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Creatine Plays a Direct Role as a Protein Modifier in the Formation of a Novel Advanced Glycation End Product

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Pentosidine, a cross-link structure between lysine and arginine residues, is one of the major advanced glycation end products (AGE). It is formed by the reaction of ribose with lysine and arginine. The pentosidine concentration produced by *in vitro* incubation of plasma obtained from uremic patients was reported to be higher than in normal plasma, indicating that uremic plasma contains an enhancer(s) for pentosidine formation [Miyata, T., Ueda, Y., Yamada, Y., Izuhara, Y., Wada, T., Jadoul, M., Saito, A., Kurokawa, K., and Strihou, C.Y. (1998) *J. Am. Soc. Nephrol.* 9, 2349–2356]. Since our preliminary study using a monoclonal anti-pentosidine antibody identified creatine as the most effective enhancer, the purpose of the present study was to clarify the mechanism by which creatine contributes to pentosidine formation. Lysine was incubated with ribose in the presence of creatine and analyzed by reverse phase high performance liquid chromatography. A novel fluorescent peak ($\lambda_{ex/em}$ = 335/385 nm) was detected at 8 min, under conditions at which the authentic pentosidine (lysine was incubated with ribose in the presence of arginine under identical conditions) eluted at 12 min. Structural analyses of this compound revealed a pentosidine-like structure in which the arginine residue was replaced by creatine. This novel AGE-structure, named here creatine-derived pentosidine (C-pentosidine), was detected in the plasma of patients on hemodialysis. These results indicate that creatine increases the formation of C-pentosidine but not authentic pentosidine. This study indicates that creatine plays a direct role as a protein modifier in C-pentosidine formation, although the clinical significance of C-pentosidine is still unknown.

Key words: advanced glycation end product, AGE-related disorders, creatine, pentosidine, protein modification.

Proteins are known to undergo non-enzymatic modification by reducing sugars such as glucose. In the early stages of the Maillard reaction, the aldehyde groups of reducing sugars react with amino groups of N-terminal amino acids or epsilon lysine to form Schiff bases, which are then converted to Amadori compounds *via* 1,2-enaminol. In the advanced stages, these Amadori compounds are exposed to more complex reactions such as oxidation, dehydration and condensation to form advanced glycation end products (AGE) that are characterized by fluorescence, browning, and intra- or inter-molecular cross-linking properties.

Immunohistochemical studies using anti-AGE antibodies have demonstrated the presence of AGE-modified proteins in several human tissues under pathological conditions, including the kidneys of patients with diabetic nephropathy (*1*) and chronic renal failure (*2*), atherosclerotic lesions of arterial walls (*3*), amyloid fibrils in hemodialysis-related amyloidosis (*4, 5*), and actinic elastosis of photoaged skin (*6*). These findings suggest the potential involvement of AGE-modification in the pathogenesis of age-related disorders.

Pentosidine, a main AGE compound, is formed from a cross-linking reaction between lysine and arginine residues of proteins and reducing sugars. Pentosidine is a stable fluorescent AGE-structure upon acid hydrolysis, and its plasma levels increase in aging (*7, 8*), diabetes (*9, 10*), renal diseases (*11–18*), and chronic rheumatoid arthritis (*19, 20*). Pentosidine also accumulates in the skin and kidneys of diabetic patients (*21–23*), in the brains of patients with Alzheimer's disease (*24*), and in dialysis-related amyloidosis (*5*). These data suggest a potential link between pentosidine and pathophysiological processes *in vivo*.

Miyata *et al.* reported an interesting observation: When plasma obtained from uremic patients was incubated *in vitro* at 37°C for 24 h, the amount of pentosidine produced was significantly higher (3.4-fold) than that produced in plasma from normal subjects, which led them to propose that the plasma of uremic patients contains a compound

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Abbreviations: AGE, advanced glycation end products; Boc, *tert*-butoxycarbonyl; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride; ELISA, enzyme-linked immunosorbent assay; FAB-MS, fast atom bombardment mass spectrometry; HD, hemodialysis; HFBA, heptafluorobutyric acid; HRP, horse radish peroxidase; HSA, human serum albumin; KLH, keyhole limpet hemocyanine; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing Tween 20; pentosidine, 2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)-amino]-3*H*-imidazo[4,5-*b*]pyridin-4-ium-4-yl}hexanoate.

that enhances pentosidine formation (*11*). The original purpose of the present study was to identify the enhancer compound(s). To this end, we set up an *in vitro* model system for pentosidine formation; bovine serum albumin (BSA) was incubated with ribose in the presence or absence of the compound to be tested, and then pentosidine was detected by enzyme-linked immunosorbent assay (ELISA) using a monoclonal anti-pentosidine antibody. We first tested several endogenous compounds present in human serum or urine as candidates for enhancer compounds. This *in vitro* ELISA assay for pentosidine formation revealed that creatine followed by urea and creatinine could contribute to pentosidine formation. In order to clarify the contributing mechanism, especially for creatine, we then set up an *in vitro* experiment in which lysine and ribose were incubated with creatine or arginine (as a control), followed by HPLC analyses. This gave a somewhat expected result; incubation of lysine and ribose with arginine gave authentic pentosidine, incubation of lysine and ribose with creatine, however, gave a compound different from pentosidine. The isolation and chemical analyses successfully led to the identification of the structure of this compound in which creatine was incorporated instead of arginine. This new pentosidine-like structure named "creatine-derived pentosidine (C-pentosidine)," was detected in the plasma of patients undergoing hemodialysis. Taken together, the present study discloses that creatine is directly involved as a protein modifier in the generation of C-pentosidine, a newly found AGE-compound.

