Covalently cross-linked proteins are among the major modifications caused by the advanced Maillard reaction. So far, the chemical nature of these aggregates and their formation pathways are largely unknown. Synthesis and unequivocal structural characterization are reported for the lysine-arginine cross-links N²-2-[[4(5S)-4-ammonio-5-oxido-5-oxopentyl]amino]-5-[[2S,3R]-2,3,4-trihydroxybutyryl]-3,5-dihexylo-4H-imidazol-4-yldiene]-1-lysinate (DOGDIC 12), N²-2-[[4(5S)-4-ammonio-5-oxido-5-oxopentyl]amino]-5-[[2S,3R]-2,3,4-trihydroxypropyl]-3,5-diheptyl-4H-imidazol-4-yldiene]-1-lysinate (DOPDIC 13), and 6-[[6(S)-2-[[4(5S)-4-ammonio-5-oxido-5-oxopentyl]amino]-6-hydroxy-5,6,7,7a-tetrahydro-4H-imidazo[4,5-b]pyridin-4-yl]-1-norleucinate (pentosinate 10). For these compounds, as well as for glucosepane 9 and pentosidine 11, the formation pathways could be established by starting from native carbohydrates, Amadori products, and 3-deoxyosones, respectively. Pentosinate 10 was unequivocally proven to be an important precursor of pentosidine 11, which is a well established fluorescent indicator for advanced glycation processes in vivo. The Amadori products are shown to be the pivots in the formation of the various cross-links 9–13. The bicyclic structures 9–11 are directly derived from aminoketoses, whereas 12 and 13 stem from reaction with the 3-deoxyosone. All products 9–13 were identified and quantified from incubations of bovine serum albumin with the respective 3-deoxyosone or carbohydrate. From these results it seems fully justified to expect both glucosepane 9 and DOGDIC 12 to constitute important in vivo cross-links.

The Maillard reaction or “nonenzymatic browning” is a complex series of reactions between reducing carbohydrates with lysine side chains and N-terminal amino groups of proteins. In the first step of this process, rather labile Schiff bases are formed which as a rule rearrange to the more stable Amadori products. The Amadori compounds are slowly degraded, in complex reaction pathways via dicarbonyl intermediates, to a plethora of compounds (1, 2) subsumed summarily under the term “advanced glycation end products” (AGEs); this overall reaction sequence proceeds both in vitro and in vivo. In long lived tissue proteins, such as collagen and lens crystallins, these chemical modifications accumulate with age and hence may contribute to pathophysiologies associated with aging and long term complications of diabetes and atherosclerosis. A major consequence of the advanced Maillard reaction is the formation of covalently cross-linked proteins. On the basis of various model reactions, different mechanisms for cross-linking of amino acid side chains in proteins have been discussed (3–14). So far, pentosidine 11 (Fig. 1) (15), fluorophore LM-1 (16, 17), crossline (18), MOLD (19), and GOLD (20, 21) have been detected in vivo. Some of the cross-links (e.g. fluorophore LM-1 and GOLD) are formed via post-translational modification of proteins by products of carbohydrate oxidation; they can therefore be designated as “advanced glycoxidation end products” (AGOE) thus representing a subgroup of AGEs. The other cross-links (e.g. MOLD) are derived from carbohydrate-protein reactions that do not involve an oxidation step. The latter formation pathway is operative also in the formation of the cross-linking unit glucosepane 9 recently being identified from reactions of bovine serum albumin (BSA) with D-glucose (1) (22). For better understanding the impact of the Maillard reaction on aging and diabetes, and for developing effective therapeutic methods to prevent AGE accumulation in tissues, it is an absolute prerequisite to establish definitively the chemical nature of the major protein cross-links and to elucidate their formation. The structural similarity between glucosepane 9 and pentosidine 11 suggests a parallelism in the respective formation pathways, although generation of 11, contrary to 9, requires an oxidation process. Despite the fact that up to now more than 100 papers dealing with the prominent AGE 11 have been published, no reaction scheme for its generation from pentoses has so far been established.

