Isolation and Characterization of Glyoxal−Arginine Modifications

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5-(4,5-Dihydroxy-2-imino-1-imidazolidinyl)norvaline (**1**) was identified as the only product of the early reaction of arginine with glyoxal, which was slowly degraded to N^5-[[(carboxymethyl)amino]-(imino)methyl]ornithine (**3**, N^7-carboxymethylarginine). No other structures could be detected within a range of pH 4−8 and 20−50 °C in reaction conditions. The rates of formation for both products increased with pH and temperature. In equilibrium, the vincinal diol groups of **1** were 86% trans configured. The formation of **1** was reversible, as could be shown by cis-trans isomerization of the separated isomers and by regeneration of arginine in the presence of the α-dicarbonyl trapping reagents, *o*-phenylenediamine and aminoguanidine. Both **1** and **3** were converted to 5-(2-imino-5-oxo-1-imidazolidinyl)norvaline (**2**) only under strong acidic conditions.

Keywords: *Glyoxal−arginine modifications; Maillard reaction; imidazoles; N^7-carboxymethylarginine; advanced glycation endproduct*

INTRODUCTION

Short-chained α-dicarbonyl compounds such as glyoxal are of major importance as mutagens and very potent protein modifiers. Therefore, considerable effort has been undertaken to quantify glyoxal in various foods such as wine, yogurt, and fats with levels up to 1.8 mg/L (*1*−*3*). In general, the detection methods described require transformation into a stable derivative with trapping reagents such as *o*-phenylenediamine. Glyoxal was also identified in vivo. Blood plasma levels were found to increase significantly in uremic patients with chronic kidney failure, to concentrations up to 221 μg/L (*4*). Three mechanisms of formation have been elucidated for glyoxal. First, this carbonyl evolves during the Maillard reaction of reducing sugars with amines. Major concentrations are formed at a very early stage prior to the formation of the Amadori product (*5*). Most likely, the first-formed Schiff base adduct is oxidized and then fragmented (*6*). A catalytic effect of hydrogenphosphate anions and of transition metal ions on this reaction has been reported (*7*). Glyoxal has also been shown to be formed directly from autoxidation of glucose (*8*). Second, glyoxal is formed during autoxidation of unsaturated fatty acids via mechanisms including hydroperoxide formation and β-fragmentation (*9*). Investigations concerning the synthesis of the glyoxal-lysine adduct N^ϵ-carboxymethyllysine revealed that in vivo the autoxidation of fats contributes at least the same amount of glyoxal as the degradation of sugars (*10*). Third, in vivo, glyoxal can be generated by serine degradation in the presence of myeloperoxidase at sites of inflammation (*11*). The detection of glyoxal upon irradiation of acetaldehyde, acetone, propanal, and acrolein points toward other mechanisms, which might be important in foods (*12*).

Glyoxal, as a highly reactive α-dicarbonyl, reacts with the ϵ-amino group of lysine to form N^ϵ-carboxymethyllysine and amide, imidazole (GODIC) and imidazolium (GOLD) cross-links (*13*−*15*). Also, the synthesis of vesperlysine A seems to depend on glyoxal, although the mechanism was not totally clarified (*16*). In contrast, for the reaction of glyoxal with the guanidino group of arginine contradictory results have been published. Whereas Glass and Pelzig (*17*) reported the formation of a single structure N^7,N^8-(1,2-dihydroxyethyl-1,2-yl)-arginine in glyoxal−arginine reaction mixtures, Schwarzenbolz et al. (*18*) have published the formation of exclusively 5-(2-imino-5-oxo-1-imidazolidinyl)norvaline (Glarg). The latter structure was also verified in incubations of ribose and collagen (*19*). In contrast, Iijima et al. (*20*) identified N^7-carboxymethylarginine in incubations of glucose with collagen after enzymatic hydrolysis and linked the formation of this structure to Glarg. However, no direct evidence for N^7-carboxymethylarginine synthesis from glyoxal was given. To clarify this obvious confusion of several compounds published for the reaction of arginine with glyoxal, we started to reinvestigate this reaction including isolation and full characterization of all structures involved and elucidation of their mechanistic relationship.

MATERIALS AND METHODS

Materials. Chemicals of highest quality available were obtained from Aldrich (Steinheim, Germany) and Fluka (Neu-Ulm, Germany), unless otherwise indicated.

Syntheses. *5-(4,5-Dihydroxy-2-imino-1-imidazolidinyl)norvaline* (**1**). To a solution of 1.0 g (5.9 mmol) L-arginine in 59 mL of H_2O was added 1 mL (8.9 mmol) of 40% glyoxal solution, the pH was adjusted to 7.4 with 0.1 N HCl, and the solution was incubated 4 h at 37 °C. The solution was concentrated to about 10 mL and subjected to ion exchange chromatography. The column was first washed with H_2O and basic material was then eluted with 3 N HCl. After removal of solvents, the residue was subjected to preparative HPLC. Fractions with material having R_f 0.35 + 0.45 (TLC) were combined and freeze-dried to give a colorless amorphic material (514 mg, 13.6%, **1** x 2HFBA salt based on elemental analysis).

Accurate mass (mean of 8 measurements ± standard deviation): *m*/*z* 233.1248 ± 0.0012 $[M + H]^+$ (233.1250, calcd

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for $C_8H_{17}N_4O_4$). HPLC anal: (12 °C) t_R 15.9, 16.4, 18.1, 19.4, 23.0 min. In LC/MS analysis, all peaks revealed the same *m*/*z* 233.

For NMR studies of the *trans*-isomers **1 a**+**b**, 185 mg of above material was subjected to preparative column chromatography. Fractions with material having R_f 0.45 were combined and freeze-dried to give a colorless oil (42 mg, **1 a**+**b** as CH_3COOH salt).

For cis-trans isomerization experiments, **1 a**+**b** and **1 c**−**e** were separated from 500 μg of above colorless amorphic material by TLC. Two bands with R_f 0.35 (**1 c**−**e**) + 0.45 (**1 a**+**b**) were scraped off and products were extracted from the silica gel with HPLC-solvent A. The resulting suspensions were filtered through 0.45-μm centrifuge filters.