EXPERIMENTAL PROCEDURES

Chemicals—BSA, human serum albumin (HSA), and keyhole limpet hemocyanine (KLH) were purchased from Sigma (St. Louis, MO). L-Lysine, L-arginine, creatine, creatinine, urea, and Dowex cation exchange resin (50 W ×8 100–200 mesh) were purchased from Wako Pure Chemical (Osaka). TiterMaxGold™ was purchased from CytRx Corp. (Norcross, GA). CF11 fibrous cellulose powder was purchased from Whatman (Kent, UK). Microtiter plates (96-well, Nunc Maxisorp) were purchased from Nalge Nunc International (Rochester, NY). Horseradish peroxidase–labeled anti–mouse IgG (γ) antibody was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Other chemicals were of the best grade available from commercial sources.

Preparation of Pentosidine—Diacetyl pentosidine was synthesized as described previously (*25*). Briefly, 2.19 g of D-ribose, 2.5 g of N^{α}-acetyl lysine and 3.69 g of N^{α}-acetyl arginine were dissolved in 58 ml of 100 mM of sodium phosphate, the pH was adjusted to 9.0 by the addition of 5 M sodium hydroxide, and the mixture was stirred for 48 h at 65°C. The reaction mixture was diluted fivefold with water and passed through 800 ml of Diaion HP-20 (Mitsubishi Chemical, Tokyo) cation exchange resin. The column was eluted with 2 liters of water, 5% methanol, 10% methanol, and 20% methanol, followed by elution with 2 liters of 40% methanol. Our analytical HPLC system described below detected *N,N'*-diacetyl pentosidine in the fraction eluted with 40% methanol, which gave 267.1 mg of crude product. The crude product was then purified by a modification of the HPLC system reported by Grandhee and Monnier (*25*). The amount of purified compound was 40.6 mg and its identity to pentosidine was confirmed by NMR analyses (*7, 25*).

Preparation of Monoclonal Anti-Pentosidine Antibody—The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University School of Medicine. Diacetylpentosidine-conjugated HSA was prepared by the EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride] method described previously (*24*). BALB/c mice were injected subcutaneously with a single dose of 0.1 mg of pentosidine-conjugated HSA (pentosidine-HSA) in 50% TiterMaxGold™ as adjuvant. Titers to pentosidine-HSA in immunized mouse sera were determined by an ELISA method as described below. Eight weeks after the initial injection, the immunized mouse with the highest titer to pentosidine-HSA was given an intraperitoneal boost of 0.1 mg of pentosidine-HSA. Three days later, splenic lymphocytes from the immunized mouse were fused with myeloma P3U1 cells by polyethylene glycol, and then incubated for three days in medium containing aminopterin, hypoxanthine, and thymidine with 10% fetal calf serum. The supernatant fluid of each culture was screened for its reactivity to pentosidine-HSA and pentosidine-KLH by ELISA. Two antibody-producing wells were identified as pentosidine-HSA and pentosidine-KLH–positive and EDC-treated HSA negative. Upon successive subcloning, two cell lines were finally cloned. These cell lines were injected intraperitoneally into BALB/c mice. Each antibody (IgG_1) was purified from ascitic fluid on a protein G-conjugated Sepharose column. One of these monoclonal antibodies, named 1C12, was used in the present study.

ELISA—ELISA was performed at room temperature in two different systems: noncompetitive ELISA and competitive ELISA. In the noncompetitive ELISA, each well of a 96-well microtiter plate was incubated for 1 h with 0.1 ml of the sample to be tested or its corresponding control sample in 50 mM carbonate buffer (pH 9.7), and washed three times with 0.4 ml of phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). Each well was then blocked for 1 h with 0.2 ml of 0.5% gelatin in 50 mM carbonate buffer (pH 9.7). Each well was washed three times with PBS-T and incubated for 1 h with 0.1 ml of anti-pentosidine antibody (1 μg/ml). The wells were then washed three times with PBS-T and incubated for 1 h with 0.1 ml of HRP-labeled anti–mouse IgG antibody, followed by reaction with 1,2-phenylenediamine dihydrochloride. The reaction was terminated by the addition of 0.1 ml of 1 M sulfuric acid, and the absorbance at 492 nm was read on a micro-ELISA plate reader (TECAN Spectra Fluor Plus). For competitive ELISA, each well of a 96-well microtiter plate was coated with 0.1 ml of 100 ng/ml of pentosidine-HSA, blocked with 0.2 ml of 0.5% gelatin, and washed three times with PBS-T. To each well, 0.05 ml of various amounts of samples to be tested and 0.05 ml (50 ng/ml) of anti-pentosidine antibody were added. After standing at room temperature for 1 h, the wells were washed three times with PBS-T and incubated for 1 h with 0.1 ml of HRP-labeled anti–mouse IgG antibody. The wells were washed, and amount of antibody bound to the wells was determined by reaction with 1,2-phenylenediamine dihydrochloride as described above.

