Studies on Radical Intermediates in the Early Stage of the Nonenzymatic Browning Reaction of Carbohydrates and Amino Acids

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Determination of the color intensity of heated mixtures of L-alanine and carbohydrate degradation products revealed furan-2-carboxaldehyde and glycolaldehyde as by far the most effective color precursors. EPR studies demonstrated that furan-2-carboxaldehyde generated colored compounds exclusively via ionic mechanisms, whereas glycolaldehyde led to color development accompanied by intense radical formation. In agreement with literature data, these radicals were also detected in heated mixtures of L-alanine and pentoses or hexoses, respectively, and were identified as 1,4-dialkylpyrazinium radical cations by EPR as well as LC/MS measurements. Studies on the mechanisms of radical formation revealed that under the reaction conditions applied, glyoxal is formed as an early product in hexose/L-alanine mixtures prior to radical formation. Reductones then initiate radical formation upon reduction of glyoxal and/or glyoxal imines, formed upon reaction with the amino acid, into glycolaldehyde, which was found as the most effective radical precursor. LC/MS measurements gave evidence that these pyrazinium radicals cations are not stable but are easily transformed into hydroxylated 1,4-dialkyl-1,4-dihydropyrazines upon oxidation and hydrolysis of intermediate diquarternary pyrazinium ions. Besides other types of color precursors, these intermediates might be involved in the formation of colored compounds in the Maillard reaction.

Keywords: *1,4-Dialkylpyrazinium radical cation; nonenzymatic browning; Maillard reaction; glyoxal; glycolaldehyde; deoxyosones; Amadori product; oxidation*

INTRODUCTION

Concerning the nonenzymatic browning occurring during thermal processing of foods such as roasted coffee or bread crust, surprisingly little is known about the reaction mechanisms leading to the formation of colored compounds from carbohydrates and amino acids.

More than 40 years ago, Hodge (1953) proposed the formation of 1-deoxy-2,3-diulose and 3-deoxy-2-osulose from the Amadori rearrangement product, the early reaction intermediate from carbohydrates and amino acids. Due to their reactivity, these dioxo intermediates undergo fragmentation, cyclization, and condensation reactions leading to advanced Maillard reaction products such as furans, 2*H*-furan-3-ones, 2*H*-pyran-3-ones, and 2*H*-pyran-4-ones or, upon reaction with amino compounds, to structurally related pyrroles, 2*H*-pyrrolin-3-ones, or pyridinium betains. As documented by a series of model studies, such advanced Maillard reaction intermediates were found to serve as penultimate precursors of colored compounds (Severin and Krönig, 1972; Ledl and Severin, 1978; Hofmann, 1997a,b, 1998a–c; Hofmann and Heuberger, 1998).

Besides Hodge's reaction pathway, model experiments elucidated an alternative reaction route leading to color formation in a very early stage of the Maillard reaction, prior to the Amadori rearrangement (Hayashi et al., 1977, 1985; Hayashi and Namiki, 1980; Namiki and Hayashi, 1981, 1983). A mechanism was reported involving cleavage of the carbohydrate skeleton with liberation of glycolaldehyde imine, which, upon dimerization and oxidation, formed a 1,4-dialkylpyrazinium radical cation, the structure of which is given in Figure 1, as a key intermediate in a very early stage of the Maillard reaction. Because EPR measurements revealed the radical cation formation immediately before the browning development, Namiki and Hayashi (1983) proposed this alternative reaction as a key step in the formation of colored compounds prior to the Amadori rearrangement. However, the mechanisms leading to the color development are as yet not clearly understood.

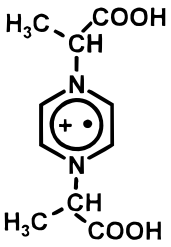

Figure 1. Structure of 1,4-bis(1-carboxy-1-ethyl)pyrazinium radical cation.

The purpose of the present investigation was, therefore, to study the color formation from certain Maillard reaction intermediates and to gain insights into the

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10.1021/jf980626x CCC: $18.00
Published on Web 01/22/1999

radical-assisted mechanisms occurring in nonenzymatically browned carbohydrate/amino acid mixtures.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: ethylamine, N^{α}-acetyl-L-lysine, L-alanine, glucose, xylose, furan-2-carboxaldehyde, 5-(hydroxymethyl)furan-2-carboxaldehyde, glyoxal (30% solution in water), glycolaldehyde, triethyloxonium tetrafluoroborate, 1,2-dichloroethane, pyrazine, 1,2-diaminobenzene, 1-hydroxycyclohexan-2-one, pyrrole-2-carboxaldehyde, 2-oxopropanal (40% in water), butane-2,3-dione, glycerinaldehyde, 2-hydroxy-3-butanone (Aldrich, Steinheim, Germany). The furan-2-carboxaldehyde was freshly distilled prior to use. 1,2-Diaminobenzene was twice recrystallized from methanol.

The water used for the EPR experiments was stirred with an ion-exchange resin (Amberlite MB-1; Merck, Darmstadt, Germany) for at least 24 h prior to use, to remove adventitious metal ions. Acetylformoin (Hofmann, 1998d), methylenereductinic acid (Hofmann, 1998d), and [$^{13}C_4$]-butane-2,3-dione (Schieberle and Hofmann, 1997) were synthesized as recently reported. 3-Deoxyhexos-2-ulose was prepared from glucose following a method described in the literature for the synthesis of 3-deoxypentos-2-ulose (Hofmann and Schieberle, 1998a).

Syntheses. *1,4-Diethylpyrazinium Diquaternary Salt.* Following a procedure of Curphey and Prasad (1972), a suspension of triethyloxonium tetrafluoroborate (50 mmol) in 1,2-dichloroethane (20 mL) was stirred under nitrogen while a solution of pyrazine (20 mmol) in 1,2-dichloroethane (10 mL) was added over a 5 min period. The reaction mixture was then refluxed for 40 min, cooled to room temperature, and filtered, yielding the crude target compound as a white solid. Recrystallization from acetonitrile/1,2-dichloroethane afforded pure 1,4-diethylpyrazinium diquaternary tetrafluoroborate (15 mmol; 75% in yield): ^{1}H NMR (500 MHz, CF_3COOH) δ 1.93 (t, 6H, ^3J = 7.8 Hz, 2 × $-CH_2CH_3$), 5.23 (q, 4H, ^3J = 7.8 Hz, 2 × $-CH_2$CH3), 9.87 (s, 4H, 4 × $-$N=$CH-$);^{13}C NMR (360 MHz, CF_3COOH) δ 17.5 (2 × $-CH_2CH_3$), 65.8 (2 × $-CH_2CH_3$), 149.0 (4 × $-$N=$CH-$).

2-(2,3,4-Trihydroxybutyl)quinoxaline. A mixture of 3-deoxyhexos-2-ulose (20 mmol) and 1,2-diaminobenzene (22 mmol) was stirred in water (30 mL) for 12 h in the dark under an atmosphere of argon. The mixture was then extracted with methylene chloride (5 × 20 mL); the organic layer was dried over Na_2SO_4, concentrated to ~1 mL, and, then fractionated by preparative thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using acetonitrile/water (9 + 1, by vol) as the mobile phase. The band at R_f = 0.3−0.4 was scraped off, suspended in methanol, and filtered, affording the target compound as a colorless oil after removal of the solvent in vacuo (9 mmol; 45% in yield): LC/MS (ESI) 235 (100; $[M + 1]^+$), 217 (15; $[M + 1 - H_2O]^+$), 257 (8; $[M + Na]^+$);^{1}H NMR (360 MHz; CD_3OD) δ 3.16 [dd, 1H, ^2J = 15.04 Hz, $-CH_aH_b$C(OH)−], 3.43 [dd, 1H, ^2J = 15.04 Hz, ^3J = 7.96 Hz, $-CH_aH_b$C(OH)−], 3.65−3.74 (m, 2H, $-CH_2$OH), 3.85 [m, 1H, $-CH$(OH)−], 4.14 [m, 1H, $-CH$(OH)−], 7.76 (m, 2H, 2 × =$CH-$), 8.00 (m, 2H, 2 × =$CH-$), 8.84 (s, 1H, N=$CH-$);^{13}C NMR (360 MHz; CD_3OD) δ 38.9 ($-CH_2$), 62.7 ($-CH_2$OH), 71.4 [$-CH$(OH)−], 74.5 [$-CH$(OH)−], 127.7 (=CH−), 127.9 (=CH−), 128.8 (=CH−), 129.6 (=CH−), 140.2 [=C(C)−], 141.3 [=C(C)−], 146.3 [=C(C)−], 155.7 [=C(C)−].