5-(2-Imino-5-oxo-1-imidazolidinyl)norvaline (**2**). 0.87 g (5 mmol) of L-arginine and 1 mL (8.8 mmol) of 40% glyoxal solution were dissolved in 50 mL of H_2O, the pH was adjusted to 7.4, and the solution was incubated at 37 °C for 4 h. Then 50 mL of 12 N HCl was added and the mixture was heated at 95 °C for 4 h. Solvents were removed to complete dryness and the resulting residue was subjected to ion exchange chromatography. The column was washed with H_2O and 1 N HCl, and the target material was then eluted with 3 N HCl. After complete removal of solvents, the residue was subjected to preparative HPLC. Fractions with material having R_f 0.42 (TLC) were combined and freeze-dried to yield a colorless amorphic material (1.39 g, 43.2%, **2** x 2HFBA salt based on elemental analysis).

Accurate mass (mean of 8 measurements ± standard deviation): *m*/*z* 215.1142 ± 0.0008 $[M + H]^+$ (215.1144, calcd for $C_8H_{15}N_4O_3$). HPLC anal: (20 °C) t_R 30.7 min.

N^5-*[[(carboxymethyl)amino](imino)methyl]ornithine* (**3**). A solution of 0.60 g (0.93 mmol) **2** in 40 mL of H_2O was adjusted to pH 8.0 with 0.1 N NaOH and incubated at 37 °C for 2 d. During incubation the pH had to be readjusted frequently. The reaction was stopped by adding HCl to pH 2.5. Solvents were removed to about 3 mL and the solution was subjected to preparative HPLC. Fractions with material having R_f 0.30 (TLC) were combined and freeze-dried to yield a colorless amorphic material (0.31 mg, 50.4%, **3** x 2 HFBA salt based on elemental analysis).

Accurate mass (mean of 8 measurements ± standard deviation): *m*/*z* 233.1246 ± 0.0003 $[M + H]^+$ (233.1250, calcd for $C_8H_{17}N_4O_4$). HPLC anal: (20 °C) t_R 28.0 min.

Model Reactions. In general, experiments were conducted in 0.1 M phosphate buffer after sterile filtration.

N^α-t-BOC-Arginine−*Glyoxal Incubations*. For rate determination, incubations were conducted under the following conditions: 37 °C with pH 6.0, 7.0, 7.4, 8.0, and 9.0; pH 7.4 at 29, 37, 42, and 50 °C. For the general survey of the product spectrum, incubations at 37 °C were also conducted at pH 2.0, 3.0, 4.0, and 5.0. To stop incubations and for removal of the protection group, aliquots were taken up in a 100-fold excess of 3 N HCl. After 35 min at room temperature, H_2O was added in a 1000-fold excess to final dilution for HPLC analysis.

Imidazolinone **2** *Incubations*. Incubations were stopped by dilution with phosphate buffer pH 3.0 to concentrations suitable for final HPLC analysis. Incubations were also performed in the presence of *o*-phenylenediamine and aminoguanidine (both 30 mM), respectively, with no effect on CMA **3** synthesis.

Cis−*trans Isomerization*. TLC-isolated fractions of **1 a**+**b** and **1 c**−**e** were diluted with phosphate buffer of appropriate pH before incubation (see Results section). Isomerization was stopped by dilution with phosphate buffer pH 3.0. Samples were analyzed immediately by HPLC.

Dihydroxyimidazolidine **1** *- o-phenylenediamine/aminoguanidine Incubations*. The reaction conditions are given later in the legends to Figure 6. For arginine, dihydroxyimidazolidine **1**, and CMA **3** analysis, samples were diluted with solvent A prior to HPLC. For the quinoxaline analysis, samples were diluted with water, and 2-ethyl-3-methylquinoxaline was added as the internal standard. After extensive extraction with EtOAc, the organic layer was dried and evaporated, and the residue derivatized with a mixture of pyridine and *N*,*O*-bis-(trimethylsilyl)acetamide (1:1). After 1 h an aliquot was subjected to HRGC−FID. 2-Ethyl-3-methylquinoxaline was synthesized according to Glomb and Tschirnich (unpublished). For the triazine analysis, samples were injected to HPLC without postcolumn derivatization, and the effluent was monitored with a UV detector (Hitachi 655A, Merck) set at 208 nm. Quantitation was based on an external 2-amino-1,3,4-triazine standard (*5*). In addition, the identity of the peaks measured for the quinoxaline and triazine were verified by HRGC−MS.

Chromatography. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) with *n*-butanol/H_2O/HOAc/pyridine 4:2:3:3 as the mobile phase. Visualization of separated material was achieved with ninhydrine. Preparative column chromatography was performed on silica gel 60, 63−200 μm (Merck, eluent *n*-butanol/H_2O/HOAc/pyridine 10:2:3:3, 2 cm i.d. × 13 cm) and ion exchange chromatography on Dowex 50 WX 8, 50−100 mesh (H^+-form, 2 cm i.d. × 10 cm). Solvents were all chromatographic grade. From the individual chromatographic fractions solvents were removed under reduced pressure.

High-Performance Liquid Chromatography (HPLC). *Analytical systems*. A Jasco (Groβ-Umstadt, Germany) ternary gradient unit 980-PU-ND, with degasser, autosampler 851-AS, column oven CO-200, and fluorescence detector 920-FP, was used. The effluent was monitored at 340 nm for the excitation and 455 nm for the emission. Chromatographic separations were performed on stainless steel columns (VYDAC 218TP54, 250 × 4.6 mm, RP18, 5 μm, Hesperia, CA) using a flow rate of 1.0 mL min^{-1}. The mobile phase used was water (solvent A) and MeOH/water (7:3, v/v; solvent B). To both solvents (A and B), 1.2 mL/L heptafluorobutyric acid (HFBA) was added. Prior to the detector, a postcolumn derivatization reagent was added at 0.5 mL min^{-1}. This reagent consisted of 0.8 g of *o*-phthaldialdehyde, 24.7 g of boric acid, 2 mL of 2-mercaptoethanol, and 1 g of Brij 35 in 1 L of H_2O adjusted to pH 9.8 with KOH. Samples were injected at 0% B and run isocratic for 35 min, the gradient then changed to 100% B in 5 min, and held at 100% B for 10 min. The column temperature was 12 °C for the cis-trans isomerization experiments and 20 °C for all other experiments.

Preparative System. A Besta HD 2-200 pump (Wilhelmsfeld, Germany) was used at a flow rate of 18 mL min^{-1}. Elution of material was monitored by TLC and analytical HPLC. Chromatographic separations were performed on stainless steel columns (VYDAC 218TP1022, 250 × 25 mm, RP18, 10 μm). The mobile phase used was solvent A identical to the analytical HPLC system. Combined fractions were freeze-dried in a Savant-Speed-Vac Plus SC 110 A combined with a Vapor Trap RVT 400 (Life Sciences International, Frankfurt, Germany).