We now report on the elucidation of the formation pathways for AGOE, advanced glycoxidation end product; BSA, bovine serum albumin; COSY, correlation spectroscopy; 3-DOG, 3-deoxyglucose; 3-DOP, 3-deoxypentosulose; DTPA, diethylenetriaminopentaacetic acid; gs-HMBC, gradient-selected heteronuclear single quantum coherence; LC-(ESI)MS, coupled liquid chromatography - electrospray ionization mass spectrometry; LC-NMR, coupled liquid chromatography - nuclear magnetic resonance spectroscopy; MWCO, molecular weight cut-off; SIM, selected ion monitoring; TOCSY, total correlation spectroscopy; PBS, phosphate-buffered saline; DOGDIC 12, N²-2-[[4(5S)-4-ammonio-5-oxido-5-oxopentyl]amino]-5-[[2S,3R]-2,3,4-trihydroxybutyryl]-3,5-diheptyl-4H-imidazol-4-yldiene]-1-lysinate; DOPDIC 13, N²-2-[[4(5S)-4-ammonio-5-oxido-5-oxopentyl]amino]-5-[[2S,3R]-2,3,4-trihydroxypropyl]-3,5-diheptyl-4H-imidazol-4-yldiene]-1-lysinate; HPLC, high performance liquid chromatography; t-Boc, tert-butoxycarbonyl; H/D, hydrogen/deuterium; pentosinate 10, 6-[[6(S)-2-[[4(5S)-4-ammonio-5-oxido-5-oxopentyl]amino]-6-hydroxy-5,6,7,7a-tetrahydro-4H-imidazo[4,5-b]pyridin-4-yl]-1-norleucinate; TFA, trifluoroacetic acid.
glucospane 9 as well as for pentosidine 11 and on the structural characterization of its precursor 6-(6S)-2-[(4S)-4-amino-5-oxido-5-oxopentyl]amino]-6-hydroxy-5,6,7,7a-tetrahydro-4H-imidazol[4,5-b]pyridin-4-yl]-t-norleucine (10, Fig. 1). Additionally, two novel cross-links derived from the 3-deoxyosones 7 and 8, respectively, were independently synthesized and their structures unequivocally established: N\(^{6}\)-[2-[(4S)-4-amino-5-oxido-5-oxopentyl]amino]-5-[(2S)-2,3,4-trihydroxybutyl]-3,5-dihydro-4H-imidazol-4-ylidene]-l-lysine (12) and N\(^{6}\)-[2-[(4S)-4-amino-5-oxido-5-oxopentyl]amino]-5-[(2S)-2,3,4-dihydro-3,5-dihydro-4H-imidazol-4-ylidene]-l-lysine (13). All products 9–13 were identified and quantified from incubations of BSA with the respective 3-deoxyosane or carbohydrate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Milli-Q water (purified to 18 M\(^{2}\) cm; Millipore, Eschborn, Germany) was used in the preparation of all solutions. HPLC grade methanol and acetonitrile were employed for LC, LC-MS, and LC-NMR. For preparative HPLC, solvents were degassed by flushing with helium. D-Ribose (Na\(_{2}\)HPO\(_{4}\), Merck; D-arabinose (Fisher, Neum, Germany) was used in the preparation of all solutions. HPLC grade methanol and acetonitrile were employed for LC, LC-MS, and LC-NMR. For preparative HPLC, solvents were degassed by flushing with helium.

**Synthesis of N\(^{6}\)-6-{2-[(4S)-4-Amino-5-oxido-5-oxopentyl]amino}-5-[(2S)-2,3,4-trihydroxybutyl]-3,5-dihydro-4H-imidazol-4-ylidene]-l-lysine (DOPDIC 13)—**N\(^{6}\)-t-Boc-l-lysine (3.04 g, 12.3 mmol), N\(^{6}\)-t-Boc-t-arginine (2.28 g, 8.3 mmol), and 3-DOP 8 (515 mg, 3.9 mmol, prepared according to Refs. 22 and 23, respectively). The mixture was kept at 50 °C for 12 h and purified by preparative HPLC (gradient B) with t\(_{R}\) 14.8 min yielded, after lyophilization, N\(^{6}\)-tert-butoxycarbonyl]-N\(^{6}\)-[(2S)-2,3,4-trihydroxybutyl]-amino]-4-carboxybutyllamino]-5-[(2S)-2,3,4-trihydroxybutyl]-3,5-dihydro-4H-imidazol-4-ylidene]-l-lysine (DOPDIC 13). This compound (75.4 mg, 0.114 mmol) was dissolved in aqueous 3 N HCl (3 ml) and kept at ambient temperature for 30 min. The pH was adjusted to 7 by slowly adding solid NaHCO\(_{3}\), the volume finally filled up to 5 ml, and the mixture was subjected to preparative HPLC (gradient B). Fractions with t\(_{R}\) 3.8 min and t\(_{R}\) 6.5 min yielded, after lyophilization, 13a-3 HCOOH (13.0 mmol, 0.023 mmol, 0.06%) and 13b-3 HCOOH (15.8 mmol, 0.026 mmol, 0.07%), respectively; UV(H\(_{2}\)O): \(\lambda_{\text{max}}(\text{nm})\) (eq g) 13a, 242 (4.11), 13b, 240 (4.20); LC-ESI/MS (gradient B): 13a, \(m/z\) 205.0, \(m/z\) 417 (100, [M + H\(^{+}\)]), 372 (3), 271 (8); 13b, \(m/z\) 20.8 min, \(m/z\) 417 (100, [M + H\(^{+}\)]), 372 (3), 271 (5); accurate mass (mean of 10 measurements \(\pm\) S.D.): 13a, \(m/z\) 417.2463 \(\pm\) 0.0008 [M + H\(^{+}\)]; 13b, \(m/z\) 417.2462 \(\pm\) 0.0008 [M + H\(^{+}\)]; for NMR data see Table I.