2-Methyl-3-(1,2,3-trihydroxypropyl)quinoxaline. A mixture of glucose (196 mmol), alanine (183 mmol), and 1,2-diaminobenzene (148 mmol) in phosphate buffer (800 mL; 0.5 mol/L, pH 6.8) was refluxed for 12 h. After cooling to room temperature, the reaction mixture was extracted with methylene chloride (5 × 200 mL), and the combined organic layers were dried over Na_2SO_4 and, after concentration, separated by column chromatography (35 × 400 mm) on silica gel (150 g, silica gel 60, Merck), which was conditioned with ethyl acetate. After application of the crude material onto the column, chromatography was performed with ethyl acetate (400 mL), followed by ethyl acetate/methanol (50:50, v/v; 500 mL), affording the target compound as a crude product. Further fractionation by preparative thin-layer chromatography on silica gel (20 × 20 cm; 0.5 mm; Merck) using acetonitrile/water (95:5, v/v) as the mobile phase revealed the target compound in a band at R_f = 0.5−0.6, which was scraped off and suspended in methanol. Filtration and concentration afforded the target compound as white crystals (64 mmol; 33% in yield): LC/MS (ESI) 235 (100; $[M + 1]^+$), 217 (11; $[M + 1 - H_2O]^+$), 257 (9; $[M + Na]^+$);^{1}H NMR (360 MHz; CD_3OD) δ 2.86 (s, 3H, $-CH_3$), 3.85−3.95 (m, 2H, $-CH_2$OH), 4.09 [m, 1H, $-CH$(OH)−], 5.11 [d, 1H; =CCH(OH)−], 7.73 (m, 2H, =$CH-$), 7.95 (m, 1H, =$CH-$), 8.08 (m, 1H, =$CH-$);^{13}C NMR (360 MHz; CD_3OD) δ 23.1 ($-CH_3$), 65.2 ($-CH_2$OH), 72.4 [$-CH$(OH)−], 76.8 [$-CH$(OH)−], 129.2 (=CH−), 130.2 (=CH−), 130.8 (=CH−), 131.5 (=CH−), 142.4 [=C(C)−], 142.6 [=C(C)−], 155.8 [=C(C)−], 158.6 [=C(C)−].

N-(1-Deoxy-D-fructos-1-yl)-L-alanine. Powdered anhydrous glucose (300 mmol) was refluxed for 10 min in anhydrous methanol (400 mL), then L-alanine (500 mmol) was added, and heating was continued for 4 h. After the addition of malonic acid (90 mmol), the solution was refluxed for an additional 1 h, then cooled to room temperature, and concentrated to ~200 mL in vacuo, and unreacted L-alanine was filtered off. Dropwise addition of acetone to the ice-cooled filtrate yielded the Amadori rearrangement product as a hydroscopic solid. Concentration of the mother liquor, recooling, and addition of acetone yielded additional crude product. Both crops were combined, dissolved in a mixture of water (80 mL) and ethanol (200 mL), and fractionated by cation-exchange chromatography (220 mL; Amberlite IR120, Aldrich). Unreacted carbohydrate was eluted with a mixture of water (120 mL) and ethanol (280 mL), followed by water (100 mL). After elution with an aqueous ammonium hydroxid solution (0.2 mol/l) and freeze-drying, the *N*-(1-deoxy-D-fructos-1-yl)-L-alanine was obtained as a white powder (42 mmol; yield = 14%): LC/MS 252 (100, $[M + 1]^+$), 234 (45, $[M + 1 - H_2O]^+$), 274 (13, $[M + Na]^+$), 216 (10, $[M + 1 - 2H_2O]^+$), 525 (9, $[M_2 + Na]^+$), 188 (5, $[M + 1 - H_2O - HCOOH]^+$); ^{1}H NMR (360 MHz in D_2O; DQF-COSY) of the β-fructopyranose form, δ 1.52 (d, 3H,$^3J_{8,7}$ = 7.1 Hz, CH_3-), 3.21 (d, 1H,$^2J_{1a,1b}$ = 12.9 Hz, $-CH_aH_b$N), 3.34 (d, 1H, $^2J_{1b,1a}$ = 12.9 Hz, $-CH_aH_b$N), 3.76 [d, 1H, $^3J_{3,4}$ = 9.8 Hz, $-CH$(OH)−], 3.78 (dd, 1H, $^3J_{6a,5}$ = 2.0 Hz, $^2J_{6a,6b}$ = 12.8 Hz, $-CH_aH_b$O), 3.82 [d, 1H, $^3J_{7,8}$ = 7.1 Hz, $-CH(CH_3)-$], 3.89 [dd, 1H, $^3J_{4,5}$ = 3.3 Hz, $^3J_{4,3}$ = 9.8 Hz, $-CH$(OH)−], 4.00 [m, 1H, $^3J_{5,4}$ = 3.3 Hz, $^3J_{5,6a}$ = 2.0 Hz, $^3J_{5,6b}$ = 1.2 Hz, $-CH$(OH)−], 4.02 (dd, 1H, $^2J_{6b,6a}$ = 12.8 Hz, $^3J_{6b,5}$ = 1.2 Hz, $-CH_aH_b$O); ^{13}C NMR (360 MHz in D_2O; DEPT, HMQC, HMBC) of the β-fructopyranose form, δ 19.9 (CH_3, $-CH_3$), 54.2 (CH_2, $-CH_2$N), 63.5 [CH, $-CH(CH_3)-$], 66.1 (CH_2, $-CH_2$O), 74.2 [CH, $-CH$(OH)−], 74.6 [CH, $-CH$(OH)−], 75.2 [CH, $-CH$(OH)−], 97.4 [C, OC(OH)−], 179.4 (C, $-COOH$).

Determination of the Color Dilution (CD) Factor. To measure the browning intensity of heated mixtures of carbohydrates or carbonyl compounds, respectively, in the presence of alanine, CD factors were determined as recently described (Hofmann, 1998e). Aliquots of the reaction mixtures were diluted step by step (1 + 1; by vol) until a color difference between the sample (5 mL) and two blanks (tap water; 5 mL) in a glass vial (1 cm i.d.) could just be visually detected using a triangle test.

Preparation of Reaction Mixtures for EPR Measurements. Binary mixtures of an amino compound (6 mmol) and a carbohydrate or carbonyl compound (6 mmol), respectively, were heated in phosphate buffer (4 mL; 0.5 mmol/L; pH 7.0) in closed vials at 95 °C. After rapid cooling, an aliquot of each reaction mixture was analyzed by EPR spectroscopy.

Quantification of Glycolaldehyde and Glyoxal in Heated Aqueous Glucose/Alanine Solutions. Mixtures of glucose (6 mmol) and L-alanine (6 mmol) were refluxed in phosphate buffer (pH 7.0; 0.5 mol/L; 4 mL). After the reaction times, given in Figure 8 and Tables 2 and 3, aliquots of the mixtures were withdrawn and glycolaldehyde and glyoxal were quantified after derivatization with ethoxamine and 1,2-diaminobenzene, respectively, following closely a procedure described recently (Hofmann, 1998f).

