AT base pairs, so that hybridization becomes purely a function of the length of the oligonucleotide (*8*). The combined use of a competition assay with tetramethyl ammonium chloride may make it possible to obtain near-universal conditions for specific single-point mutation detection.

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Quantification of the Cross-Link Pentosidine in Serum from Normal and Uremic Subjects

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Pentosidine is a fluorescent cross-link compound that accumulates in human tissues from uremic and diabetic patients. Using SP-Sephadex C-25 pretreatment before reversed-phase HPLC, we developed a method for quantifying pentosidine in the acid hydrolysate of serum. We examined concentrations of pentosidine in serum from 98 patients with end-stage renal disease requiring hemodialysis and from 33 normal control subjects. The mean (± SD) concentration of pentosidine was significantly greater in serum from uremic patients than that from control subjects (1267 ± 695 nmol/L vs 77 ± 40 nmol/L, $P = 0.0001$). There was a significant correlation between serum pentosidine concentrations and age in control subjects ($r = 0.453$, $P < 0.01$), but not in uremic patients. Serum pentosidine significantly increased with the duration of hemodialysis treatment ($r = 0.272$, $P < 0.05$). The greater pentosidine concentrations in uremic patients may be caused by the increased synthesis of pentosidine (perhaps because of the retention of pentosidine precursors), by the retention of pentosidine molecule itself in end-stage renal failure, or both.

Indexing Terms: *chromatography, reversed-phase · hemodialysis · renal function · age-related effects*

Proteins in long-lived tissues are known to be modified by reducing sugars posttranscriptionally. Pentosidine, characterized by Sell and Monnier (*1*) in the human dura mater collagen, is a bifunctional condensation product of arginine, lysine, and ribose, and is formed through the Maillard reaction. Recent studies reveal that pentosidine accumulates in human tissues with age and that its formation is affected by the acceleration of this process in uremic and diabetic conditions (*2, 3*). Although several researchers have investigated the amount of pentosidine in tissues, until recently only one study has dealt with pentosidine in body fluids. Odetti et al. (*4*) developed a combined reversed-phase ion-exchange HPLC method to quantify pentosidine in a hydrolysate of serum and reported that serum pentosidine was increased in diabetes and extremely high in uremia. However, their method is complex, requiring two HPLC systems, and the procedures are tedious.

Previously, we developed a method to quantify pentosidine in hydrolysates of human urine, using SP-Sephadex C-25 pretreatment before HPLC, and found that urinary pentosidine increased exponentially with age in healthy subjects (*5*). In the present study, we applied this method to serum and quantified the pentosidine in serum from control and uremic subjects.

Subjects and Methods

Subjects. Serum samples were obtained from 98 uremic patients, ages 31 to 90 years, who had end-stage renal disease requiring hemodialysis. Seven of these patients were diabetic. For a control group, serum samples were obtained from 33 healthy subjects, ages 18 to 57 years, with no history of renal disease or diabetes

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mellitus and no current known diseases. The procedures followed were in accordance with the principles of the Declaration of Helsinki in 1975, as revised in 1983. All samples were stored at −30 °C until use.

Reagents. The following reagents were obtained from Wako Pure Chemical Industries, Osaka, Japan: HPLC-grade acetonitrile (MeCN); sequenation-grade *n*-heptafluorobutyric acid (HFBA); reagent-grade HCl; and reagent-grade perchloric acid (PCA).[4] All the water used was purified with a Milli-Q water purification system (Millipore, Bedford, MA).

Preparation of samples. Serum samples (0.5 mL) were precipitated with an equal volume of 100 g/L PCA on ice and centrifuged at 1000 × *g* for 15 min. Each sample of supernate, residue, and serum was hydrolyzed in 6 mol/L HCl (final concentration) at 110 °C for 20 h in a sealed glass tube purged with argon. Hydrolysates were filtered with 0.45-μm (pore size) membrane filters (DISMIC-25cs; Toyo Roshi Ltd., Tokyo, Japan). Hydrolysate (125 μL) was mixed with 10 mL of water and applied to a 0.8 × 1.0 cm SP-Sephadex C-25 column (H^+ form; Pharmacia LKB, Uppsala, Sweden) that had been equilibrated with water. The column was washed with 20 mL of 0.1 mol/L HCl and eluted with 5 mL of 1.0 mol/L HCl. The eluate was evaporated under reduced pressure in a TC-8 concentrator (Taitec, Tokyo, Japan), and the residue was dissolved in 200 μL of 10 mL/L HFBA. The solutions were stored at −30 °C until the HPLC analysis.