**Model Reactions—**b-Glucose (11, 15 mg, 0.1 mmol), p-t-Boc-m-xylene (2a, 15 mg, 0.1 mmol), p-t-Boc-m-xylene (2b, 15 mg, 0.1 mmol), p-t-Boc-m-2a (15 mg, 0.1 mmol), 3-DOP 7 (316 mg, \(\pm\) 48%, 0.9 mmol, prepared according to Ref. 24) were dissolved in water (10 ml), and the pH was adjusted to 7 by adding solid NaHCO\(_{3}\), the volume finally filled up to 5 ml, and the mixture subjected to preparative HPLC (gradient C). Fractions with t\(_{R}\) 20.5 min, \(m/z\) 417 (100, [M + H\(^{+}\)]), 372 (3), 271 (8); 13b, \(m/z\) 20.8 min, \(m/z\) 417 (100, [M + H\(^{+}\)]), 372 (3), 271 (5); accurate mass (mean of 10 measurements \(\pm\) S.D.): 13a, \(m/z\) 417.2463 \(\pm\) 0.0008 [M + H\(^{+}\)]; 13b, \(m/z\) 417.2462 \(\pm\) 0.0008 [M + H\(^{+}\)]; for NMR data see Table I.
has been validated by 1H, 1H-COSY, 1H, 13C-COSY, 1H, 1H-TOCSY, and spectra. No absolute configuration was determined for the diastereoisomers from the gs-HMBC and gs-HSQC measurements.

The arrows in the structural formulae indicate the characteristic 1H and 13C NMR data of DOGDIC (ppm), chemical shift for the indicated hydrogen/carbon.

| TABLE I | Hydrogen/carbon assignment |
|---|---|
| 12a | 12b | 12a,b | 13a | 13b | 10a |
| Solvent | 12a | 12b | 12a | 12b | 12a,b | 13a | 13b | 10a |
| D$_2$O | D$_2$O | D$_2$O | D$_2$O | D$_2$O | 0.05 % TFA in D$_2$O |
| R$_3$ | $^2$CH$_2$O$_4$ | $^2$CH$_2$O$_4$ | $^2$CH$_2$O$_4$ | $^2$CH$_2$O$_4$ | $^2$CH$_2$O$_4$ | $^2$CH$_2$O$_4$ | $^2$CH$_2$O$_4$ | $^2$CH$_2$O$_4$ |
| $^1$H NMR | $^1$H, $^1$H-COSY | $^1$H, $^1$H-COSY | $^1$H, $^1$H-COSY | $^1$H, $^1$H-COSY | $^1$H, $^1$H-COSY | $^1$H, $^1$H-COSY | $^1$H, $^1$H-COSY | $^1$H, $^1$H-COSY |
| H-5 | 4.80 | 4.90 | 4.85 | 4.89 | 4.53 |
| H-6 | 1.99 | 1.99 | 1.99 | 1.84 | 1.31 |
| H-6 | 2.35 | 2.35 | 2.16 | 1.94 | 2.93 |
| H-7 | 3.70 | 3.73 | 3.78 | 3.82 | 4.31 |
| H-8 | 2.27 | 2.27 | 2.00 | 2.00 | 2.00 |
| H-8 | 3.76 | 3.76 | 3.54 | 3.56 | 3.53 |
| H-9 | 3.76 | 3.76 | 3.54 | 3.56 | 3.53 |
| H-10 | 3.41 | 3.44 | 3.42 | 3.45 | 4.35 |
| H-10 | 3.41 | 3.44 | 3.42 | 3.45 | 4.35 |
| H-11 | — | — | — | — | 3.55 |
| H-12 | 1.68 | 1.67 | 1.68 | 1.68 | 1.70 |
| H-13 | 1.44 | 1.45 | 1.42 | 1.43 | 1.42 |
| H-14 | 1.87 | 1.88 | 1.80 | 1.86 | 1.80 |
| H-15 | 3.72 | 3.72 | 3.72 | 3.72 | 4.87 |
| H-16 | 3.37 | 3.35 | 3.37 | 3.36 | 3.31 |
| H-17 | 1.72 | 1.72 | 1.71 | 1.72 | 1.72 |
| H-18 | 1.92 | 1.92 | 1.89 | 1.88 | 1.93 |
| H-19 | 3.77 | 3.76 | 3.76 | 3.76 | 3.88 |
| HCCO | 8.44 | 8.43 | 8.42 | 8.42 | 8.42 |

