxypyridinium may be lost during the acetone precipitation procedure.

The amount of 3-hydroxypyridinium excreted in 24 h may vary with age. 3-Hydroxypyridinium values of nM-leucine equivalents per 24 h in a 13-year-old female and 22-year-old male (controls) were 551 and 85 respectively.

Urine from patients with Paget's disease of the bone was selected for studies of dihydroxylysinonorleucine, since this cross-link is abundant in bone collagen and bone collagen degradation is extremely active in this disease. Urinary [^{3}H]dihydroxylysinonorleucine-containing peptides were found in retained fractions of UM-0.5 membrane (~500-mol.wt. cut-off), and none in retained fractions of PM-30, PM-10 and UM-2 membranes, indicating a molecular weight of peptides in the region of approx. 500–900. The identity of dihydroxylysinonorleucine was established by the periodate treatment (see the Materials and methods section) of the isolated cross-link, which produced [^{3}H]proline and [^{3}H]hydroxynorvaline, as shown by amino acid analyses. These products suggest the presence of the chemically stable form of the cross-link, dihydroxylysinonorleucine, in human urine. No attempt was made to quantify dihydroxylysinonorleucine per 24 h urine sample or per g of protein.

The isolation of 3-hydroxypyridinium and dihydroxylysinonorleucine is the first report of collagen cross-links in human urine. Assays of urinary 3-hydroxypyridinium and dihydroxylysinonorleucine should be useful in the diagnosis and therapeutic assessments of a variety of osteoclastic diseases in man.

We gratefully acknowledge the excellent technical assistance of Ms. Mariko Ono and the guidance of Dr. J. Frederick Woessner, Jr. This investigation was supported in part by the American Heart Association of Greater Miami and the Florida Affiliate, and by U.S. Public Health Service Research Grant HL-17909.

References

Boucek, R. J., Noble, N. L. & Gunja-Smith, Z. (1979) *Biochem. J.* **177**, 853–860

Eyre, D. R. & Oguchi, M. (1980) *Biochem. Biophys. Res. Commun.* **92**, 403–410

Fujimoto, D. & Moriguchi, T. (1978) *J. Biochem. (Tokyo)* **83**, 863–867

Fujimoto, D., Akiba, K. & Nakamura, N. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1124–1129

Fujimoto, D., Fujii, M., Abe, E. & Suda, T. (1979) *Biochem. Biophys. Res. Commun.* **91**, 24–28

Gunja-Smith, Z. & Boucek, R. J. (1981) *Biochem. J.* **193**, 915–918

Robins, S. P. & Bailey, A. J. (1973) *FEBS Lett.* **33**, 167–171

Robins, S. P. & Bailey, A. J. (1975) *Biochem. J.* **149**, 381–385