Pentosidine was isolated from human articular cartilage. Methods for purification and characterization are described elsewhere (*6*).

[4] Nonstandard abbreviations: HFBA, *n*-heptafluorobutyric acid; MeCN, acetonitrile; and PCA, perchloric acid.

Instruments and chromatographic procedure. The HPLC system consisted of a Model CCPM pump, a Model FS-8010 spectrofluorometer, a Model AS-8010 autosampler, and a Model SC-8010 super system controller (all from Tosoh, Tokyo, Japan). We used an 8 mm × 10 cm column prepacked with Radial-Pak C_{18} (10-μm particle size, type 8C1810μ; Waters Associates, Milford, MA). The mobile phase was MeCN and 40 mmol/L HFBA (27:73, by vol); the flow rate was 1.0 mL/min. The volume of sample injected was 160 μL. To detect pentosidine, we measured the fluorescence at 385 nm (excitation at 335 nm). The minimum amount of pentosidine detectable (signal-to-noise ratio = 4) was ~0.2 pmol per injection under these conditions.

Statistical analysis. Statistical analyses were performed with a StatView II program on a Macintosh computer (Apple Computers, Cupertino, CA). The statistical significance and correlation were determined with nonparametric statistics by Mann–Whitney *U* tests and the Spearman rank correlation method, respectively. *P* values <0.05 were considered significant.

Results

Assay Procedures

Figure 1 shows the chromatograms of serum samples in several conditions. Pentosidine was not detected in the supernates of sera from control subjects after precipitation with 50 g/L PCA. However, it was detected in low concentrations in the PCA supernates of sera from uremic patients. Pentosidine was also detected in the PCA residues of sera from both control subjects and uremic patients. The amount of pentosidine in the supernate as a percentage of the total pentosidine in the

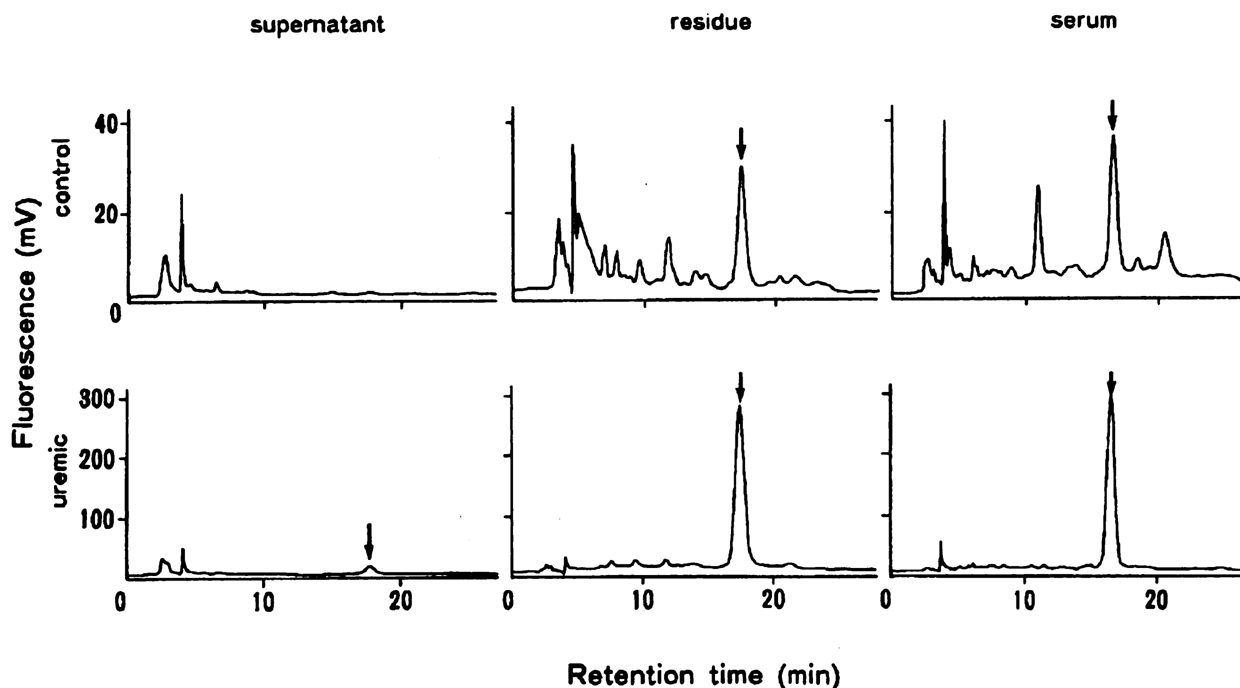

Fig. 1. Chromatograms of hydrolysates of serum samples from a control subject (*upper panels*) and a uremic subject (*lower panels*)
(*Left*) serum supernate after precipitation with PCA; (*middle*) the serum precipitate; (*right*) serum hydrolysate without precipitation. *Arrows* indicate pentosidine peak