* The arrows in the structural formulae indicate the characteristic carbon-proton long-range coupling connectivities from the gs-HMBC spectra. No absolute configuration was determined for the diastereoisomers 12a and b as well as 13a and b. Hydrogen/carbon assignment has been validated by $^1$H, $^1$H-COSY, $^1$H-$^1$C-COSY, $^1$H, $^1$H-TOSCY, gs-HSQC, gs-HMBC, and gs-HQC-TOCSY measurements.

$^a$ (ppm), chemical shift for the indicated hydrogen/carbon.

$^b$ J (Hz), coupling constant between the indicated protons.

$^c$ No coupling constant can be determined, due to overlapping multiplets.

$^d$ Coupling constant determined via gs-HSQC recorded with 4096 data points in F2 and 512 increments in F1; Fourier transformation size 8000 (F2) × 1000 (F1).

$^e$ Assignment may have to be reversed.

$^f$ No $^1$C chemical shift can be determined from the HSQC spectrum, due to H/D exchange.
The two diastereoisomers with \([M + H]^+\) at \(m/z\) 647 were isolated from 3-DOG 7 incubation, the protective groups cleaved off with 3 \(\times\) HCl at room temperature and the products purified by preparative HPLC. Accurate mass determination of the obtained compounds gave \([M + H]^+\) at \(m/z\) 647.2572, corresponding to a loss of two \(t\)-Boc groups from \(m/z\) 647 and an elemental composition of \(C_{25}H_{42}N_6O_2\). The NMR data, compiled in Table I, unequivocally prove formation of \(N^\alpha-[2-[(4S)-amino-5-oxido-5-oxopentyl]amino]-5-[(2S,3R)-2,3,4,5-tetrahydroxybutyl]-3,5-dihydro-4H-imidazol-4-ylidene]-L-lysinate (12), existing as a pair of diastereoisomers 12a and 12b. For this compound, we have coined the abbreviation DOGDIC, representing the term deoxyglucose-derived imidazoline cross-link; this acronym is in accordance with those for the glycolyl and methylglyoxal products GODIC and MODIC (29).

**Formation of the Pentose-derived Cross-links**—We had found previously (22) that reaction of pentoses with \(N^\alpha-t\)-Boc-L-lysine and \(N^\alpha-t\)-Boc-L-arginine yields the \(t\)-Boc derivatives of the prominent cross-link pentosidine 11 as well as of two compounds with quasimolecular ions being 20 and 38 Da higher, respectively, than that for the \(t\)-Boc derivative of 11 \([M + H]^+\) at \(m/z\) 579. The proposed structures for these products, however, had not been confirmed. We therefore reinvestigated this subject and incubated \(\alpha\)-ribose (2a) with the \(t\)-Boc-protected amino acids at pH 7.4 and 60 °C in both 1 M phosphate and 10 mM hydrogen carbonate buffer, according to our experiences from the hexose experiments on the influence of the reaction medium on product formation. In fact, use of the different buffers severely changed the compound ratios. Whereas generation of the \(t\)-Boc derivative of 11 and the product with \([M + H]^+\) at \(m/z\) 599 is clearly favored in the concentrated phosphate buffer relative to the compound with \([M + H]^+\) at \(m/z\) 617, the reverse holds for the incubation in hydrogen carbonate medium. Since these quasimolecular ion signals are both 30 Da lower than those for the \(t\)-Boc derivatives of glucosepane 9 or DOGDIC 12, representing the mass difference between pentoses and hexoses, it seems reasonable to expect homologous structures. This assumption is supported by the fact that reaction of \(N^\alpha-(\text{tert-butoxycarbonyl})-N^\alpha-(1-deoxy-\alpha-D-ribofuranos-1-yl)-L-lysine (t-Boc-4a, i.e. Amadori product of \(\alpha\)-ribose (2a) and \(N^\alpha-t\)-Boc-L-lysine) predominantly yields the \(t\)-Boc derivative of 11 and the compound with \([M + H]^+\) at \(m/z\) 599, whereas incubation of 3-deoxypentosulose (8, 3-DOP) gave only the product with \([M + H]^+\) at \(m/z\) 617. These results are completely analogous to those of the hexose experiments.