Preparation of Ribose-Modified BSA—To prepare ribose-modified BSA, BSA (2 mg) was incubated in 1.0 ml of 100 mM sodium phosphate at 60°C for 1 week with 0.5 mmol of

ribose in the absence or presence of 10 μmol of creatine, creatinine, or urea. Before incubation, the pH of each sample was adjusted at 7.4 with sodium hydroxide. Then, these samples were dialyzed at 4°C overnight against PBS and used for ELISA assay. For HPLC analysis, 0.05 ml of each sample (0.1 mg protein) was hydrolyzed at 110°C for 24 h in a sealed tube with 0.5 ml of 6 M hydrochloric acid in the presence of 0.05% 2-mercaptoethanol. Solvent was removed *in vacuo*, and then the residue was dissolved in 0.5 ml of 20 mM hydrochloric acid and filtered through a 0.45 μm millex filter (Millipore). An aliquot of hydrolysate (5 μl) was analyzed by the analytical HPLC system. Briefly, amounts of C-pentosidine or pentosidine were determined by fluorescence ($\lambda_{ex/em}$ = 335/385 nm) with a Shiseido Capcellpak C_{18} UG80 (5 μm) column (4.6 × 250 mm) at a flow rate of 1 ml/min at 40°C by a Hitachi HPLC system equipped with a model L-7100 pump, L-7300 column oven, L-7200 autosampler, L-7485 fluorescence detector, and DG660 on-line degasser (GL Science) with mobile phase A, 20% acetonitrile containing 0.2% heptafluorobutyric acid (HFBA), and mobile phase B, 100% acetonitrile containing the same amount of HFBA. For analytical and column-cleaning purposes, a step-wise gradient was used as follows: mobile phase A from 0 to 20 min for analysis, mobile phase B from 20 to 40 min for column cleaning, and then mobile phase A from 40 to 60 min for the next analysis.

Purification and Identification of C-Pentosidine—Preparation of C-pentosidine was carried out on an equimolar basis with 250 mM of each of creatine, Boc-lysine and ribose. Briefly, creatine (0.6 g), Boc-lysine (1.0 g), and ribose (0.6 g) were dissolved in 16 ml of 50 mM sodium phosphate with the pH adjusted to 9.0 by the addition of 5 M sodium hydroxide, and the samples were then stirred for 48 h at 60°C. The sample was loaded onto a glass column filled with 650 ml of Dowex cation exchange resin equilibrated with water in the same way as reported by Grandhee and Monnier (*25*). The resin was washed with 1 liter of water and 1 M pyridine, followed by elution of the C-pentosidine–containing material with 2 liters of 1 M pyridine acetate (pH 6.0). The eluted solution was concentrated to dryness, redissolved in 10 ml of HPLC eluent (20% acetonitrile-water), applied to a preparative scale HPLC (Kanto Chemical Mightysil RP-18 GP 250-20, 20 × 250 mm) and eluted at a flow rate of 8 ml/min at room temperature with monitoring of the absorbance at 335 nm. Other HPLC conditions were similar to those described above. In a parallel experiment, an aliquot of the collected fractions containing C-pentosidine was confirmed by analytical HPLC. Aqueous solution (40 ml) was lyophilized and 6.4 mg of C-pentosidine was obtained as a pale yellow powder.

Structure Analyses by NMR and FAB-MS—The nuclear magnetic resonance (NMR) spectrum of C-pentosidine was measured in a JEOL (Akishima, Tokyo) ECP-300 (300 MHz) spectrometer. C-Pentosidine (3 mg) was dissolved in 0.5 ml of deuterium oxide as a solvent. 3-(Trimethylsilyl)-1-propanesulfonic acid was used as an internal standard. The molecular weight was determined by fast atom bombardment (FAB) mass spectrometry with a JEOL JMX-500. The sample (0.5 mg) was dissolved in 0.01 ml of DMSO and mixed with an equal volume of glycerol and 2-nitrobenzyl alcohol as a matrix.

Time-Course Study of C-Pentosidine Formation In Vitro—Boc-lysine and ribose were dissolved with creatine or Boc-arginine on an equimolar basis in 250 mM phosphate buffer (pH 7.4). After the pH was adjusted to 7.4 with 0.1 M sodium dihydrogen phosphate (pH 4.7) or 1 M sodium hydroxide (pH 14), the mixtures were incubated at 37°C for 7 days. Aliquots were removed at various times to measure pH and the amount of C-pentosidine or pentosidine by analytical HPLC.

Detection of C-Pentosidine in Human Plasma—Human plasma samples were withdrawn after obtaining a signed consent form. Plasma samples were collected from 5 inpatients with renal dysfunction who underwent hemodialysis at a local medical facility. Control plasma samples were obtained from healthy volunteers aged 24–34 years. None of the subjects was taking any medication or had any recognizable disease or history of renal disease or diabetes mellitus. The pretreatment column was prepared as follows, according to the methods of Yoshihara *et al.* (*26*) who determined pentosidine in human urine and plasma. The washing solvent was made of a mixture of 1-butanol, acetic acid, and water (8:1:1, v/v). A CF11 slurry was prepared by preparing a 5% (w/v) suspension of CF11 fibrous cellulose powder in the washing solvent. The pretreatment column was prepared by adding 15 ml of CF11 slurry to a glass column (8.0 mm ID × 24.0 mm) stopped with fiberglass. Plasma samples (0.25 ml each) were hydrolyzed at 108°C for 18 h in vacuum-sealed test tubes with an equal volume of concentrated hydrochloric acid to detect creatine-derived pentosidine analogue. An aliquot of hydrolysate (0.5 ml) was mixed with 0.5 ml of CF11 slurry, acetic acid and 4 ml of 1-butanol, then loaded onto the pretreatment column. After washing the column with 30 ml of the washing solvent, pentosidine and its analogue were eluted with 20 ml of 50 mM hydrochloric acid and evaporated to dryness under reduced pressure. The dry residue was then dissolved in 0.5 ml of HPLC eluent and an aliquot of each sample was applied to the analytical HPLC. Data were evaluated by Student's *t*-test. The difference were judged to be significant at $p < 0.05$.