supernate and residue ranged from 1.8% to 7.9% (mean ± SD, 4.7% ± 1.6%) in the uremic patients (n = 12). The right-hand panels of Figure 1 show chromatograms of the serum hydrolysates without precipitation by PCA. There was almost no difference between hydrolysates of serum with precipitation by PCA and without precipitation, although the peak for pentosidine in the hydrolysate without precipitation by PCA was larger than that with precipitation. Therefore, we directly hydrolyzed serum without precipitation for further studies of quantification of pentosidine in serum from control subjects and uremic patients.

A calibration line was constructed by assaying seven serum standards generated by adding 40 pmol–4 nmol of pentosidine to 0.5 mL of serum from a healthy subject. Linear-regression analysis showed the linearity of the calibration curve (r = 0.999). The analytical recovery of added pentosidine was 95.5% (SD 2.9%) at 500 nmol/L, with similar recovery at 50 nmol/L (n = 5 each). The intraassay CV was 3.6% (n = 5) and the interassay CV was 6.3% (n = 5) for a pentosidine concentration of 66 nmol/L.

Clinical Studies

The concentrations of pentosidine in serum from control subjects and uremic patients are shown in Figure 2. The values of pentosidine in the patients were extremely high compared with those in the control subjects (mean ± SD: 1267 ± 695 vs 77 ± 40 nmol/L, P = 0.0001). There was no difference in serum pentosidine concentrations between diabetic and nondiabetic patients (1199 ± 584 vs 1273 ± 706 nmol/L).

The correlation between age and the concentrations of serum pentosidine was significant in control subjects (r = 0.453, P <0.01) (Figure 3), but not in uremic patients (data not shown).

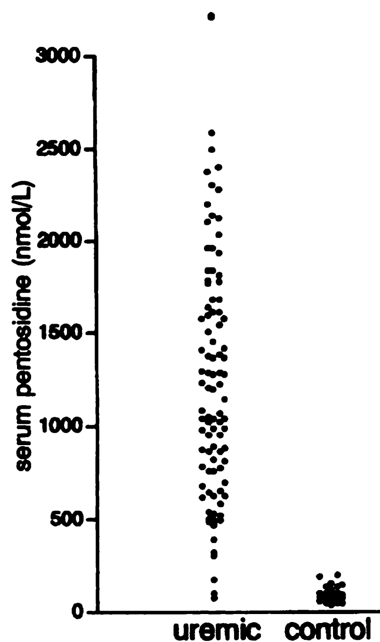

Fig. 2. Concentrations of serum pentosidine in uremic patients and control subjects

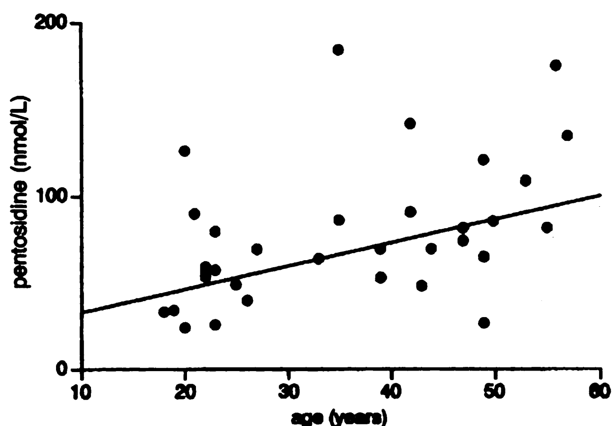

Fig. 3. Age-related change of serum pentosidine in control subjects. The *line* indicates the linear regression equation: pentosidine (nmol/L) = 1.404 (years) + 26.729 (r = 0.453, P <0.01)

In uremic patients, serum pentosidine weakly but significantly increased with the duration of hemodialysis (r = 0.272, P <0.05) (Figure 4).