Quantification of 1-Deoxy-D-*erythro*-2,3-hexodiulose and 3-Deoxyhexos-2-ulose in a Heated Glucose/Alanine Solution. Mixtures of glucose (6 mmol) and L-alanine (6 mmol) were refluxed in phosphate buffer (pH 7.0; 0.5 mol/L; 4 mL). After the reaction times, given in Figure 9, a fourth of the mixtures was withdrawn and diluted with water (1 mL), 1,2-diaminobenzene (2 mmol) was added, and the mixtures were maintained for 3 h at 30 °C. After dilution with water, the mixtures were analyzed by RP-HPLC using a solvent gradient starting with a mixture (10:90, v/v) of acetonitrile and ammonium formate buffer (pH 3.5; 20 mmol/L) and increasing the acetonitrile content to 30% within 50 min. By monitoring the effluent at $\lambda = 320$ nm, 1-deoxy-D-*erythro*-2,3-hexodiulose and 3-deoxyhexos-2-ulose were detected as the derivatives 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline and 2-(2,3,4-trihydroxybutyl)quinoxaline at the retention times of 14.3 and 15.9 min, respectively. Quantification of these derivatives was performed by using the synthetic compounds as external standards.

Quantification of *N*-(1-Deoxy-D-fructos-1-yl)-L-alanine. A mixture of glucose (6 mmol) and L-alanine (6 mmol) was refluxed in phosphate buffer (pH 7.0; 0.5 mol/L; 4 mL) for 10 min. An aliquot of the mixture was diluted with water and analyzed by HPLC using an aminopropyl material as the stationary phase and acetonitrile/water (70:30, v/v) as the mobile phase. By monitoring the effluent with a refractive index detector, the Amadori product ($R_f = 10.4$ min) was quantified using the synthetic *N*-(1-deoxy-D-fructos-1-yl)-L-alanine as external standard.

Determination of Reducing Substances in a Heated Glucose/Alanine Solution. Mixtures of glucose (6 mmol) and L-alanine (6 mmol) were refluxed in phosphate buffer (4 mL; 0.5 mol/L; pH 7.0). After the reaction times, given in Figure 11, fourths of the cooled mixtures were diluted with an aqueous solution of oxalic acid (1 mL; 2% in water). After addition of diethyl ether (2 mL), the mixtures were titrated with an aqueous solution of 2,6-dichlorophenol−indophenol sodium (DCIP, 0.7 mmol/L water) while the solutions were vigorously shaken. The end-point of the titration was reached as the colorless organic layer turned to a slight rose color. On the basis of the consumption of DCIP for a definite amount of ascorbic acid, the results, given in Figure 11, were calculated as ascorbic acid equivalents (aae).

Determination of the Radical Precursor Concentration. Solutions of glucose (6 mmol) and L-alanine (6 mmol) in phosphate buffer (4 mL; 0.5 mmol/L; pH 7.0) were heated in closed vials at 95 °C for the reaction times given in Figure 12. To locate the radical precursor, the mixtures were cooled to room temperature, ascorbic acid (1 mmol) was added, and, after 5 min of incubation at room temperature, each reaction mixture was analyzed by EPR spectrosopy.

Incubation of Reaction Mixtures with Reductones. A mixture of L-alanine (2 mmol) and glyoxal (2 mmol) was heated in phosphate buffer (2 mL; 0.5 mol/L, pH 7.0) for 3 min at 95 °C. After cooling, the reductone (2 mmol), given in Table 4, was added, and the mixture was maintained for 10 min at room temperature and was then applied to the EPR cavity.

EPR Spectroscopy. The EPR spectra were recorded on an ESP 300 spectrometer (Bruker, Rheinstetten, Germany). The experimental parameters were as follows: modulation amplitude, 1 G; sweep rate, 2.4 Gs^{-1} at a frequency of 9.75 GHz and a gain of 1×10^4. All spectra were recorded at room temperature.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Kontron, Eching, Germany) consisted of two pumps (type 422), a gradient mixer (M 800) and a Rheodyne injector (100 μL loop). The effluent was monitored either by a diode array detector (DAD; type 440, Kontron), operating in a wavelength range between 220 and 500 nm, or by a refractive index detector (SE-11, Shodex; Erma Optical Works Ltd.). Separations were performed on a stainless steel column packed with RP-18 material (ODS-Hypersil, 250 × 4.6 mm, 5 μm, Shandon, Frankfurt, Germany) or an aminopropyl phase (Nucleosil 100-5 NH_2; 250 × 4 mm; 5 μm, Macherey-Nagel, Dueren, Germany) with a flow rate of 0.6 or 1.2 mL/min, respectively.

Table 1. Color Development and Radical Formation in Binary Mixtures of L-Alanine and Carbohydrates or Carbohydrate Degradation Products, Respectively

carbonyl compound	CD factor[a]	rel radical formation (%)
glucose	16	4[b]
xylose	64	8[b]
N-(1-deoxy-D-fructos-1-yl)-L-alanine	8	1[b]
glycolaldehyde	1024	100[c]
glyoxal	128	4[c]
furan-2-carboxaldehyde	1024	0[c]
pyrrol-2-carboxaldehyde	256	0[c]
2-oxopropanal	256	0[c]
butane-2,3-dione	128	na[d]
5-(hydroxymethyl)furan-2-carboxaldehyde	2	na
glycerinaldehyde	2	na
2-hydroxy-3-butanone	2	na

[a] The CD factor was applied to compare the color intensities of the reaction mixtures, which were heated for 15 min at 95 °C. [b] For EPR measurements the mixture was heated for 10 min at 95 °C. [c] For EPR measurements the mixture was heated for 2 min at 95 °C. [d] na, not analyzed.

Liquid Chromatography/Mass Spectrometry (LC/MS). An analytical HPLC column (Nucleosil 100-5C18, Macherey and Nagel) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray ionization (ESI). After injection of the sample (2.0 μL), analysis was performed using a gradient starting with a mixture (90:10, v/v) of acetonitrile and water and increasing the acetonitrile content to 100% within 15 min.

UV−Vis Spectrocopy. UV−Vis spectra were obtained by means of a U-2000 spectrometer (Colora Messtechnik GmbH, Lorch, Germany). To follow the color formation in the reaction mixtures, the absorption at 420 nm was determined in appropiate dilutions of the reaction mixtures.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^1H and ^{13}C spectra were recorded by means of a Bruker-AM-360 spectrometer using the acquisition parameters described recently (Hofmann, 1997a).