High-Resolution Gas Chromatography (HRGC−FID/MS). HRGC−FID was performed on a VEGA 6000 (Fisons Instruments, Mainz, Germany); quartz capillary column (60 m, 0.32 mm i.d., DB-5, 1.0 μm, He, 35 cm/s; J&W Scientific, Cologne, Germany); injection port, 270 °C; detector, 270 °C; temperature program, after injecting the samples at 120 °C, the temperature of the oven was raised at 5 °C min^{-1} to 170 °C and held for 20 min, then raised at 5 °C min^{-1} to 280 °C, and held for 15 min. For HRGC−MS analysis, the HRGC was connected to a MAT ITD 700 (Finnigan, Bremen, Germany); transfer line, 280 °C; EI at 70 eV.

Accurate Mass Determination. For accurate mass determination (*21*), a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer equipped with an ESI interface was employed: ESI+; source temperature, 80 °C; capillary, 3.0 kV; cone voltage, 20 V. The data were collected in the multichannel acquisition (MCA) mode with 128 channels per *m*/*z* unit using 13 scans (6 s) with 0.1 s reset time. The resolution was 650 (10% valley definition). The sample was dissolved for analysis in water/MeCN (1:1) containing poly(ethylene glycol) 200 (0.1 μg/μL) as reference material, ammonium formate (0.1%), and formic acid (0.2%); the sample concentration was similar to that of PEG 200. The solution was introduced into the ESI source at a flow of 5 μL/min. With a *m*/*z* 190−285 scan range, 5 reference peaks could be used

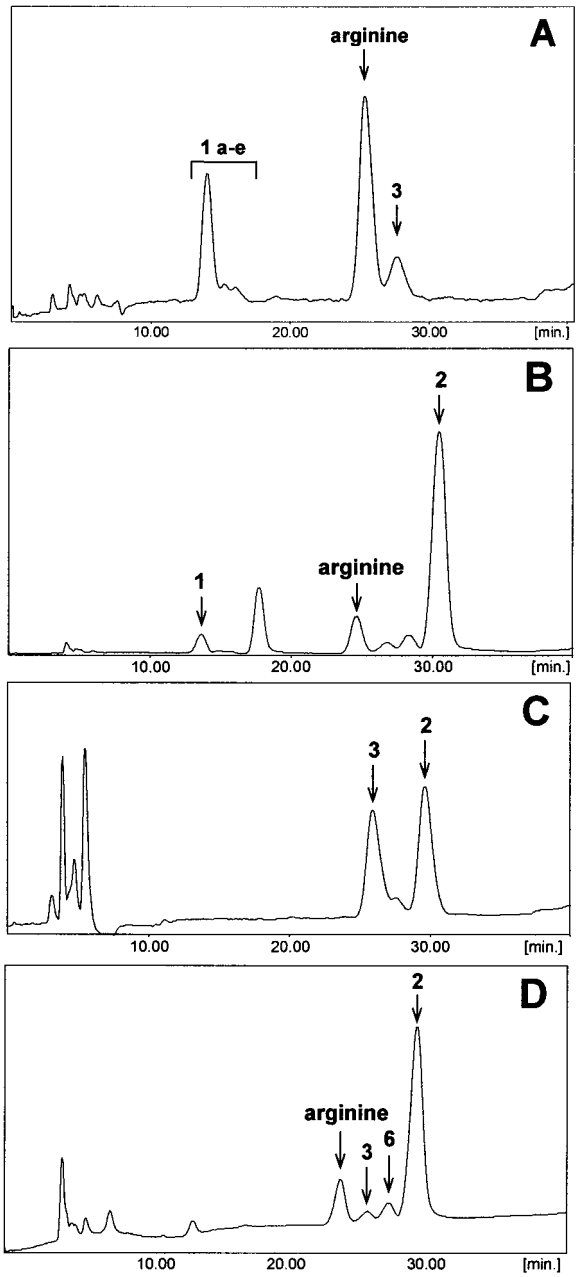


Figure 1. HPLC chromatograms obtained after workup of reaction mixtures from(A) incubations of N^{α}-*t*-BOC-arginine with glyoxal at 37 °C/pH 7.4/14 d; (B) degradation of dihydroxyimidazolidine **1** during acid protein hydrolysis (6 N HCl/110 °C/deaerated/1 h); (C) degradation of imidazolinone **2** (20 °C/pH 7.0/24 h); (D) degradation of N^{7}-carboxymethylarginine **3** during acid protein hydrolysis (6 N HCl/110 °C/deaerated/24 h).

for calibration: *m*/*z* 195.1234, 212.1498, 239.1495, 256.1760, and 283.1757.

Magnetic Resonance Spectroscopy (NMR). NMR spectra were recorded on a Brucker DRX 500 instrument (Rheinstetten, Germany). Chemical shifts are given relative to external Me_4Si.