RESULTS

Immunoreactivity and Characterization of Anti-Pentosidine Antibody—A monoclonal anti-pentosidine antibody (1C12) was prepared using pentosidine-HSA as described under "EXPERIMENTAL PROCEDURES." Non-competitive ELISA showed that the antibody reacted with pentosidine-HSA and pentosidine-KLH but not with EDC-treated HSA (data not shown). In the next step, we examined its immunoreactivity by a competitive ELISA system. As shown in Fig. 1, the reaction of the antibody with pentosidine-HSA was effectively inhibited by free pentosidine; the concentration for 50% inhibition (IC_{50}) was 6 nM. L-Arginine and L-lysine, starting materials for pentosidine formation, were not recognized by the antibody. Furthermore, other compounds, such as creatine, creatinine, *N*-monomethylarginine, and *N*-propylpyridinium bromide, did not compete with the antibody for binding to pentosidine-HSA. However, 2-aminobenzimidazole effectively competed with the antibody for binding to pentosidine-HSA in a fashion similar to that of free pentosidine with an IC_{50} of 2-aminobenzimidazole of 90 nM. These results suggest that the anti-pentosidine antibody recognizes the 9-member heterocyclic ring structure of pentosidine, which is common to both pentosidine

and 2-aminobenzimidazole.

Effect of Creatine, Creatinine or Urea on Pentosidine Formation In Vitro—BSA was incubated at 60°C for one week with ribose in the presence or absence of urea, creatine or creatinine, and the amounts of pentosidine formed in the reaction mixtures were determined by non-competitive ELISA. As shown in Fig. 2, pentosidine formation detected by anti-pentosidine antibody was significantly increased by urea, creatine, and creatinine. Enhancement by creatine was 2.5-fold that of the control value (without addition), suggesting the possibility that creatine can increase the formation of pentosidine during the incubation of BSA with ribose.

Formation of Pentosidine or a Pentosidine-Like Compound by Incubation of Boc-Lysine and Ribose with Creatine—To determine the mechanism of creatine-enhanced pentosidine formation *in vitro*, Boc-lysine was incubated with ribose at 60°C for 48 h in the presence of either creatine or Boc-arginine (as a control), and the reaction mixtures were analyzed by competitive ELISA. As expected, the reaction mixture of Boc-lysine and ribose with Boc-arginine competed with pentosidine-HSA for interaction with the anti-pentosidine antibody in a dose-dependent manner (Fig. 3). Although the reaction was weaker, the reaction mixture of Boc-lysine and ribose with creatine showed a similar dose-dependent competition (Fig. 3). Under the same conditions, the same mixtures without incubation had no effect on the immunoreactions (Fig. 3). Since it is expected that the incubation of Boc-lysine and ribose with Boc-arginine should lead to the generation of pentosidine, incubation of Boc-lysine and ribose with creatine might result in the formation of pentosidine or a pentosidine-like structure, which is recognized by the anti-pentosidine antibody. To test this notion, the reaction mixture of Boc-lysine and ribose with creatine was subjected to HPLC analysis.

Detection of Creatine-Derived Pentosidine (C-Pentosidine) by HPLC—HPLC analysis was performed using a detection system with excitation at 335 nm and emission at 385 nm, the characteristic fluorescence spectrum of pentosidine. As expected, a specific fluorescent peak was eluted at 12.27 min from the reaction mixture of Boc-lysine and ribose with Boc-arginine (Fig. 4A), identical to that of authentic pentosidine (Fig. 4C). Under the same HPLC conditions,

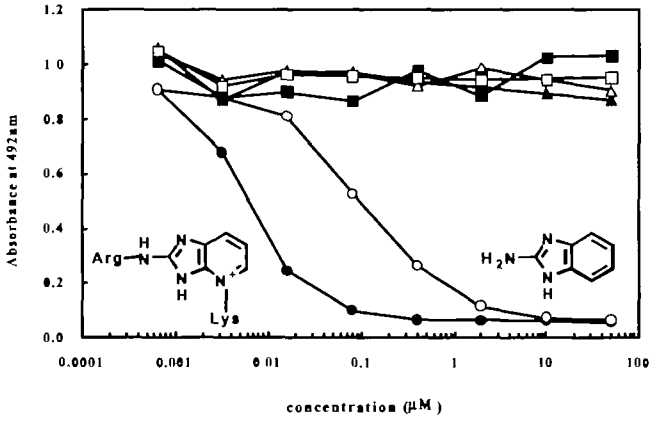

Fig. 1. **Immunoreactivity of the anti-pentosidine antibody.** Assays were performed at room temperature by competitive ELISA. Each well of a 96-well microtiter plate was coated for 1 h with 0.1 ml of 100 ng/ml of pentosidine-HSA, and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Each well was blocked with 0.2 ml of 0.5% gelatin and washed with PBS-T. To each well were added 0.05 ml of various amounts of compounds to be tested and 0.05 ml (50 ng/ml) of anti-pentosidine antibody, followed by incubation for 1 h. Each well was then washed three times with PBS-T and the amounts of antibody bound to the wells were determined by HRP-conjugated anti-mouse IgG, followed by reaction with 1,2-phenylenediamine dihydrochloride and determination of absorbance at 492 nm as described under "EXPERIMENTAL PROCEDURES." Tested compounds were pentosidine (●), 2-aminobenzimidazole (○), creatine (▲), creatinine (△), arginine (■), and lysine (□). The data shown represent typical results obtained in five separate experiments.