RESULTS AND DISCUSSION

Namiki and Hayashi (1981) were the first to study sytematically the correlation between color formation and radical generation in the course of Maillard-type reactions using β-alanine. On the basis of their results and to gain further insights into the intermediates involved in the color formation during thermal processing of foods, the effectiveness of certain carbohydrate degradation products as color precursors was reinvestigated in the presence of the more food-relevant α-amino acid L-alanine. Aqueous solutions of carbohydrates and carbohydrate degradation products, respectively, which are potentially involved in the nonenzymatic browning, were, therefore, heated in mixtures with L-alanine and the intensity of the color produced was measured. Because browned Maillard mixtures consist of a multiplicity of colored compounds varying in their absorption maxima, it is not reliable to measure the color intensity by a determination of the extinction coefficient at only one wavelength. CD factors, which were recently introduced for the visual evaluation of the color intensity of colored solutions (Hofmann, 1998b,e), were, therefore, chosen to compare the color intensity of the heated Maillard mixtures in the entire wavelength range of the visible light, thus covering all browning products. The data, summarized in Table 1,

revealed glycolaldehyde and furan-2-carboxaldehyde with by far the highest effectiveness in color formation, followed by pyrrole-2-carboxaldehyde and 2-oxopropanal with somewhat lower browning abilities. In contrast to its high browning effectiveness determined in the presence of β-alanine (Namiki and Hayashi, 1981), glyoxal was not such a good color precursor in the presence of the α-amino acid L-alanine. This difference might be due to the possible Strecker reaction of L-alanine and glyoxal, whereas β-alanine has to react via different pathways. In comparison to furan-2-carboxaldehyde, 5-(hydroxymethyl)furan-2-carboxaldehyde was a very ineffective color precursor in the reaction with L-alanine, because a 500-fold lower color intensity was determined (Table 1). In comparison to glycolaldehyde, xylose and glucose also showed only weak color formation when heated in the presence of L-alanine. Thermal treatment of the Amadori rearrangement product *N*-(1-deoxy-D-fructos-1-yl)-L-alanine generated by a factor of 2 fewer browning products compared to the corresponding glucose/alanine mixture (Table 1), indicating that the color formation in carbohydrate/amino acid mixtures might not exclusively run via the Amadori product. This finding is well in line with data reported earlier for the Amadori product of β-alanine by Namiki and Hayashi (1981).

The most effective color precursor systems were then analyzed for free radical formation by EPR spectroscopy. As shown in Table 1, in the glycolaldehyde-containing mixture an intense radical was detected, which, in accordance with data reported by Hayashi et al. (1977), could be identified as 1,4-bis(1-carboxy-1-ethyl)pyrazinium radical cation (Figure 1). The same free radical was detected in the heated mixtures containing either the carbohydrates *N*-(1-deoxy-D-fructos-1-yl)-L-alanine or glyoxal, respectively, however, by a factor of >10 lower in intensity. These data demonstrate that the radical was formed independently from the carbohydrate moiety in a very early stage of the Maillard reaction of α-amino acids, predominantly prior to the Amadori rearrangement. The data are well in line with those obtained for β-alanine-containing carbohydrate solutions (Namiki and Hayashi, 1983). In contrast, free radicals could not be detected in heated solutions of L-alanine containing furan-2-carboxaldehyde or 5-(hydroxymethyl)furan-2-carboxaldehyde. The fact that glycolaldehyde and furan-2-carboxaldehyde were by far the most effective color precursors, whereas the free radical was observed only in the presence of glycolaldehyde, suggested that colorant formation from advanced Maillard reaction intermediates such as furan- or pyrrol-2-carboxaldehyde might run predominantly via ionic mechanisms, whereas browning reactions of glycolaldehyde are probably associated with radical intermediates.

Because thermal treatment of furan-2-carboxaldehyde/L-alanine solutions led to a very intense color development (Table 1), we recently investigated the main colored reaction products, among which red 1*H*-pyrrol-3(2*H*)-ones were identified (Hofmann, 1997a,b, 1998a). ^{13}C-labeling experiments revealed that these chromophores were generated by amino acid-induced opening of the furan ring, followed by several condensation reactions (Hofmann, 1998g), fitting well with the lack of free radical formation in the heated furan-2-carboxaldehyde/L-alanine mixture (Table 1).

To study whether also the ε-amino group of lysine might be involved in radical formation, we heated neutral aqueous solutions of N^{α}-acetyl-L-lysine in the presence of glucose or glycolaldehyde, respectively. A rapid brown colorization of the reaction mixture was observed. EPR spectroscopy revealed an intense free radical, which, due to the striking similarity with those obtained from a computer-simulated spectrum, was assumed to be the 1,4-bis[5-(acetylamino)-5-carboxy-1-pentyl)pyrazinium radical cation (Figure 2). The proposed structure was further confirmed by means of LC/MS measurements indicating a molecular ion of *m*/*z* 424. LC/MS^2 experiments demonstrated a loss of 172, most likely resulting from the elimination of 5-(acetylamino)-5-carboxy-1-pentene from the 5-(acetylamino)-5-carboxy-1-pentyl side chain of the radical cation (data not shown).

Studies on Radical-Assisted Mechanisms in the Nonenzymatic Browning Reaction. In their experiments Hayashi et al. (1977) as well as Namiki and Hayashi (1983) detected 1,4-dialkylpyrazinium radical cations as an early reaction intermediate just before color developed. They proposed that the one-electron oxidized product, the 1,4-dialkylpyrazinium diquaternary salt (diquat), should be the color precursor. Diquats are known to be very unstable in polar solvents (Curphey and Prasad, 1972; Namiki and Hayashi, 1983) and were, therefore, assumed to give important information on the role of free radical products in nonenzymatic browning.

To study its role in color formation in water, we synthesized the 1,4-diethylpyrazinium diquat. The diquat was extraordinarily labile and upon dissolving turned colored immediately. Color formation was accompanied by an immediate formation of 1,4-diethylpyrazinium radical cations (Figure 3), thereby confirming data reported by Curphey and Prasad (1972) as well as Hayashi et al. (1977). As displayed in Figure 4, heating of a neutral aqueous solution of the 1,4-diethylpyrazinium diquat at 95 °C gave the highest concentration of radical cations immediately upon dissolving. During the first 10 min the radical intensity decreased rapidly to ∼50%, whereas increasing the reaction time to 60 min led only to a slight decrease in the radical concentration (Figure 4). Monitoring the visible absorption in the heated diquat solution at 420 nm within a period of 60 min revealed a drastic increase in color development during the first 10 min. The color development then slowly approximated a maximum value within the following 50 min. On the basis of the opposite change in the concentration of the radical and the browning products, it might be assumed that color development from diquat is associated with the decomposition of the radical cations. This is well in line with the redox reaction equilibrium between the 1,4-dialkylpyrazinium radical cation and its one-electron oxidation product 1,4-dialkylpyrazinium diquat proposed by Namiki and Hayashi (1983).

To study the mechanism of this redox process in more detail, a freshly prepared solution of diquat in water was analyzed by LC/MS. Due to the double positively charged diquat, a molecular ion at *m*/*z* 69 has to be expected; however, this ion was lacking in the LC/MS spectrum. As displayed in Figure 5A, a base peak at *m*/*z* 155 (100%) and an ion at *m*/*z* 138 (35%) were monitored by LC/MS. The ion at *m*/*z* 138 is well in line with the formation of the 1,4-diethylpyrazinium radical cation, which was identified by EPR spectroscopy. LC/