RESULTS

Isolation and Characterization of Reaction Products. In the early reaction of glyoxal with N^{α}-*t*-BOC-arginine a single product **1** was formed very quickly, which was then slowly degraded to compound **3** (Figures 1A and 4). No other structures could be detected at pH 4−8 and 20−50 °C. 5-(4,5-Dihydroxy-2-imino-1-imidazolidinyl)norvaline (**1**, dihydroxyimidazolidine) was isolated from aqueous solutions of L-arginine with glyoxal at pH 7.4 by a combination of ion exchange chromatography to remove excess of nonbasic material and of ion pair chromatography on reverse-phase material for final purification. As depicted in Figure 2B the resulting material consisted of 5 structures, which were further separated by preparative chromatography on silica gel with a butanol/water/acetic acid/pyridine eluent into **1 a**+**b** and **1 c**−**e** (Figure 2A, C). In coupled high performance liquid chromatography−electrospray ionization mass spectrometry (HPLC−ESI−MS) all 5 structures revealed the same molecular mass of *m*/*z* 233 for the M+1 pseudo molecular ion. Accurate mass determination proved the elemental composition of the structures to be the simple addition product of glyoxal and arginine. Final structural evidence was achieved by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), as well as heteronuclear multiple quantum coherence-(HMQC) and heteronuclear multiple bond correlation-(HMBC) NMR experiments, which are summarized for the major fraction **1 a**+**b** in Table 1. The data unequivocally identified the structures as the two possible *trans*-isomers of the dihydroxyimidazolidines. Especially the $^{3}J_{5-H,C-8}$ correlation showed that the ring structures are N^{δ}-endocyclic. In the ^{1}H NMR, protons 7-H and 8-H appeared as singlets, which means that the $^{3}J_{7-H,8-H}$ coupling constant is extremely small. As this can be explained only by a dihedral angle of about 90°, the diol configuration of **1 a**+**b** has to be trans. Because of the fixed stereochemistry at C-2, the *trans*-isomer exists as a pair of diastereomers. They could be separated by HPLC and give rise to a doubling of the ^{13}C NMR signals for C-2, C-4, C-5, and C-8 ($\Delta\delta$ 0.03−0.11 ppm). Thin-layer chromatography (TLC) of the complete mixture of **1 a**−**e** led to separation of two spots at R_f 0.35 for **1 c**−**e** and R_f 0.45 for **1 a**+**b**. If boric acid was added, the lower spot was retarded to R_f 0.1, while the upper one remained unchanged. This indicated that **1 c**−**e** possess a vicinal *cis*-diol structure and can form stable complexes with borate, thus altering their chromatographic properties. **1 c**+**d** always had the same concentration, indicating their structures to be the two possible *cis*-isomers. The ^{1}H NMR of the total mixture of **1** differed from the one for **1 a**+**b** in two additional signals at 5.25 and 5.45 ppm, their relative abundance exactly matching the one in HPLC for **1 c**+**d** and **1 e**, respectively. The C−H bonds of the *cis*-configured diols can deviate from coplanarity by, at most, 30°; this should result in a relatively large $^{3}J_{7-H,8-H}$ coupling constant. However, for the actual structures both these protons are fortuitously isochronous. Thus, only one singlet can be observed, and no coupling constant can be determined. Similar data have been published for other 2-amino-4,5-dihydro-4,5-dihydroxy-1-alkylimidazolines (*22*). The small signal at 5.45 ppm was assigned 7-H and 8-H of **1 e**. The molecular mass and the *cis*-diol configuration, which was assigned by NMR and TLC experiments, indicated the N^{δ}-exocyclic structure N^{5}-(4,5-dihydroxy-4,5-dihydro-1*H*-imidazol-2-yl)ornithine (**1 e**, dihydroxyimidazolidine).

For the isolation of 5-(2-imino-5-oxo-1-imidazolidinyl)-norvaline (**2**, imidazolinone) reaction mixtures of glyoxal and L-arginine were incubated as described for **1** and, after acidification with HCl, heated at 95 °C for several hours. The spectral data proved the structure to be

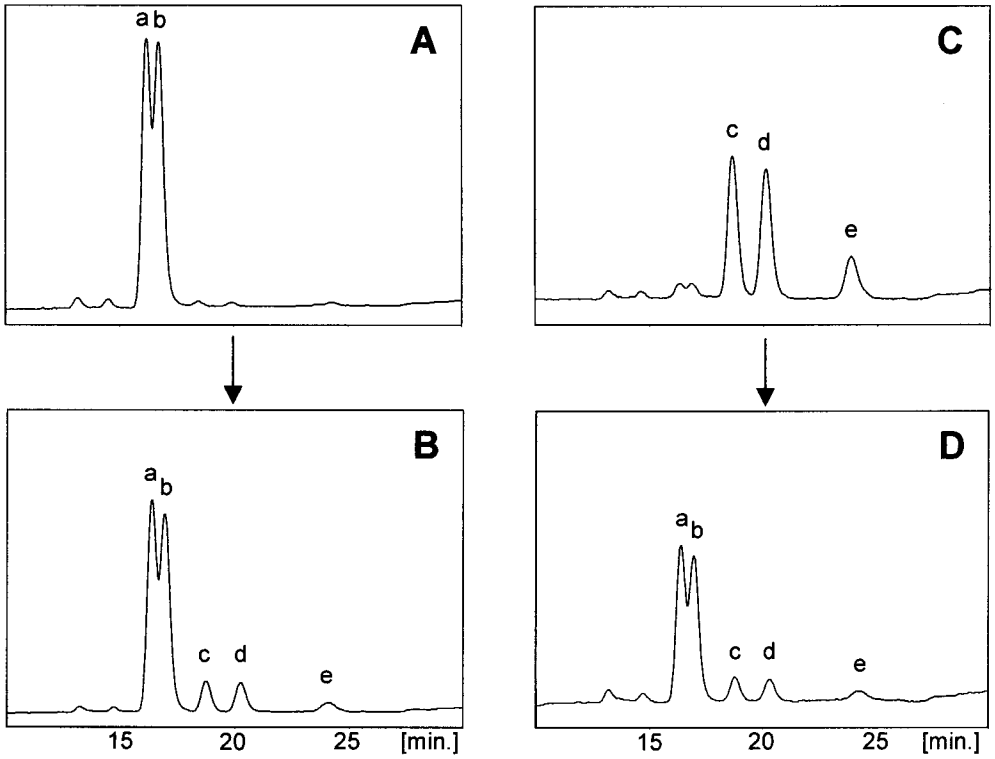
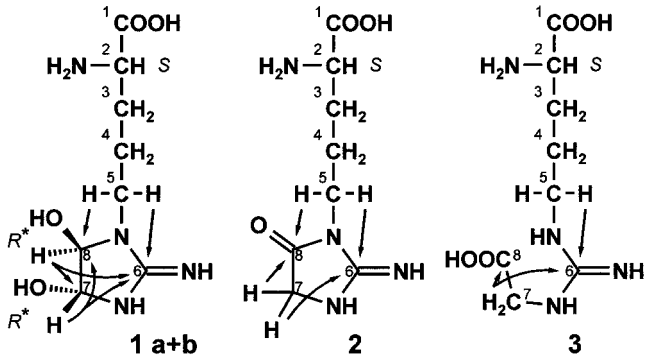


Figure 2. Cis-trans isomerization of dihydroxyimidazolidine **1** at 37 °C/pH 7.4/21 h; starting from *trans*-isomers **1 a**+**b** (A → B) and from *cis*-isomers **1 c**−**e** (C → D).