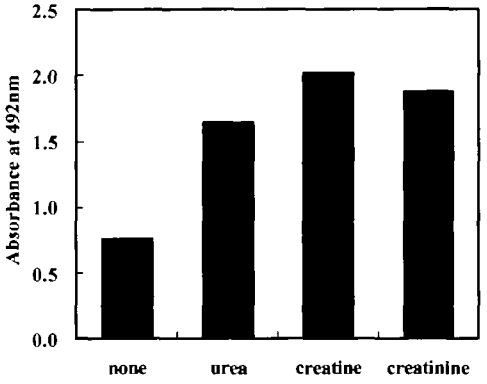

Fig. 2. **Effect of creatine, creatinine, or urea on pentosidine formation *in vitro*.** BSA (2 mg/ml) was incubated at 60°C for 1 week with ribose (500 mM) in the presence or absence of 10 mM of urea, creatine or creatinine in 1.0 ml of 100 mM phosphate buffer (pH 7.4). The samples were then dialyzed at 4°C overnight against PBS, and the pentosidine-content was determined by noncompetitive ELISA. Each well of a microtiter plate was coated for 1 h with 0.1 ml of sample (5 μg/ml), washed three times with PBS-T and blocked with 0.2 ml of 0.5% gelatin for 1 h, followed by washing with PBS-T. To each well was added 0.1 ml of anti-pentosidine antibody (100 ng/ml). After reaction for 1 h, each well was washed three times with PBS-T and the amounts of antibodies bound to the wells were determined by HRP-conjugated anti-mouse IgG as described under "EXPERIMENTAL PROCEDURES."

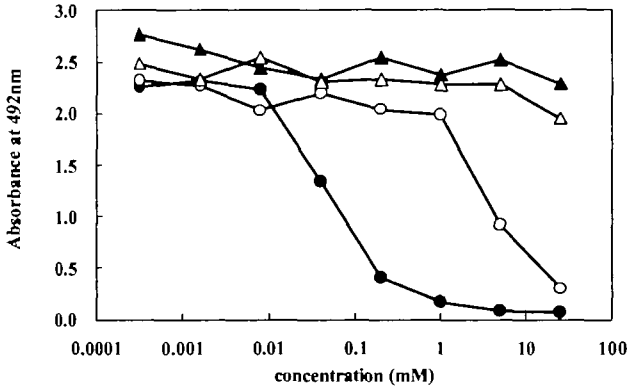

Fig. 3. **Formation of pentosidine or pentosidine-like compounds by incubation of Boc-lysine and ribose with creatine.** Boc-lysine (0.25 mmol) and ribose (0.25 mmol) were incubated at 60°C for 48 h with 0.25 mmol of creatine (○) or Boc-arginine (●) in 1.0 ml of sodium phosphate (50 mM, pH 9.0). The formation of pentosidine or pentosidine-like compounds was determined by competitive ELISA as described in the legend to Fig. 1. The corresponding samples (creatine (△) or Boc-arginine (▲)) without incubation were subjected to competitive ELISA.

the reaction mixture of Boc-lysine and ribose with creatine yielded a fluorescent peak different from that of authentic pentosidine (Fig. 4B). This fluorescent peak was further purified by HPLC, from which it had a retention time of 8.16 min with 99.2% purity (Fig. 4D). These results indicate that the incubation of Boc-lysine and ribose with creatine leads to the generation of a compound different from pentosidine. Since this compound was derived from creatine and reacted with the anti-pentosidine antibody, it was named creatine-derived pentosidine (C-pentosidine).

Immunoreactivity of C-Pentosidine with Anti-Pentosidine Antibody—The immunoreactivity of the purified C-pentosidine with the anti-pentosidine antibody was examined by competitive ELISA. The immunoreactivity of the antibody with pentosidine-HSA was inhibited by C-pentosidine in a dose-dependent manner with an IC_{50} of 1 μM, which is 1,000 times lower than authentic pentosidine because the IC_{50} for the authentic pentosidine is 6 nM (Fig. 5). The positive recognition of C-pentosidine by the antibody suggests that C-pentosidine has a structure identical or similar to the 9-member heterocyclic ring structure of pentosidine, which both pentosidine and 2-aminobenzimidazole have in common (Fig. 1).

Structural Identification of C-Pentosidine by NMR and FAB-MS—The results of structural analysis of C-pentosidine by proton NMR are shown in Fig. 6. Three sets of peaks appeared in the aromatic proton region, expressing chemical shifts and coupling patterns identical to those of authentic pentosidine. It is, therefore, suggested that this compound possesses a heterocyclic structure containing a quaternary nitrogen atom. Furthermore, a specific peak, termed the *N*-methyl peak, derived from creatine, and the Boc group, derived from Boc-lysine, appeared at 3.3 and 1.4 ppm, respectively. It is likely, therefore, that the structure of C-pentosidine resembles that of pentosidine, because Boc-lysine and creatine are incorporated into the structure. Figure 7 presents the results of FAB-MS and shows a molecular ion peak at *m/z* 649 for C-pentosidine, which accompanies HFBA as a counter anion used in HPLC purification, corresponding to the exact mass number *m/z* 436.2196 $[M]^+$ calculated as $C_{20}H_{30}N_5O_6$ for C-pentosidine