The products with \([M + H]^+\) at \(m/z\) 617 were obtained from the reaction of 3-DOP 8 with \(N^\alpha-t\)-Boc-L-lysine and \(N^\alpha-t\)-Boc-L-arginine. The protective groups were cleaved off in acidic medium, and the two diastereoisomers 13a and 13b of \(N^\alpha-[2-[(4S)-amino-5-oxido-5-oxopentyl]amino]-5-[(2S,2,3,5-dihydroxypropyl]-3,5-dihydro-4H-imidazol-4-ylidene]-L-lysinate (13) were isolated by preparative HPLC. Accurate mass determination \((m/z\) 417.2463) proved the expected elemental composition \(C_{25}H_{42}N_6O_2\); the NMR data for 13a,b are given in Table I. Analogous to the acronym DOGDIC for the hexaside derivative, we designated 13 as DOPDIC.

In contrast to glucosepane 9, the postulated pentose analog 10 was very difficult to obtain. On the one hand, reaction of \(\alpha\)-ribose (2a) or other pentoses with \(N^\alpha-t\)-Boc-L-lysine and \(N^\alpha-t\)-Boc-L-arginine in phosphate buffer gave a much lower yield for the desired product with \([M + H]^+\) at \(m/z\) 599 compared with that for the \(t\)-Boc derivative of 9 from the corresponding \(\alpha\)-glucose incubation. On the other hand, the compound with \([M + H]^+\) at \(m/z\) 599 proved unstable in the course of work up, especially at high pH values. It was thus isolated only in small amounts, the \(t\)-Boc groups were eliminated as described, and the resulting 6-((6S)-2-[(4S)-4-
ammonio-5-oxido-5-oxopentyl[amino]-6-hydroxy-5,6,7,7a-tetrahydro-4H-imidazo[4,5-b]pyridin-4-yl]-l-norleucinate (10) was purified by preparative HPLC. As for glucosepane 9, the pair of diastereoisomers 10a and 10b could be separated chromatographically. However, interconversion between 10a and 10b is much faster than that observed for 9a,b (22). Since it can be at least retarded in acidic medium, we used a trifluoroacetic acid (0.05% in D2O)-acetonitrile gradient for LC-NMR measurements. This coupling allows for immediate recording of the spectra subsequent to chromatographic separation and was the only possibility to obtain proper 1H data for each diastereoisomer. Based on these data and with respect to the very low product amounts, 13C chemical shifts and connectivities were only established for 10a (Table I), definitely proving the bicyclic structure and the homology to glucosepane 9 (see under "Structural Assignments"). The elemental composition C17H31N6O5 of 10 was confirmed by accurate mass determination showing m/z 399.2353 for [M + H]+. Because of the saturated six-membered ring in 10, we named this compound pentosinane according to the name pentosidine for 11. Pentosinane 10 proved to suffer almost quantitative H/D exchange at C-5 (the numbering does not follow IUPAC rules and is given in Table I) when stored for about 48 h in acidic D2O but apart from that it is quite stable. If the pH of the D2O solution, however, is raised to 7.4 and the mixture kept at 50 °C, 10 is rapidly degraded. Fig. 2 shows the total ion current of LC-(ESI)MS runs after 0, 2, and 21 h of incubation, respectively. Chromatogram B shows signals for all involved compounds: 14a,b, the intermediate 15, and the AGOE pentosidine 11. Compound 14 is quantitatively transformed to 11 within 21 h.

15 and pentosidine 11, starting from the pentosinane derivatives 10 and 14, respectively, is summarized in Fig. 4.

Conservation of the Original Carbohydrate Backbone during Formation of the Cross-links 9–13—The fact that glucosepane 9, DOGDIC 12, pentosinane 10, pentosidine 11, and DOPDIC 13 all are built up with the original carbon chain of the carbohydrate retained was unequivocally proven by reactions of an equimolar mixture of D-glucose (1) and D-glucose-13C6 with Nα-t-Boc-L-lysine and Nα-t-Boc-L-arginine. Only t-Boc derivatives for 9, 12, and 13, which had either the natural 13C/L label or an isotope pattern characteristic for the incorporation of 13C6 and 13C4, respectively, were detected by LC-(ESI)MS. Involvement of C2 and/or C4 sugar degradation products, such as glycoaldehyde and glyceraldehyde, in the reaction pathways of 9–13 (Fig. 1) would result in a scrambling of the 13C and 12C labels in the cross-link and therefore can be definitely excluded. Formation of minor amounts of the pentose-derived compounds 10, 11, and 13 in the glucose incubations can be rationalized by the well established generation of D-arabinose (2c) in the course of autoxidative glycosylation (6).