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Dihydroxyimidazolidine 1 a+b, Imidazolinone 2, and N^7-carboxymethylarginine 3 (in D_2O)[a]



	1 a + b	2	3
		^{1}H NMR δ [ppm]	
2-H	3.57 (t, 1 H, J = 5.2 Hz)	4.02 (t, 1 H, J = 6.4 Hz)	3.88 (t, 1 H, J = 6.3 Hz)
3-H	1.72 (m, 2 H)	1.90 (m, 2 H)	1.78 (m, 2 H)
4-H	1.57 (m, 2 H)	1.73 (m, 2 H)	1.52 (m, 2 H)
5-H	3.26 (t, 2 H, J = 7.1 Hz)	3.65 (t, 2 H, J = 7.0 Hz)	3.07 (t, 2 H, J = 6.8 Hz)
7-H	4.98 (s, 1 H)	4.18 (s, 2 H)	3.86 (s, 2 H)
8-H	4.94 (s, 1 H)		
		^{13}C NMR δ [ppm]	
C-1	174.19	172.82	171.50
C-2	54.18/54.23[b]	53.57	52.33
C-3	27.35	28.06	26.79
C-4	22.89/22.92[b]	24.12	23.65
C-5	41.00/41.03[b]	40.28	40.40
C-6	156.79	160.14	156.18
C-7	83.09	48.78	42.42
C-8	88.76/88.87[b]	174.56	172.27

[a] δ, chemical shift; J, coupling constant; hydrogen/carbon assignments are verified by HMQC and ^{13}C-DEPT measurements; selected $^2J_{H,C}$ and $^3J_{H,C}$ correlations from HMBC experiments are marked by arrows; the prefixes *R** denote the relative configuration at the respective stereogenic centers. [b] Carbon chemical shifts for the respective two diastereoisomers.

identical to Glarg published by Schwarzenbolz et al. (*18*, Table 1). By the use of deuterated dimethyl sulfoxide these authors were able to determine the protonation of the nitrogens and the location of the positive charge.

To isolate **3**, aqueous solutions of **2** were adjusted to pH 8 and incubated 2 days at 37 °C. Accurate mass determination showed the material to be the addition product of arginine and glyoxal. The HMBC experiments indicated no $^3J_{5-H,C-8}$ correlation. Compared to **1** and **2** this indicates that the molecule does not exist as a ring, but as an open-chain structure. This conversion of **2** to **3** was further supported by high field shifts for the signals of 5-H from 3.65 to 3.07 ppm, because of hydrolysis of the amide moiety, and of 7-H from 4.18 to 3.86 ppm because of the ring opening. Also, the high field shift for C-7 from 48.8 to 42.4 ppm has been published for analogous creatine/creatinine systems (*23*). Taken together, the spectral data verified **3** to be N^5-[[(carboxymethyl)amino](imino)methyl]ornithine (N^7-carboxymethylarginine) and seemed to be identical to work just published by Iijima et al. (*20*), although no detailed NMR data were given.

Kinetic Studies. The detailed HPLC analysis of isolated dihydroxyimidazolidine **1**, as well as of **1** in glyoxal−arginine incubations, revealed a ratio of 6.14:1 for the *trans*-isomers **1 a**+**b** to the *cis*-isomers **1 c**−**e**. If both isomer fractions were incubated separately at 37 °C and pH 7.4, the equilibrium of the total isomer mixture was quickly reached again (Figure 2). Incubations conducted at various pH conditions showed that the rate of isomerization increased with higher pH values (trans-cis ratio measured in incubations conducted at 20 °C, start ratio 0.07:1; ratio after 1 h at pH 3.0, 0.07:1; pH 4.0, 0.07:1; pH 5.0, 0.11:1; pH 6.0, 0.23:1; pH 7.0, 0.41:1; pH 7.4, 0.92:1; pH 8.0, 3.17:1). Under all circumstances the ratio of **1 c**+**d** to **1 e** has never changed.

The stability of **1**, **2**, and **3** was studied under acid protein hydrolysis conditions (6 N HCl, 110 °C). As shown in Figures 3A and 1B, dihydroxyimidazolidine **1** was completely transformed into imidazolinone **2** within the first 2 h. **2**, in turn, was slowly degraded to arginine, reaching about 20% after 24 h. Under the same conditions, N^7-carboxymethylarginine **3** also reacted to imidazolinone **2**, but turnover was much slower. Simultaneously, an additional minor unstable compound **6** was generated (Figures 3B and 1D). Thus, acid hydrolysis is not appropriate to investigate the formation of dihydroxyimidazolidine **1** and N^7-carboxymethylarginine **3** in glyoxal-protein mixtures. However, if the reaction conditions are kept well controlled, imidazolinone **2** might be used as a specific tool to gain insight into the overall extent of glyoxal−arginine modifications. Stability was also tested under conditions published for enzymatic hydrolysis by Schwarzenbolz et al. (*18*). The method employs successively a combination of pepsin, pronase E, prolidase, and aminopeptidase first in 0.02 N HCl and then in 2 M Tris buffer pH 8.2. Under these conditions dihydroxyimidazolidine **1** was rather stable, but about 10% was converted to arginine and about 5% was converted to N^7-carboxymethylarginine **3**. In contrast, imidazolinone **2** was almost completely degraded to N^7-carboxymethylarginine **3**. Starting from **3** about 10% was transformed to imidazolinone **2**. Detailed studies revealed that cyclization takes place during the first acidic step.

The concentrations of arginine, dihydroxyimidazolidine **1**, and N^7-carboxymethylarginine **3** were monitored in glyoxal−arginine reaction mixtures by HPLC at temperatures of 25−50 °C and pH values of 6.0−9.0 (Figure 4). Until all glyoxal was used up, the formation

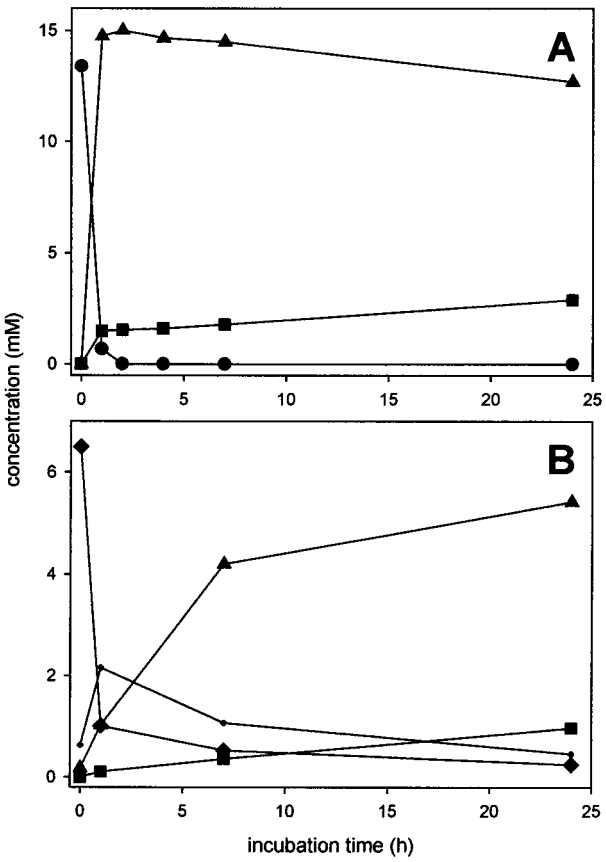


Figure 3. Degradation of dihydroxyimidazolidine **1** (A) and N^7-carboxymethylarginine **3** (B) under acid protein hydrolysis conditions (6 N HCl, 110 °C, deaerated). ●, Dihydroxyimidazolidine **1**; ■, arginine; ▲, imidazolinone **2**; ◆, N^7-carboxymethylarginine **3**; ·, unknown compound **6**.