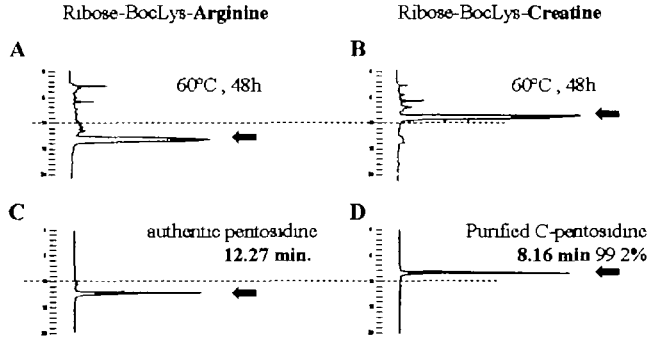

Fig. 4. **Detection of creatine-derived pentosidine (C-pentosidine) by HPLC.** Boc-lysine (0.25 mmol) and ribose (0.25 mmol) were incubated at 60°C for 48 h with creatine (0.25 mmol) in 1.0 ml of 50 mM of sodium phosphate (pH 9.0) and aliquots of sample were subjected to HPLC analysis as described under "EXPERIMENTAL PROCEDURES." The major fluorescent peak was eluted at a retention time of 8.2 min (B). Upon further purification of the fluorescent peak by an analytical HPLC system, a single peak was eluted at a retention time of 8.16 min (D). In a parallel experiment, Boc-lysine and ribose were incubated with Boc-arginine under the same conditions and aliquots were analyzed by the same HPLC system, resulting in the elution of a major fluorescent peak (A) at 12.27 min, which was indistinguishable from that of authentic pentosidine (C).

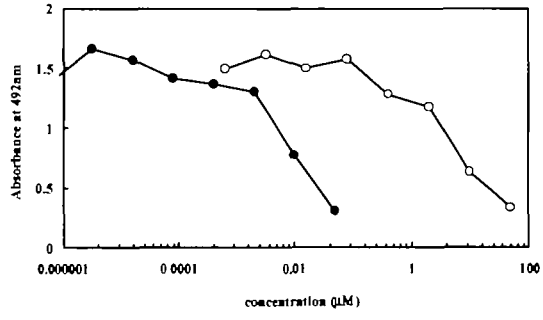

Fig. 5. **Immunoreactivity of C-pentosidine with anti-pentosidine antibody.** The immunoreactivity of C-pentosidine purified by HPLC in Fig. 4 with the anti-pentosidine antibody was determined by competitive ELISA (○) as described in the legend to Fig. 1. Under identical conditions, the authentic pentosidine was subjected to the same ELISA (●).

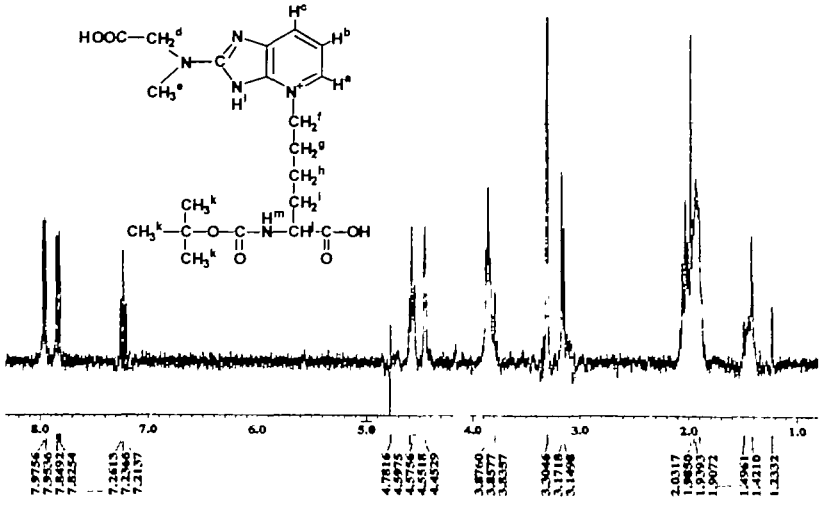

Fig. 6. **^{1}H-NMR spectrum of C-pentosidine.** The proton NMR spectrum was measured in a JEOL ECP-300 (300 MHz) spectrometer using D_2O as a solvent. Three sets of peaks appeared in the aromatic proton region, expressing chemical shifts and coupling patterns identical to those of authentic pentosidine. Specific peaks appearing at 3.3 ppm and 1.4 ppm were assigned as *N*-methyl, derived from creatine, and the methyl group of Boc, derived from Boc-lysine, respectively. δ 7.96 (d, 1H, H^c, J = 6.6 Hz), 7.84 (d, 1H, H^a, J = 7.2 Hz), 7.24 (t, 1H, H^b, J = 7.2 Hz), 4.57 (m, 2H, H^f), 4.45 (s, 1H, H^l), 3.86 (s, 2H, H^g), 3.30 (s, 3H, H^e), 3.16 (s, 2H, H^d), 2.15–1.8 (m, 2H, H^i), 1.99 (s, 9H, H^k), 1.49–1.42 (m, 2H, H^h).

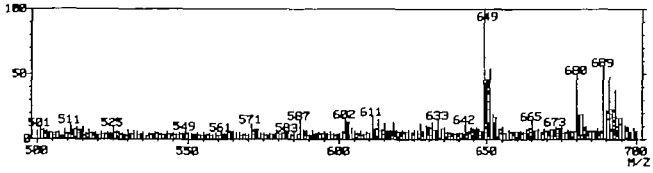

Fig. 7. **FAB-MS spectrum of C-pentosidine.** About 0.5 mg of sample was dissolved in 0.01 ml DMSO and mixed with an equal volume of glycerol and 2-nitrobenzyl alcohol as a matrix. The molecular ion peak at *m/z* 649 was assigned to C-pentosidine, which was accompanied by HFBA as a counter anion, corresponding to the exact mass number *m/z* 436.2196 $[M]^+$ calculated as $C_{20}H_{30}N_6O_6$ for C-pentosidine and *m/z* 212.9787 $[M]^-$ as $C_4F_7O_2$ for HFBA, respectively.