Discussion

Pentosidine was not detected in the PCA supernates of sera from control subjects. This indicates that most pentosidine is protein-bound in serum in healthy subjects. Because we could detect pentosidine in the PCA supernates of sera from uremic patients, both free pentosidine and protein-bound pentosidine may increase in uremic patients. We previously observed (*7*) that 80–90% of pentosidine is present in the free form in urine from normal subjects and diabetic patients, the amount of urinary excretion being about 29 μg (77 nmol) per day in the normal subjects. Therefore, protein-bound pentosidine in serum is probably catabolized to free pentosidine and excreted in urine. If pentosidine is a nonthreshold substance for glomerular filtration in the kidney and if the normal value for pentosidine clearance is >100 mL/min (*8*), the concentration of free pentosidine in serum should be <0.5 nmol/L (77 nmol/1440 min

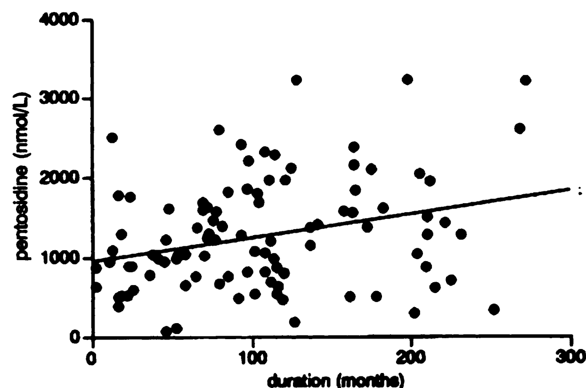

Fig. 4. Correlation between the duration of hemodialysis and pentosidine concentration
Pentosidine (nmol/L) = 2.81 (months) + 967.939 (r = 0.271, P <0.05)

divided by 100 mL/min). This concentration (0.5 nmol/L) corresponds to 0.025 pmol per injection in our method described above. Because this amount of pentosidine is below the detection limit of our method (~0.2 pmol per injection), we were unable to investigate the origin of the free pentosidine in urine.

The correlation between pentosidine and age in control subjects implies that the formation of pentosidine in serum increases with age. However, in uremic patients requiring hemodialysis, we saw no significant correlation between pentosidine and age but found a significant correlation between pentosidine and the duration of hemodialysis treatment. Apparently, the effect of uremia on the accumulation of pentosidine in serum is greater than the effect of aging.

We also saw no difference in serum pentosidine concentrations between diabetic and nondiabetic uremic patients. However, the number of diabetic patients was few and the hemodialysis treatment of diabetic patients was shorter than for nondiabetic patients (mean, 58.5 vs 110.3 months, respectively).

Although Monnier et al. (*9*) reported that pentosidine was increased in tissues and serum in uremic patients, the mechanism of the increase is unclear. Serum pentosidine is also increased in diabetes, but to a lesser extent than in uremia (*4, 9*). Therefore, the formation of pentosidine might increase in serum under uremic and diabetic conditions. However, in our study, the mean concentrations of serum pentosidine were much higher (16-fold) in uremic patients than in healthy control subjects. Hricik et al. (*10*) reported that kidney and kidney–pancreas transplantation were accompanied by a dramatic reduction of plasma pentosidine in patients with end-stage renal disease. However, the correction was incomplete by 2 years after transplantation, and the combined kidney–pancreas transplantation offered no advantage over kidney transplantation alone. Thus, Hricik et al. concluded that a metabolic abnormality leading to pentosidine persisted despite restoration of a euglycemic state. The fact that pentosidine is very high in collagen (*3*) and erythrocytes (*4*) from uremic patients suggests that pentosidine precursor sugars are retained by the diseased kidney. The accumulation of pentosidine precursors in renal failure may cause an increase in synthesis of pentosidine, reflected by increased concentrations of pentosidine in serum from uremic patients. However, the increased synthesis of pentosidine alone is insufficient to explain this extreme increase of pentosidine in uremic serum.

Most low-M_r proteins are eliminated almost exclusively through the kidney. Osteocalcin (bone Gla protein, BGP) is one of the noncollagenous proteins in bone and is secreted into the circulation. Osteocalcin has 49–50 amino acid residues and a molecular mass of 6000 Da. It, too, is markedly increased in serum from end-stage renal failure (*11*). Therefore, pentosidine, like osteocalcin, may be mostly eliminated through the kidney, and the diseased kidney may retain pentosidine in serum.

Thus, the increased concentrations of pentosidine in uremic patients may be caused by the increased synthesis of pentosidine (perhaps because of the retention of precursors), by the retention of pentosidine molecule itself in end-stage renal failure, or both.

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