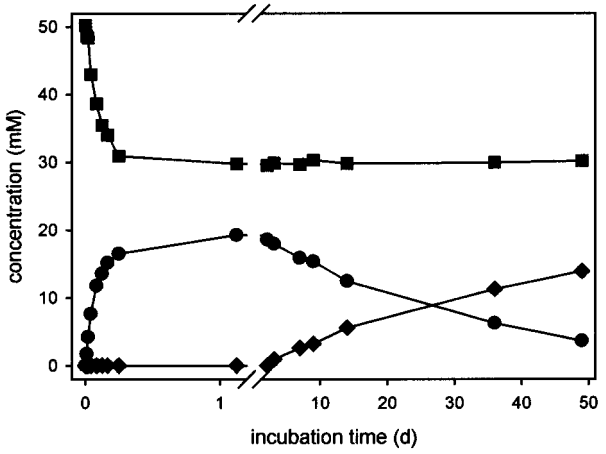


Figure 4. Reaction of N^{α}-*t*-BOC-arginine (50 mM) with glyoxal (20 mM) at 37 °C/pH 7.4. ●, Dihydroxyimidazolidine **1**; ■, arginine; ◆, N^7-carboxymethylarginine **3**.

of **1** proceeded in second order and rates increased with temperature and pH ($k[\mathrm{pH}] = e^{1.65 \cdot \mathrm{pH}} \cdot 10^{-8}$ L/(mol·s) for $T = 37$ °C, $k[T] = 4 \cdot e^{0.1204 \cdot T} \cdot 10^{-5}$ L/(mol·s) for pH = 7.4). Dihydroxyimidazolidine **1** was then converted to N^7-carboxymethylarginine **3** in a much slower reaction. At 37 °C and pH 7.4 the half-life for **1** was about 20 days. As for the formation of **1**, rates of hydrolysis to **3** increased with temperature and pH.

In no case could imidazolinone **2** be detected in the incubations described above. It was only generated from **1** in acidic solutions, especially under elevated temper-

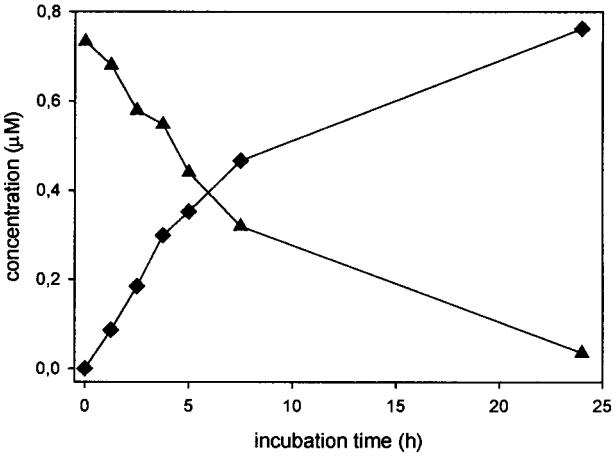


Figure 5. Hydrolysis of imidazolinone **2** (▲) to N^7-carboxymethylarginine **3** (◆) at 37 °C/pH 7.4.

atures. At neutral to basic pH values **2** hydrolyzed quantitatively to N^7-carboxymethylarginine **3** (Figures 5 and 1C), the rate of hydrolysis increasing with pH (half-life of **2** incubated at 20 °C at pH 4, 41 d; pH 5, 31 d; pH 6, 7.3 d; pH 7, 2.6 d; pH 8, 0.8 d).

Reaction with Carbonyl Trapping Reagents. To test whether dihydroxyimidazolidine **1** is a reversible glyoxal−arginine adduct, incubations were performed in the presence of the α-dicarbonyl trapping reagents *o*-phenylenediamine and aminoguanidine. Both react with glyoxal to the stable derivatives quinoxaline and 2-amino-1,3,4-triazine, respectively. In *o*-phenylenediamine reaction mixtures dihydroxyimidazolidine **1** and arginine concentrations were measured by HPLC, whereas the quinoxaline was assessed by GC−FID. Figure 6A clearly shows that dihydroxyimidazolidine **1** was almost completely converted to arginine and glyoxal in a second-order reaction within 100 h. In contrast, reaction with aminoguanidine proceeded much slower and only less than half of the dihydroxyimidazolidine was recovered as arginine and glyoxal (Figure 6B). For this experiment all structures were analyzed by HPLC. Obviously, the trapping action of aminoguanidine was too slow to inhibit side reactions to other products, as demonstrated by the formation of N^7-carboxymethylarginine **3** and by the decline of the sum of products monitored. Other structures must evolve, but could not be detected by the HPLC system used.

DISCUSSION

Our results unequivocally show that dihydroxyimidazolidine **1** is the main product of the early reaction of glyoxal and arginine at temperatures of 20−50 °C and within a pH range of 4−8 (Figure 7). This primary structure is then degraded to the stable N^7-carboxymethylarginine **3** in a much slower reaction. Research in this field has published controversial results so far. Schwarzenbolz et al. (*18*) identified imidazolinone **2** as the single direct reaction product. However, they used strong acidic conditions (2 N HCl, 110 °C, 100 min) to deacetylate their N^{α}-acetyl-arginine−glyoxal incubations, under which both dihydroxyimidazolinone **1** and N^7-carboxymethylarginine **3** are converted completely into imidazolinone **2**. Furthermore, they reported the detection of **2** in incubations of glyoxal with β-casein after enzymatic hydrolysis without giving detailed incubation conditions. Compound **1** is rather stable