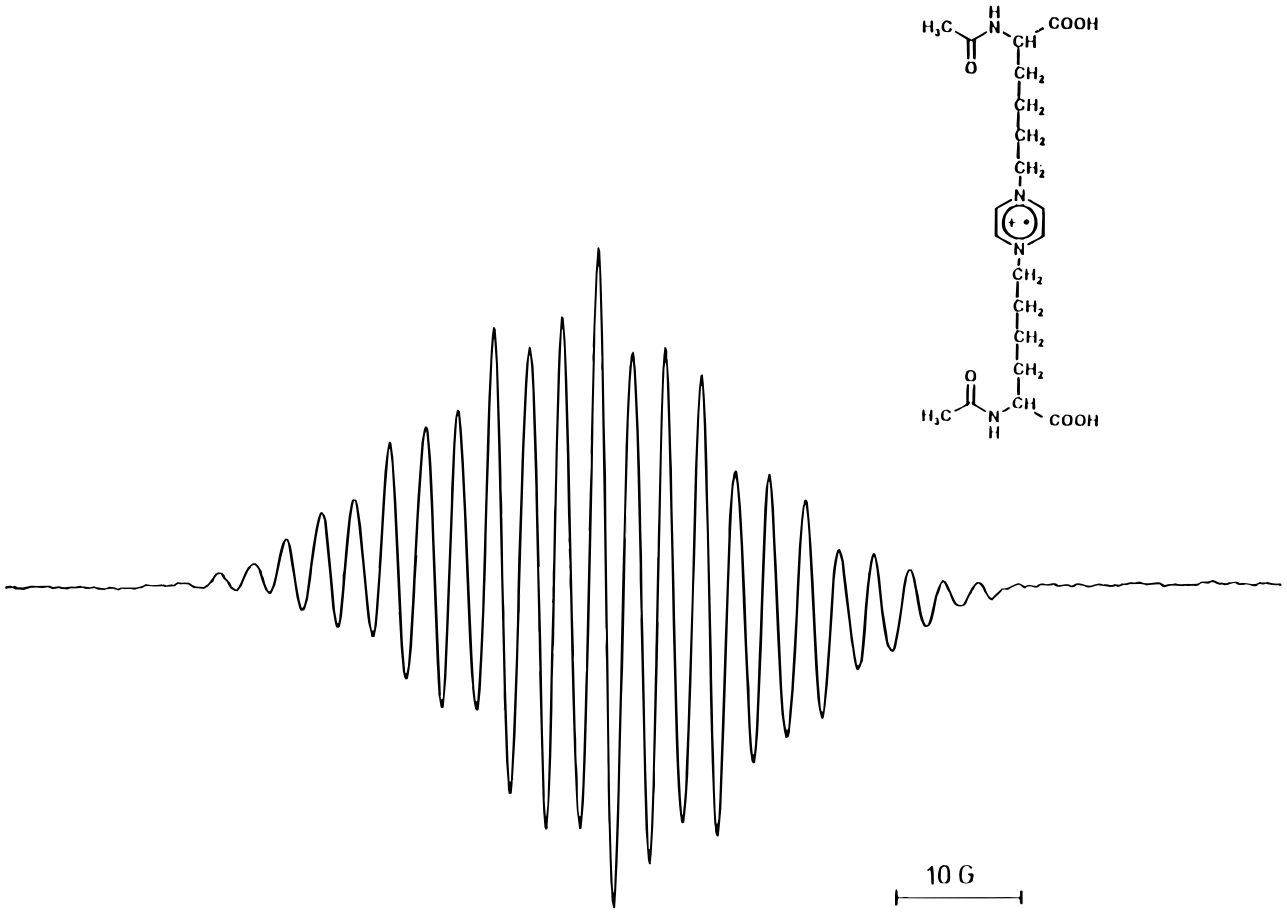

Figure 2. ESR spectrum of 1,4-bis[5-(acetylamino)-5-carboxy-1-pentyl)pyrazinium radical cation.

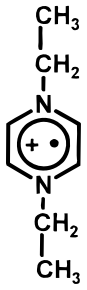

Figure 3. Structure of 1,4-diethylpyrazinium radical cation.

MS^2 of the ion at *m*/*z* 155 showed a loss of 28, yielding the ion *m*/*z* 127, most likely resulting from the elimination of ethylene from an ethyl side chain. On the basis of these data we assumed an addition of water to the diquat instantaneously upon dissolving, resulting in a 1,4-diethyl-1,4-dihydropyrazine (*m*/*z* 155). In addition, an ion at *m*/*z* 171 was detected by LC/MS (Figure 5A), most likely corresponding to a dihydroxy-1,4-diethyl-1,4-dihydropyrazine as evidenced by LC/MS^2. For further confirmation of our assumed structures, we repeated the experiment in methanol (Figure 5B). Besides the ion *m*/*z* 138 corresponding to the 1,4-diethylpyrazinium radical cation, a base peak at *m*/*z* 169 (100%) and an ion with *m*/*z* 199 were found, fitting well with the pseudomolecular ions of 2-methoxy-1,4-diethyl-1,4-dihydropyrazine and dimethoxy-1,4-diethyl-1,4-dihydropyrazine, respectively. Such alkoxy derivatives were assumed earlier by Curphey and Prasad (1972) as reaction products of diquats upon dissolving in alcohols; however, this is the first evidence for the existence of

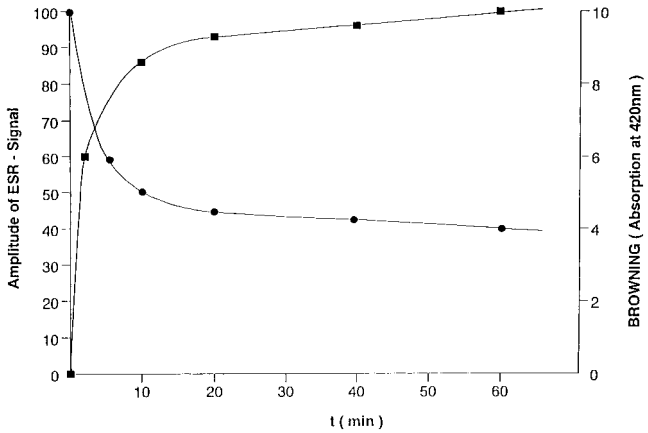

Figure 4. Time course of radical formation (●) and color development (■) in heated aqueous solutions of 1,4-diethylpyrazinium diquat (—).

hydroxylated and methoxylated dihydropyrazines formed upon dissolving diquats in water and methanol, respectively.

Curphey and Prasad (1972) proposed a reaction sequence leading to dihydropyrazines and radical cations from diquats dissolved in alcohols. Because in the present investigation these intermediates were identified by EPR spectroscopy as well as LC/MS measurements to be formed in aqueous solution, we outlined a redox reaction, partly according to that of Curphey and Prasad (1972), in Figure 6 leading to the formation of 1,4-diethylpyrazinium cation radicals in aqueous solutions of diquats. Rapid hydratization of diquat (**I**) gives the 1,4-dialkyl-2-hydroxypyrazinium cation (**II**) as an