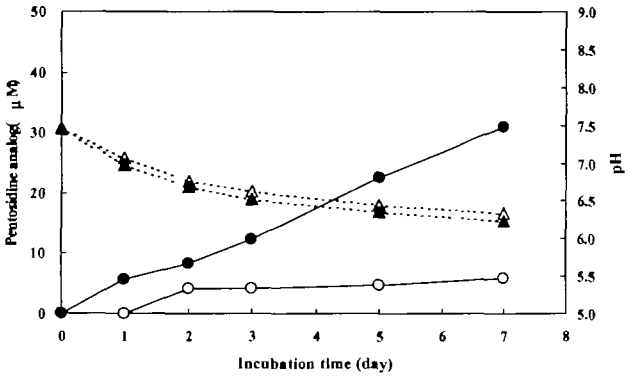

Fig. 8. **Time course of C-pentosidine formation *in vitro*.** Boc-lysine (0.25 mmol) and ribose (0.25 mmol) were dissolved with equimolar creatine (0.25 mmol) or Boc-arginine (0.25 mmol) in 1.0 ml of 250 mM phosphate buffer (pH 7.4). After the pH was adjusted to 7.4 with 0.1 M sodium dihydrogen phosphate (pH 4.7) or 1 M sodium hydroxide (pH 14), the mixture were incubated at 37°C for 7 days. The amounts of C-pentosidine (●) and pentosidine (○) at various times were determined by analytical HPLC (left axis) performed as described in the legend to Fig. 4; pH values of C-pentosidine (▲) or pentosidine (△) were also measured (right axis).

and *m/z* 212.9787 $[M]^-$ as $C_4F_7O_2$ for HFBA, respectively.

Time-Course Study of C-Pentosidine Formation In Vitro—Boc-lysine and ribose were dissolved with creatine or Boc-arginine on an equimolar basis in 250 mM phosphate buffer (pH 7.4). After the pH was adjusted to 7.4 with 0.1 M sodium dihydrogenphosphate (pH 4.7) or 1 M sodium hydroxide (pH 14), the mixtures were incubated at 37°C for 7 days. Aliquots were taken at various times to measure the pH and the amounts of C-pentosidine or pentosidine by analytical HPLC. The amounts of C-pentosidine generated under the conditions used were significantly higher than those of pentosidine (Fig. 8). The pH values of both incubation mixtures gradually decreased from 7.4 to 6.3 during incubation, but they did not differ from each other. Therefore, the difference in the amount of C-pentosidine and pentosidine can not be explained by the pH change during incubation, but rather by the difference in the chemical reactivity of creatine and Boc-arginine.

Identification of C-Pentosidine in the Plasma of Patients on Hemodialysis—Plasma samples from 5 patients with renal dysfunction who underwent HD treatment and 5 normal subjects were hydrolyzed and subjected to HPLC analysis. Major peaks were assigned as C-pentosidine or authentic pentosidine with retention times identical to those shown in Fig. 4. Plasma levels of authentic pentosidine determined by our HPLC system were 0.43 ± 0.20 nmol/ml for normal subjects and 1.46 ± 0.85 nmol/ml for HD patients (Table I). These values are similar to those reported by Izuhara *et al.* (*14*). In addition, C-pentosidine was also detected in the plasma of HD patients. Plasma C-pentosidine levels in these patients were significantly higher than in normal subjects, which were negligibly low or below detection levels under the present detection system. The maximum of C-pentosidine levels in HD patients was 0.38 nmol/ml, less than that of authentic pentosidine in HD patients (1.46 nmol/ml). However, this level is similar to that of authentic pentosidine in normal subjects (0.43 ± 0.20 nmol/ml).

TABLE I. **Concentrations of pentosidine and C-pentosidine in human plasma.**

	C-Pentosidine (nmol/ml plasma)	Pentosidine (nmol/ml plasma)
Normal subjects (n = 5)	0.06 ± 0.06*	0.43 ± 0.20**
HD patients (n = 5)	0.19 ± 0.19*	1.46 ± 0.85**

Values are mean ± standard error of five samples. Major peaks were assigned as C-pentosidine or authentic pentosidine, with retention times identical to those shown in Fig. 4. *p < 0.05, **p < 0.0005.

DISCUSSION

Pentosidine, which forms cross-link structures between lysine and arginine residues, is one of the major AGE-structures, and its involvement in several disease processes has been emphasized. The new findings of the present study are threefold: (i) Whereas incubation of lysine and ribose with arginine gave rise to authentic pentosidine, incubation of lysine and ribose with creatine resulted in the formation of a pentosidine-like compound (C-pentosidine). (ii) Chemical analysis successfully identified the structure of this compound in which the arginine of authentic pentosidine is replaced by creatine. (iii) C-pentosidine was detected in the plasma of patients on hemodialysis. These results reveal a novel aspect of creatine as an endogenous compound *in vivo* that plays a direct role as a protein modifier in the formation of C-pentosidine. In order to know the clinical significance of C-pentosidine, however, further studies are needed to determine plasma C-pentosidine levels in more clinical samples and to correlate these levels with those of authentic pentosidine.