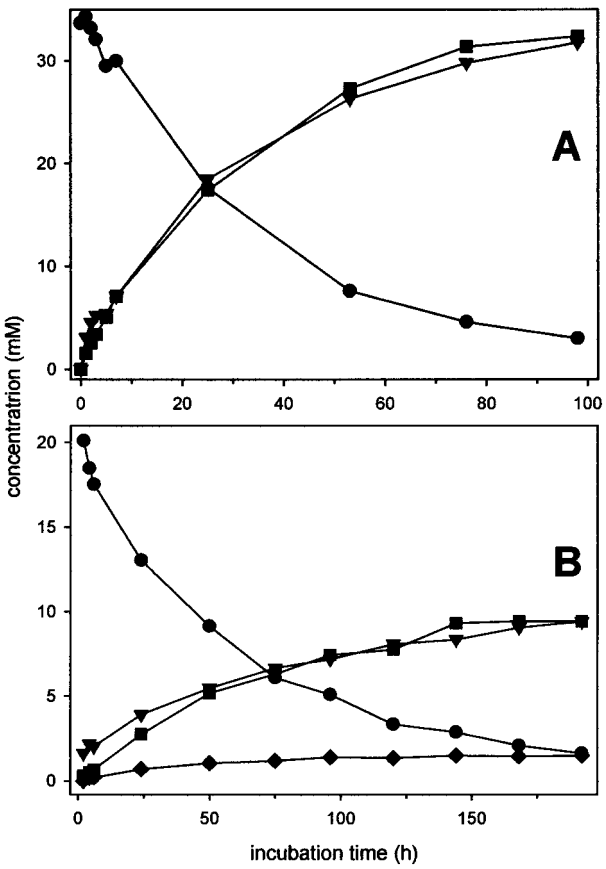


Figure 6. Degradation of dihydroxyimidazolidine **1** in the presence of trapping reagents *o*-phenylenediamine (A) and aminoguanidine (B, both 60 mM) at 37 °C/pH 7.4. ●, Dihydroxyimidazolidine **1**; ■, arginin; ▼, quinoxaline (A)/triazine (B); ◆, N^7-carboxymethylarginine **3**.

under these hydrolysis conditions in contrast to **3**. Therefore, it must be speculated that formed protein-bound N^7-carboxymethylarginine **3** has cyclized to imidazolinone **2** during enzymatic hydrolysis and led to false positive results. On the basis of our data, the detection of **2** in incubations under physiological conditions has to be evaluated as a pure artifact due to acidic workup procedures. This explains also the detection of **2** in ribose-protein incubations (*19*).

Glass and Pelzig (*17*) reported the formation of a single product in borate-buffered glyoxal−arginine incubations at pH 8−9, whereas the absence of borate resulted in a mixture of structures. As they were able to regenerate arginine from the borate product, they concluded it to be N^7, N^8-(1,2-dihydroxyethyl-1,2-yl)-arginine or dihydroxyimidazolidine **1 e**. Most likely, the mixture of structures in the absence of borate represents the full spectrum of structures **1 a**−**e**, whereas the presence of borate promotes only the formation of **1 c**−**e**. Takahashi (*24*) reported the formation of an unstable primary structure in glyoxal−arginine reaction mixtures, presumably dihydroxyimidazolidine **1**, which at pH > 7 was degraded to arginine and other products. However, structures were not further characterized.

The double hemiaminal moiety of dihydroxyimidazolidine **1** is rather stable against water elimination, which normally occurs spontaneously in amine−carbonyl reactions. In general, the stability of hemiaminals increases with increasing electronegativity of the substituents. Therefore in **1**, both hemiaminals stabilize each other through their relatively high electronegativ-

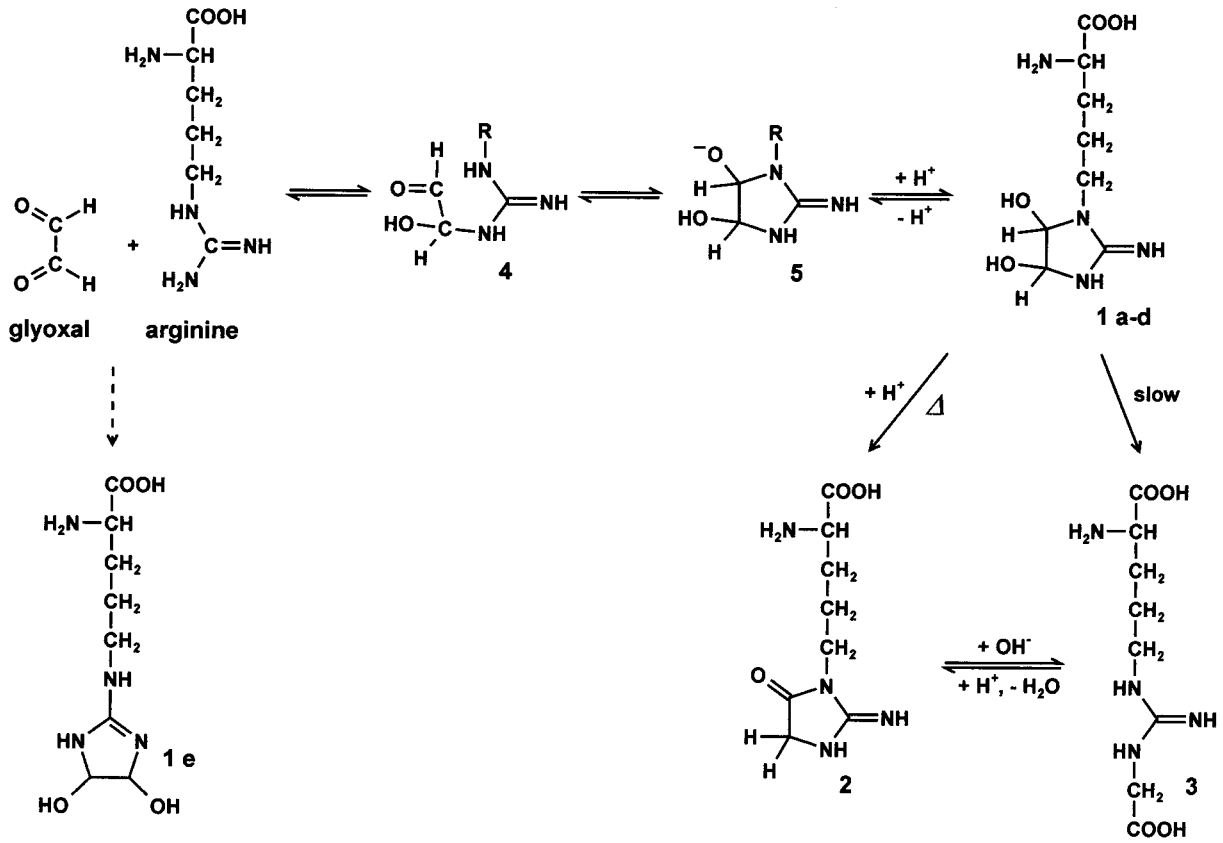


Figure 7. Mechanistic pathways for the reaction of arginine with glyoxal.