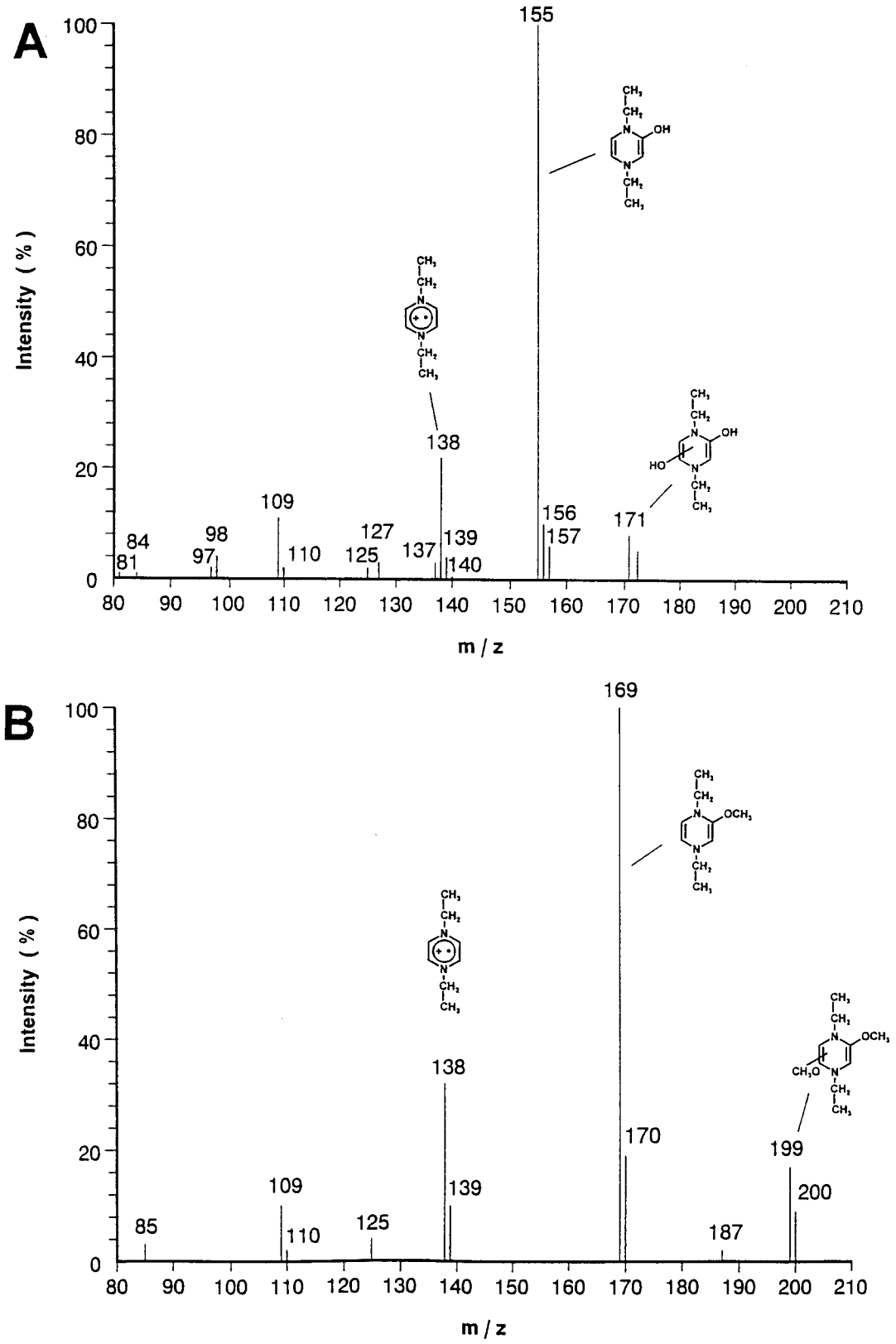

Figure 5. LC/MS of 1,4-diethylpyrazinium diquat in (A) water and (B) methanol.

early intermediate, which, upon elimination of a proton, then gives rise to the 1,4-dialkyl-2-hydroxy-1,4-dihydropyrazine (**III**). This intermediate is a reducing agent and reacts via a single-electron transfer with two molecules of diquat, yielding two molecules of the 1,4-dialkylpyrazinium radical cation (**IV**) and a 1,4-dialkyl-2-hydroxypyrazinium diquat (**V**). As outlined in the net reaction A (Figure 6), the mechanism requires that one-third of the original diquat (**I**) forms the intermediate **V**. As outlined in reaction B (Figure 6), after the addition of water, **V** might be formed by a similar sequence of steps via the intermediate 1,4-dialkyldihydroxy-1,4-dihydropyrazine. Intermediate **V** might then reduce two more molecules of diquat (**I**) and be oxidized itself into a dihydroxy diquat (**VI**).

To investigate whether these hydroxydihydropyra-

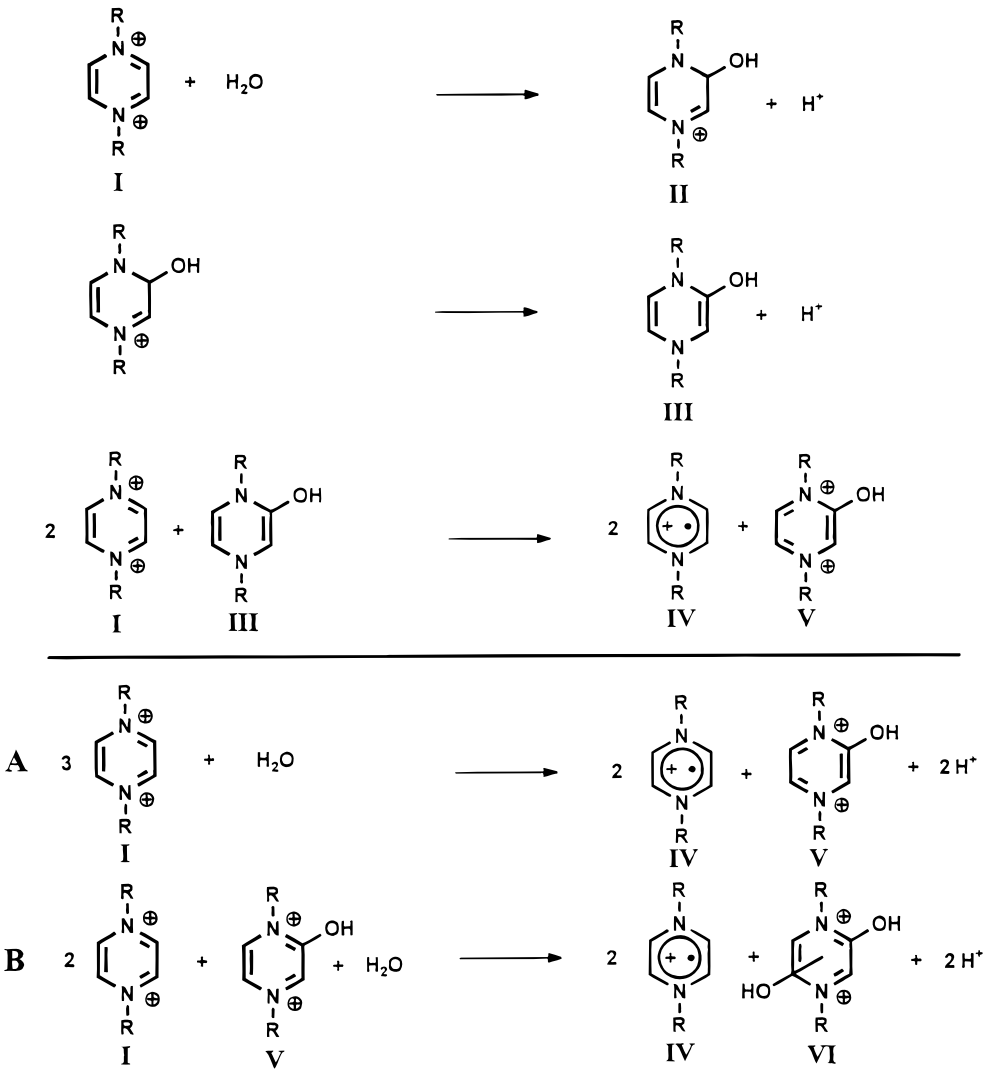

Figure 6. Mechanism proposed for reduction of diquat into 1,4-dialkylpyrazinium radical cation [partly according to Curphey and Prasad (1972)].

zines were also formed in Maillard mixtures, we measured the time course of radical cation formation and color development in the glycolaldehyde/ethylamine solution (Figure 7) and compared these results with the data obtained from the synthetic diquat. In contrast to the aqueous diquat solution, radical formation in the glycolaldehyde/ethylamine mixture rapidly increased during the first minutes of heating, then, after running through a maximum at 5 min, decreased again accompanied by an accelerated color formation. As found for the synthetic diquat, the free radicals were formed just before the color development started. On the basis of these data, it can be assumed that the formation of colored compounds in the glycolaldehyde/ethylamine mixture runs via the same intermediates as found for the synthetic diquat.

To confirm this hypothesis, we studied whether the radical cations, detected in Maillard mixtures, are able to produce the hydroxylated dihydropyrazines via the intermediate diquat, formed by oxidation. We therefore heated a mixture of glycolaldehyde and ethylamine in aqueous solution and analyzed the mixture by LC/MS. The LC/MS data (not shown) displayed the ions *m*/*z* 138, 155, and 171 as the major signals, which on the basis of LC/MS^2 measurements were proposed to correspond to 1,4-diethylpyrazinium radical cations and 1,4-diethyl-2-hydroxy- and 1,4-diethyldihydroxy-1,4-dihydropyrazines as found above in the aqueous diquat solution (Figure 5A). The evidence of hydroxylated 1,4-dihydropyrazines suggested that also in the glycolaldehyde/amine mixture diquats are formed as intermediate oxidation products of radical cations. Because the redox process between the radical cation and the diquat was found not to be influenced by oxygen (Hayashi and Namiki, 1983), we propose a disproportionation reaction of the radical cation leading to a 1,4-dihydropyrazine and the corresponding diquat.