Structural Assignments—Since compounds 10, 12, and 13 represent novel cross-links between lysine and arginine side chains, special effort was invested in unequivocally establishing their structures. For 10a and for both diastereoisomers of 12 and 13, 1H and 13C NMR chemical shifts (δ) and coupling constants (J) are compiled in Table I. All gs-HMBC spectra show only one single cross-peak for the H2-1 triplet in the downfield region, i.e. with C-2. The other two cross-peaks connect this proton resonance with the non-hetero-substituted...
C-2' and C-3' of the arginine moiety. Alternative structures with endocyclic N° nitrogen of the L-arginine side chain, in contrast, would require correlation of H-1'-1 with both quasi-carbonyl carbons (C-2 and C-4) or with C-2 and C-5 in the five-membered ring (29). The N° thus has remained exocyclic in the formation of the heterocyclic cores. The conclusion that the difference in the elemental composition (H₂O) between DOPDIC 13 and pentosinane 10 results from the closure of the six-membered ring is based on the following arguments. The gs-HMBC spectrum for 10a displays a clear correlation for one of the diastereotopic methylene protons at C-1' (H-1', 3.89 ppm) with both C-4 (179.6 ppm) and C-8 (54.5 ppm); the latter connectivity can only be explained with C-8 adjacent to the N° of the lysine moiety. In 10a, the C-8 resonance is shifted high-field by about 11 ppm relative to 13 which is rationalized best by the transformation of the alcohol group into an amine. Unfortunately, no chemical shift could be determined for C-5 due to the H/D exchange at this position occurring during the long acquisition time for the gs-HSQC spectrum. However, this substructure is properly characterized by the corresponding proton spin system remaining almost unaffected within the short acquisition time for ¹H NMR. The pronounced difference for the resonances of the diastereotopic protons at C-6 (δ 1.62 ppm) represents an independent proof for the ring closure; it is incompatible with the free rotation expected for the polyhydroxalkyl side chain in 13.

Cross-link Determination in 3-Decosylsone/Carbohydrate-BSA Incubations—To evaluate the extent of intra- and intermolecular cross-linking by the various compounds 9–13, BSA was incubated with 3-DOG 7, 3-DOP 8, D-glucose (1), and D-arabinose (2c), respectively, at pH 7.4 and 37 °C. Aliquots were taken at regular intervals and dialyzed against water using a membrane with 10-kDa MWCO. Experiments with authentic synthetic material have shown all investigated cross-links except for pentosidine 11 to be unstable under the conditions for conventional acidic hydrolysis of proteins (6 × HCl, 110 °C, 24 h). The lyophilized BSA was therefore cleaved enzymatically following a procedure developed by Glomb,² which effects close to quantitative hydrolysis even for highly cross-linked proteins. This method additionally permits direct introduction of the resulting solutions into the LC-MS system since the low buffer concentrations used for enzyme incubations do not interfere with the ion-pair chromatography.

The LC-(ESI)MS system was calibrated in the range of 3.9–2485 μg/liter of 9/liter, 3.9–2490 μg of 12/liter, 7.8–500 μg of 13/liter, and 6.2–402 μg of 11/liter, respectively. The linear calibration graphs are described by the equations area = (−1243 ± 2176) + (703 ± 20)liter/μg × c(9), area = (−899 ± 1302) + (776 ± 12)liter/μg × c(12), area = (−7052 ± 6917) + (846 ± 32)liter/μg × c(13), and area = (−6074 ± 6542) + (700 ± 37)liter/μg × c(11), the values in parentheses representing means ± confidence intervals (p = 95%). The standard error (S_p) was determined as 1702, 934, 761, and 1084, respectively. Limits of detection 13.8, 7.5, 13.0, or 12.5 μg/liter, and limits of quantitation 21.9, 11.6, 19.1, or 19.4 μg/liter for 9, 12, 13, or 11.

² M. A. Glomb, personal communication.

were calculated according to the recommendations of the Deutsche Forschungsgemeinschaft (30). Since the molar (ESI)MS response is very similar for all cross-links, we used the calibration graph for DOPDIC 13 also for the quantification of pentosinane 10. Due to the minute amount available and the chemical lability of 10, it is not practical to prepare calibration standards for this product. The concentrations of 9–13, determined for the respective incubation, can be correlated with the derivatization rate of arginine side chains in the BSA molecule as demonstrated in Figs. 5-7.