ity, further enhanced by the positive charge of the imidazolidine ring (*25*). Other 2-imino-4,5-dihydroxyimidazolidine analogues have been published for the reaction of guanidine, methyl/alkylguanidine, and N-benzyl-2-guanidino acetamide with glyoxal at pH 8 in aqueous solutions (*22, 26*). Theoretically, all reactions of arginine with α-dicarbonyl compounds will proceed via dihydroxyimidazolidines. The only compound structurally verified has been the corresponding 1,2-cyclohexanedione derivative, which was isolated as the single product from borate-buffered incubations (*27*). From reactions with other dicarbonyl compounds, only molecules analogous to imidazolinone **2** have been isolated. In general, dihydroxyimidazolidines are formed from glyoxal, presumably in two steps. One guanidino aminogroup reacts to hemiaminal **4**, which after cyclization gives the diol structure (Figure 7). The reaction depends on the unprotonated guanidino function, as the protonated form is less nucleophilic. This mechanism explains the positive correlation of reaction speed to OH concentration. However, under strongly acidic conditions, even a protonated guanidino group will react, as the electrophilic potency of glyoxal is drastically increased by protonation of the carbonyl functions (*17, 25*). Both reaction steps are reversible and in an equilibrium. The ring opening being prerequisite for cis-trans isomerization was proposed to proceed via deprotonation of one of the hydroxyl groups from dihydroxyimidazolidines (**5**, *22*), which of course is pH-dependent. This mechanism thus represents a plausible rationale for why the cis-trans isomerization is suppressed in acidic media .

Incubations of dihydroxyimidazolidine **1** with the carbonyl-trapping reagents *o*-phenylenediamine and aminoguanidine prove that the first step of the reaction of glyoxal with the guanidino function is also reversible. Similar results have been obtained from the reaction of the glyoxal-methylguanidine derivative of **1** with 2-aminoethanethiol as a trapping reagent (*22*). The authors were able to gain further evidence for their data by showing the transfer of glyoxal from dihydroxyimidazolidines between several guanidine derivatives. This reversibility therefore has to be considered, when "free" glyoxal is measured in foods or in plasma samples by carbonyl trapping reagents. On the basis of our results, this well-established methodology will assess not only free, but also arginine-bound, glyoxal in the form of dihydroxyimidazolidines. Thus, the true values for "free" glyoxal will be considerably lower.

Theoretically, two mechanisms are possible for the formation of N^7-carboxymethylarginine **3** from dihydroxyimidazolidine **1** (Figure 8). First, the imidazolidine ring opens to the open-chain form **4**, which after intramolecular disproportionation gives **3**. This mechanism is analogous to the formation of N^ϵ-carboxymethyllysine and is supported by the positive correlation of N^7-carboxymethylarginine formation with higher pH values by enhanced ring opening tendency. N^7-carboxymethylarginine formation is suppressed in the presence of *o*-phenylenediamine. This further supports the proposed mechanism, as in a first step the trapping reagent will react with the free carbonyl function of **4** and thereby forestall the reaction to N^7-carboxymethylarginine **3**. The second mechanistic possibility requires an intramolecular disproportionation of the intact ring system and subsequent hydrolysis of the resulting imidazolinone **2** to give N^7-carboxymethylarginine **3**. As the hydrolysis must be assumed to be much faster than the disproportionation, the actual concentration of imidazolinone **2** would be extremely small. On the other hand, this alternative pathway would allow N^7-car-

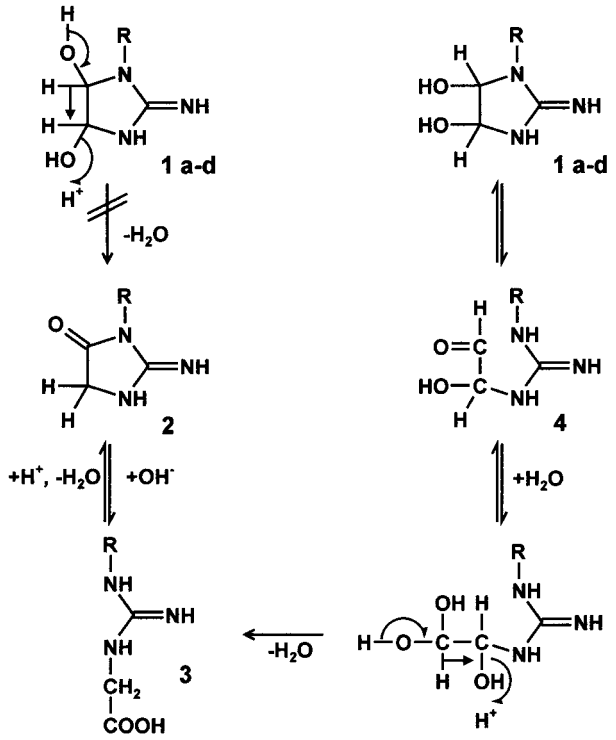


Figure 8. Mechanisms for the formation of N^7-carboxymethylarginine **3** from dihydroxyimidazolidine **1**.

boxymethylarginine formation in the presence of *o*-phenylenediamine, as no active carbonyl moieties are involved. As this is not the case, the second mechanism can be ruled out. Furthermore, incubations of imidazolinone **2** in the presence of *o*-phenylendiamine and aminoguanidine showed that hydrolysis of **2** to N^7-carboxymethylarginine **3** is not influenced by these trapping reagents at all.

Hydrolysis of imidazolinone **2** to N^7-carboxymethylarginine **3** is reversible under acidic conditions, starting at pH values smaller than two. This has already been described in the literature, e.g., for the spontaneous hydrolysis of the free base of the corresponding methylguanidine derivative in water (*28*). Our experiments concerning the hydrolytic stability of dihydroxyimidazolidine **1** and N^7-carboxymethylarginine **3** showed that differentiation and quantitation of these protein-bound structures is impossible under acid hydrolysis conditions, as both structures are converted to imidazolinone **2** and this is partially further degraded to arginine. An appropriate enzymatic hydrolysis now needs to be developed to verify the formation of **1** and **3** in modified proteins.

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