On the basis of the data obtained, a mechanism for the radical-assisted reactions is proposed in Figure 8. Condensation of two molecules of alkylaminoacetaldehyde (**I**), formed from glycolaldehyde and the amine, leads to the formation of a 1,4-dialkyl-1,4-dihydropyrazine (**IIa**), which is then oxidized to the 1,4-dialkylpyrazinium radical cation (**IIb**). Disproportionation of the radical cation results in the 1,4-dialkylpyrazinium diquaternary salt (**IIIa**) and the dihydropyrazine (**IIa**), which regenerates the radical cation upon oxidation. Rapid hydration of the diquat (**IIIa**) forms 2-hydroxy-1,4-dialkyl-1,4-dihydropyrazine (**IVa**), which, upon reduction of two molecules of diquat (**IIIa**), can produce a hydroxy diquat (**IIIb**) and regenerate two radical cations (**IIb**). Hydration of the hydroxy diquat (**IIIb**) results in the dihydroxy-1,4-dihydropyrazine (**IVb**), which, upon single-electronn transfer onto two molecules of diquat

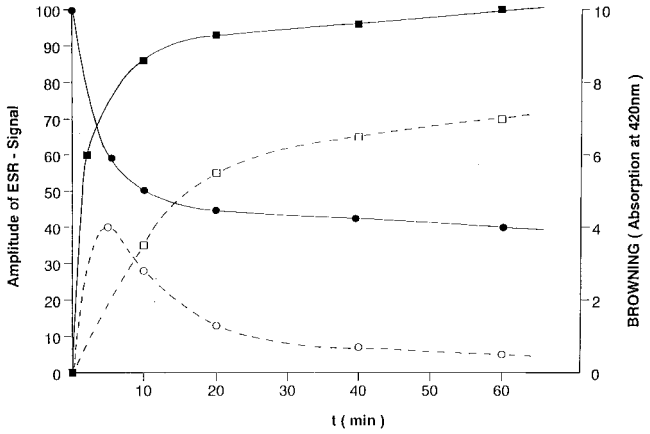

Figure 7. Time course of radical formation (●, ○) and color development (■, □) in heated aqueous solutions of 1,4-diethylpyrazinium diquat (—, solid symbols) and glycolaldehyde and ethylamine (- - -, open symbols).

(**IIIa**), might be able to regenerate the radical cation (**IIb**). Due to their strong nucleophilic character, intermediates **IVa** and **IVb** might participate in the formation of browning products by condensation with electrophiles such as aldehydes or by oligomerization with other diquat molecules. Colored oligomerization products of hydroxylated 1,4-dihydropyrazines could be observed by LC/MS in fractions of reacted glycolaldehyde/amine mixtures; however, these are as yet not identified.

These mechanisms, which take into account that the radical cation formation occurs prior to color development, are well in line with the experimental data obtained for heated glycolaldehyde/amino acid mixtures (Figure 7). The fact that the color formation increased rapidly after the radical concentration in the glycolaldehyde/amine mixture runs through a maximum (Figure 7) and no additional radical species could be observed is well documented by the reaction scheme (Figure 8), in which the radical cation is involved only as a reaction intermediate but not as the penultimate color precursor.

Yields of Glycolaldehyde/Glyoxal in Heated Carbohydrate/Amino Acid Solutions. Namiki and Hayashi (1983) proposed that the glycolaldehyde imine or the isomeric alkylaminoacetaldehyde (**I** in Figure 8) is formed by cleavage of the glucosylamine prior to the Amadori rearrangement. To gain more detailed insights into its role in the early Maillard reaction, the relative concentration changes of glycolaldehyde were followed in a heated glucose/L-alanine solution over a time period of 240 min. Because it is discussed in the literature that, in the presence of amino acids, glycolaldehyde might be oxidized to glyoxal and/or glyoxal imine (Hayashi et al., 1985; Glomb and Monnier, 1995), additionally the time course of glyoxal formation was followed. The data (Figure 9) showed that the amounts of glyoxal rapidly increased as soon as the mixture was heated. After running through a maximum of ∼100 μg/mmol of glucose after 15 min, the amount of glyoxal decreased again to about 20 or 10% after 60 or 240 min, respectively. In contrast, the concentrations of glycolaldehyde increased only slowly and approached a maximum value of ∼20 μg/mmol of glucose after 240 min. These data showed that the concentration maximum of glyoxal is reached prior to that of glycolaldehyde, indicating that, under the conditions applied here, not glycolaldehyde but glyoxal is formed as an early reaction product from carbohydrates.

To study whether glyoxal is formed by deoxyosone degradation or in an earlier stage prior to deoxyosone formation, the time course of the formation of 1-deoxy-2,3-hexodiulose and 3-deoxyhexos-2-ulose in the aqueous glucose/L-alanine solution was followed. For quantification of deoxyosones several groups (Morita et al., 1981, 1985; Beck et al., 1988; Nedvidek et al., 1992) reacted Maillard mixtures in the presence of excess amounts of 1,2-diaminobenzene to transform the reactive dicarbonyl intermediates in situ into stable quinoxaline derivatives. This in situ trapping technique leads to an accumulation of the quinoxalines, but does not offer insights into the relative changes in the concentrations of the deoxyosones. We, therefore, derivatized the deoxyosones produced with 1,2-diaminobenzene after rapid cooling of the thermally treated Maillard mixture. The data, given in Figure 10, demonstrated that high amounts of both deoxyosones were formed, among which the actual concentrations of 3-deoxyhexos-2-ulose were >4 times higher than those of the 1-deoxy-2,3-hexodiulose. The formation of 3-deoxyhexos-2-ulose and 1-deoxy-2,3-hexodiulose increased rapidly within the first 40 min and, after running through a maximum, decreased to about 75 and 30% after 120 min, respectively. Comparing the time course of deoxyosone formation (Figure 10) with the time course of glyoxal formation (Figure 9) showed that, under the reaction conditions applied here, glyoxal is produced in the carbohydrate/amino acid mixture at a very early stage of the Maillard reaction, prior to deoxyosone formation.

Several studies have been reported in the literature on glyoxal formation from carbohydrates under physiological conditions. For instance, the autoxidation of glucose (Wolff and Dean, 1987), the amine-assisted oxidation of carbohydrates (Glomb and Monnier, 1995), and the metal-catalyzed oxidative breakdown of the Amadori rearrangement product (Dunn et al., 1990) were evidenced by model experiments. The formation of glyoxal in thermally treated carbohydrate/amino acid mixtures was, however, as yet not clear. To locate the direct precursor of glyoxal, aqueous solutions of glucose, glucose/L-alanine, and *N*-(1-deoxy-D-fructos-1-yl)-L-alanine, respectively, were refluxed for 10 min, and the amounts of glyoxal generated were quantified. The results (Table 2) revealed that thermal treatment of the Amadori rearrangement product produced by far the highest amounts of glyoxal, followed by the glucose/alanine mixture, which generated glyoxal in 2.6-fold lower quantities. The enhanced glyoxal formation from the Amadori product is well in line with recent findings of Weenen and Apeldoorn (1996). In contrast, heating of glucose in the absence of amine strongly suppressed glyoxal formation, indicating that the glucose autoxidation proposed by Wolff and Dean (1987) can be excluded as effective source of glyoxal production.