From the reaction of 50 g/liter BSA with 2 mM 3-DOG 7 and 3-DOP 8, derivatization quota of 1.94 ± 0.2 mmol of 12/mol Arg and 1.53 ± 0.06 mmol of 13/mol of Arg after 14 and 7 days, respectively, could be established (Fig. 5). The bicyclic structures glucosepane 9 or pentosinane 10/pentosidine 11 were not detected; this is in accordance with the results for the cross-link syntheses described above. To prove that DOGDIC 12 is also formed with D-glucose instead of 3-DOG 7 as substrate, we re-analyzed the protein from an incubation of 100 mM D-glucose with 50 g/liter BSA, which had already been used for the quantification of glucosepane 9 (22). The graphs for 9 and 12 are shown in Fig. 6. The values given for glucosepane 9 are almost doubled compared with our previously published data; this can be ascribed to the improved enzymatic digestion procedure. DOGDIC 12 (1.29 ± 0.02 mmol/mol Arg, 56 days) attains about half the derivatization quota of 9. Incubation of 100 mM D-arabinose (2c) with 50 g/liter BSA shows pentosidine 11 as the dominant cross-link (2.23 ± 0.06 mmol/mol Arg, 29 days, Fig. 7), followed by DOPDIC 13 (1.17 ± 0.06 mmol/mol Arg, 29 days). The values obtained for pentosinane 10 (maximum 0.44 ± 0.06 mmol/mol Arg) presumably underestimate the original BSA derivatization by this compound since 10 is
expected to be in part transformed into 11 in the course of enzymatic hydrolysis (3 days, pH 7.4, 37 °C). Aliquots were taken at the indicated time intervals, the protein was isolated by ultrafiltration, enzymatically digested, and the respective cross-link quantified by LC-(ESI)MS.

DISCUSSION

Cross-linking and insolubilization are among the major biochemical changes that proteins suffer during aging. Enhancement of this process in extracellular matrix is implicated in a number of age- and diabetes-associated complications, e.g. arteriosclerosis and cataract formation. Thus, in recent years, much effort has been focused on the structural analysis of cross-linking units derived from the Maillard reaction. In this study, we have characterized the three novel cross-links glucosepane 9 and pentosidine 11 in a D-arabinose (2c)-BSA incubation. BSA (50 g/liter) and 1 (100 mM) were incubated at pH 7.4 and 37 °C. Aliquots were taken at the indicated time intervals, the protein was isolated by ultrafiltration, enzymatically digested, and the respective cross-link quantified by LC-(ESI)MS.

FIG. 7. Time course for the formation of the cross-links pentosinane 10, DOPDIC 13, and pentosinane 10 in a d-arabinose (2c)-BSA incubation. BSA (50 g/liter) and 2c (100 mM) were incubated at pH 7.4 and 37 °C. Aliquots were taken at the indicated time intervals, the protein was isolated by ultrafiltration, enzymatically digested, and the respective cross-link quantified by LC-(ESI)MS.

Fig. 6. Time course for the formation of the cross-links glucosepane 9 and DOGDIC 12 in a D-glucose (1)-BSA incubation. BSA (50 g/liter) and 1 (100 mM) were incubated at pH 7.4 and 37 °C. Aliquots were taken at the indicated time intervals, the protein was isolated by ultrafiltration, enzymatically digested, and the respective cross-link quantified by LC-(ESI)MS.

The hexose and pentose pathways are differentiated predominantly by the chemical stability of the homologous cross-links glucosepane 9 and pentosinane 10. While 9 represents a proper AGE under physiological conditions, 10 is smoothly oxidized to 15 (Fig. 4) and subsequently dehydrated to the AGOE pentosinane 10 (Figs. 2–4). The oxidation rate positively correlates with the pH value. This finding agrees well with the observation that pentosinane generation is favored at an alkaline pH, whereas concomitant generation of the latter yield specially high amounts of the bicyclic structure, whereas concomitant generation of 10 is minor. Hence, the other bicyclic structures 9–11 are formed not at all or only in negligible amounts. This was completely unexpected since from the substitution pattern of the six-membered ring in pentosinane 10 and the seven-membered ring in glucosepane 9, 3-deoxyosones would constitute reasonable precursors. Both 3-DOP 8 and 3-DOG 7 were shown to be at most a minor source for pentosine 11 (31–33). This is easily understood now that pentosinane 10 has been established as the precursor of pentosidine 11 (see below). The absence of 9–11 in the 3-deoxyosone reaction mixtures, including those with BSA, also definitely rules out DOGDIC 12 or DOPDIC 13 as precursors for glucosepane 9 or pentosinane 10 and thus invalidates the proposed hypothetical formation pathway for 9 (22). Compounds 9–11 are formed in substantial concentration only from the respective native carbohydrates or Amadori products; the latter yield specially high amounts of the bicyclic structure, whereas concomitant generation of 12 and 13 is minor. Hence, Amadori products such as 3 and 4 are the pivots of the overall reaction scheme, as outlined in Fig. 1. Grandhee and Monnier (32) reported a positive effect of phosphate salts on the formation of 11; we found the same enhancement for the formation of the other bicyclic structures 9 and 10.

The hemiketal of 3 is assumed to suffer water elimination at C-2 in the course of the nucleophilic attack of $N^+$ at C-6 with concomitant ring expansion, yielding azepanone 16.