Creatine is generated from the S-adenosylmethionine-mediated methylation of guanidino acetic acid, which is produced from glycine and arginine through the "creatine pathway." Since creatine excretion is decreased in renal disorders with a subsequent rise in plasma creatine levels, the plasma creatine level is used as a biomarker for renal function. In addition, plasma pentosidine levels correlate significantly with plasma creatine levels, as reported by Sugiyama *et al.* (*27*). Under normal conditions, plasma creatine levels range from 24 to 84 nmol/ml, whereas plasma free arginine levels vary from 44 to 120 nmol/ml. Under pathological conditions, particularly those associated with renal dysfunction, plasma creatinine levels are known to rise to more than 10 times the physiological level, in which creat-

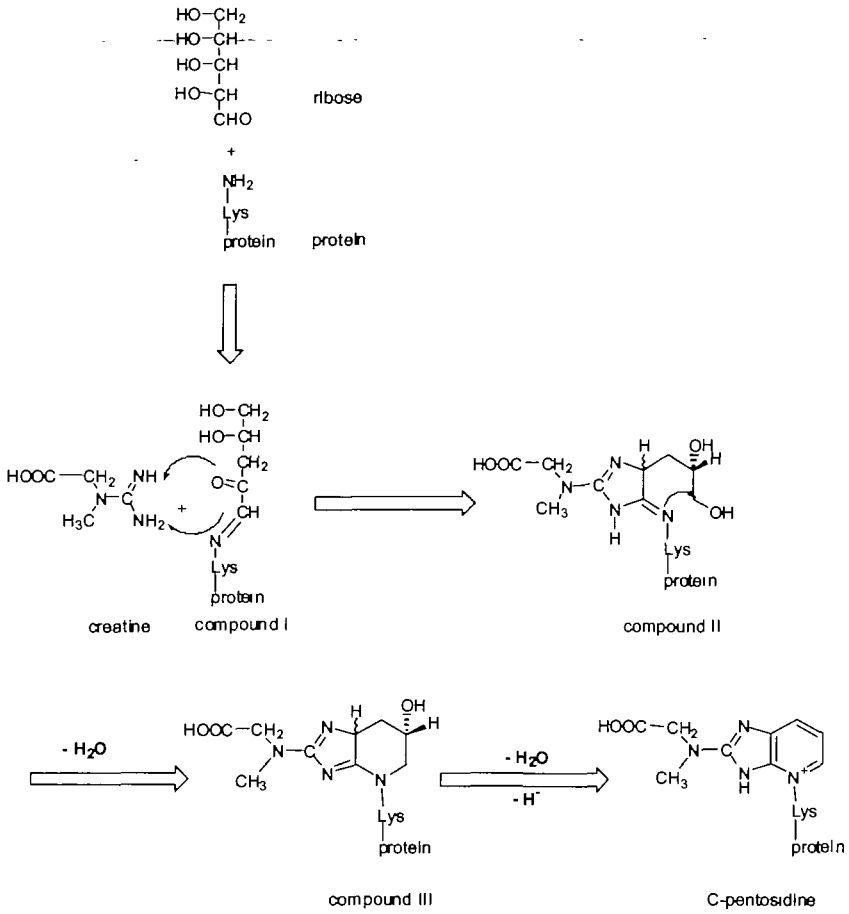

Fig. 9. **Possible synthetic pathway for C-pentosidine.** Ribose reacts with lysine to form a Schiff base (compound I) in the early stage of the Maillard reaction. The guanidino group of creatine reacts with the Schiff base to form compound II. Compound II is then converted to C-pentosidine by cyclization, dehydration, and condensation *via* compound III to form C-pentosidine. (See text for details.)

ine levels are equal to, or higher than plasma arginine levels. Under these conditions, C-pentosidine is expected to be generated *in vivo* more effectively compared with the formation of pentosidine because our *in vitro* experiments using the same concentration of arginine and creatine showed that the formation of C-pentosidine is faster than that of pentosidine (Fig. 8). However, plasma C-pentosidine levels in patients on HD were determined to be less than 30% those of pentosidine (Table I). This may reflect the fact that the concentration of protein-bound arginine (14–17 mM) in plasma is much higher than that of free creatine (24–84 μM).

Plasma pentosidine levels are often determined by HPLC analysis (*7, 9, 11, 12, 14–16, 23, 26, 27*) and ELISA using polyclonal anti-pentosidine antibodies (*5, 18*). The structure of C-pentosidine is very similar to authentic pentosidine. In fact, C-pentosidine was recognized by our monoclonal anti-pentosidine antibody (Fig. 5). Therefore, it is possible that plasma pentosidine levels could be overestimated in the presence of C-pentosidine.

Figure 9 shows the possible synthetic pathway for C-pentosidine formation. Ribose reacts with lysine residues in proteins to form Schiff bases (compound I) in the early stage of the Maillard reaction. Compound I then reacts with the guanidino group of creatine to form compound II (in a similar mechanism, compound I reacts with the guanidino group of arginine to form authentic pentosidine). Compound II is subjected to cyclization, dehydration, and condensation to form C-pentosidine *via* compound III. Although C-pentosidine structurally resembles pentosidine with creatine incorporation instead of arginine, it differs from pentosidine since C-pentosidine is not involved in its intra-molecular or inter-molecular cross-linking formation. This pathway for C-pentosidine formation is supported by studies reported by Lederer *et al.* (*28–31*): *n*-butylamine reacts with glyoxal, methylglyoxal or 3-deoxyglucosone to form Schiff base compounds, which subsequently react with creatine to give rise to imidazole derivatives, which are collectively known as "glucosepan."

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