The results reported here clearly show that both 12 and 13 are formed from the reaction of L-lysine and l-arginine side chains with the respective 3-deoxyosone 7 or 8. The corresponding methylglyoxal- and glyoxal-derived structures MODIC and GODIC have already been described in a preceding paper (29). From the reaction of 3-DOG 7 and 3-DOP 8, however, the bicyclic structures 9–11 are formed not at all or only in negligible amounts. This was completely unexpected since from the substitution pattern of the six-membered ring in pentosinane 10 and the seven-membered ring in glucosepane 9, 3-deoxyosones would constitute reasonable precursors. Both 3-DOP 8 and 3-DOG 7 were shown to be at most a minor source for pentosidine 11 (31–33). This is easily understood now that pentosinane 10 has been established as the precursor of pentosidine 11 (see below). The absence of 9–11 in the 3-deoxyosone reaction mixtures, including those with BSA, also definitely rules out DOGDIC 12 or DOPDIC 13 as precursors for glucosepane 9 or pentosinane 10 and thus invalidates the proposed hypothetical formation pathway for 9 (22). Compounds 9–11 are formed in substantial concentration only from the respective native carbohydrates or Amadori products; the latter yield specially high amounts of the bicyclic structure, whereas concomitant generation of 12 and 13 is minor. Hence, Amadori products such as 3 and 4 are the pivots of the overall reaction scheme, as outlined in Fig. 1. Grandhee and Monnier (32) reported a positive effect of phosphate salts on the formation of 11; we found the same enhancement for the formation of the other bicyclic structures 9 and 10.

The hemiketal of 3 is assumed to suffer water elimination at C-2 in the course of the nucleophilic attack of $N^+$ at C-6 with concomitant ring expansion, yielding azepanone 16.

FIG. 8. Hypothetic reaction pathway for the formation of glucosepane 9 from the Amadori product 3 via the azepanone 16.
plexed potential traces, involvement of metal ions in the oxidation of 10 is still uncertain. However, Litchfield et al. (35) reported that pentosidine 11 is formed from ρ-arabinose (2e) even with exclusion of oxygen and in the presence of the strong metal-chelating agent diethylenetriaminepentaaacetic acid, i.e. under antioxidative conditions. They found the yield for 11 to be decreased by only about 50% compared with oxidative conditions and assumed generation of 11 to proceed via an oxidation mechanism that is difficult to inhibit and might involve intermolecular redox rather than oxygen-dependent (autoxidation) reactions. This result coincides with our observation that partial transformation of pentosane 10 to 11 cannot be prevented by applying antioxidative conditions as employed for the synthesis of 10 from pentoses.

Recently, Chellan and Nagaraj (36) proposed a novel mechanism for the formation of pentosidine 11. They had observed cross-linking of non-glycated proteins by 11 from incubations with ribated proteins. Like Grandhee and Monnier previously (32), they assumed the Schiff base and the Amadori product to be crucial intermediates in the formation of 11. As actual precursors, however, they suggest short chain carbohydrates such as glycolaldehyde and glyceraldehyde from which 11 was shown to be formed. Any mechanism based on the consecutive reaction of such carbonyl intermediates, normally being present in very low concentrations, seems unlikely already from kinetic arguments. Alternatively, the native pentoses can be set free from the Schiff base of the ribated protein, for example, and then form Amadori products and subsequently pentosidine 11 (Fig. 1) on the non-glycated protein. As proven by our experiments with d-glucose-13C6 for 9–13, the original sugar carbon chain is retained in the course of cross-link formation. This fact is further emphasized by the observation that short chain carbohydrate compounds are formed from both pentoses and hexoses, but only pentoses effectively produce pentosidine 10 and pentosidine 11. Formation of 9–11 from carbohydrate fragments thus is supposed to be a minor pathway. It still remains to be clarified how the seven- and six-membered rings in 9 and 10, respectively, are actually closed at a very early stage of the Maillard reaction. A hypothetical formation pathway for 9, starting from the hemiketal of the Amadori product 3, is outlined in Fig. 8. Triggered by nucleophilic attack of N* at C-6, the C-2 hydroxy group is eliminated with concomitant ring expansion. The resulting azepanone 16 suffers a second water elimination at C-3 and finally reacts with the arginine side chain to glucosepane 9 and DOGDIC 12 therefore are expected to be of prime physiological significance. This argument is strengthened by our studies on the major cross-links in human serum albumin which will soon be reported. Like Wells-Knecht et al. (6) and Monnier et al. (38), we in fact believe that most physiologically relevant cross-links are colorless and non-fluorescent just as 9 and 12. The importance of Amadori products in protein cross-linking, which has often been doubted, therefore needs to be reconsidered.

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