To study the role of oxygen in glyoxal formation, the most effective precursor systems glucose/L-alanine and *N*-(1-deoxy-D-fructos-1-yl)-L-alanine, respectively, were heated for 10 min in the absence of oxygen. The data (Table 3) demonstrated that the lack of oxygen nearly inhibited the glyoxal formation, because by factors of 12 and 18 less glyoxal was formed from the hexose/amino acid mixture and the Amadori product, respectively, compared to that from the reaction mixture

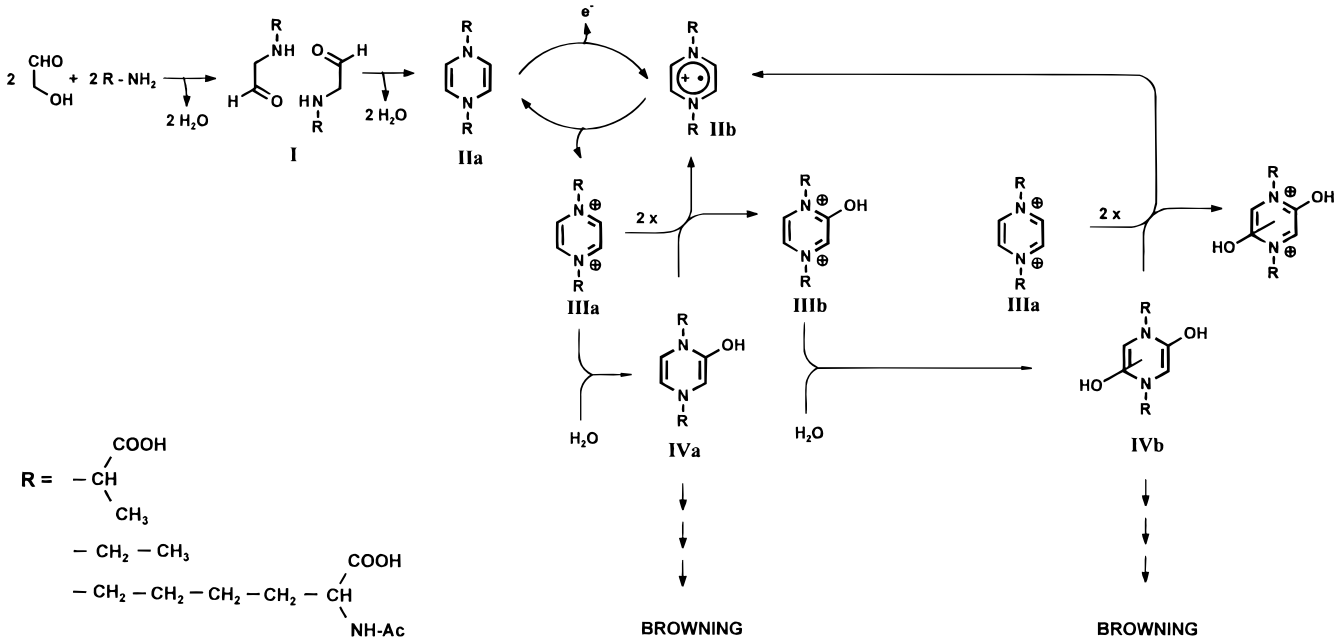

Figure 8. Reaction pathway leading to nonenzymatic browning from glycolaldehyde and amino acids.

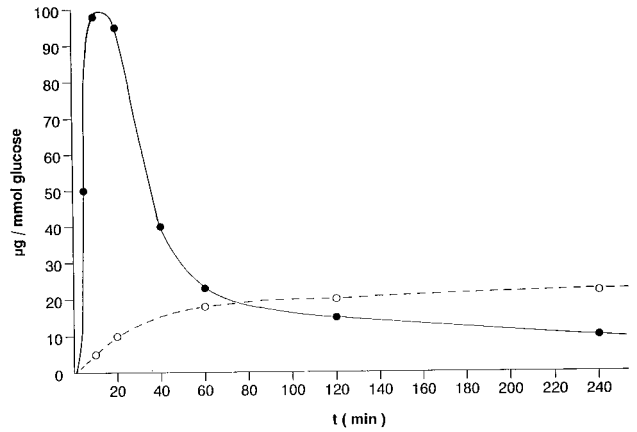

Figure 9. Time course of formation of glyoxal (—) and glycolaldehyde (- - -) in a heated aqueous solution of glucose and L-alanine.

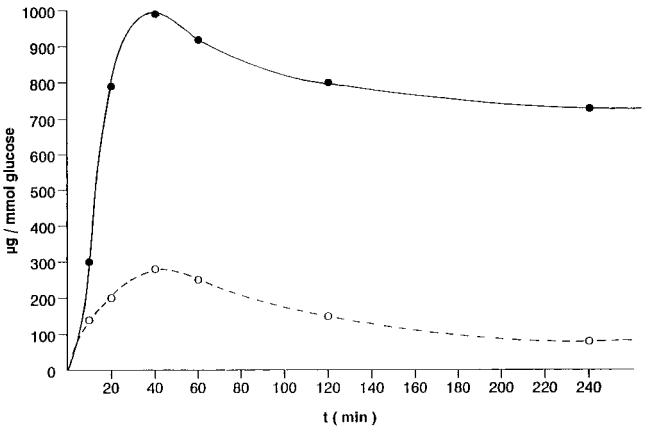

Figure 10. Time course of formation of 1-deoxy-2,3-hexodiulose (- - -) and 3-deoxyhexos-2-ulose (—) in a heated aqueous solution of glucose and L-alanine.

heated in the presence of oxygen. These data clearly indicated that under the reaction conditions applied an oxidation mechanism with oxygen is involved in glyoxal formation.

Table 2. Formation of Glyoxal in Various Hexose Precursor Systems

precursor	glyoxal µg/mmol	glyoxal %
glucose	18	0.03
glucose/L-alanine	96	0.16
N-(1-deoxy-D-fructos-1-yl)-L-alanine	253	0.42

[a] The precursors (6 mmol each) were refluxed in phosphate buffer (4 mL; pH 7.0; 0.5 mol/L) for 10 min.

Table 3. Influence of Oxygen on the Formation of Glyoxal in Various Hexose Precursor Systems

precursor	glyoxal (µg/mmol) I[a]	glyoxal (µg/mmol) II[b]
glucose/L-alanine	96	8
N-(1-deoxy-D-fructos-1-yl)-L-alanine	253	14

[a] The precursors (6 mmol each) were refluxed in phosphate buffer (4 mL; pH 7.0; 0.5 mol/L) for 10 min. [b] Nitrogen was bubbled for 30 min through the reaction mixture[a] prior to heating.

N-(1-Deoxy-D-fructos-1-yl)-L-alanine was found to be the most effective glyoxal precursor; however, quantitative data revealed that only 1.1% of the Amadori product was generated from glucose and L-alanine after 10 min of heating. These data clearly demonstrate that the predominant part of the glyoxal formed in heated glucose/alanine mixtures (Table 3) does not originate from the Amadori rearrangement product. Because the presence of the amino acid was found to be a prerequisite for an effective glyoxal formation from glucose, an oxidation of the Schiff base, presumably via the enaminol, was assumed. This oxidation is well in line with recent synthetic experiments showing that Maillard-derived enaminols are easily oxidized into the corresponding iminoketones (Hofmann and Schieberle, 1995, 1996, 1998b).

On the basis of these data, the mechanism outlined in Figure 11 is proposed for the formation of glyoxal in the early stage of the Maillard reaction. Reaction of the carbohydrate with the amino acid forms the Schiff base (**Ia**), which via the enaminol (**Ib**) rearranges into the Amadori product (**Ic**). The enaminol (**Ib**) can be oxidized,

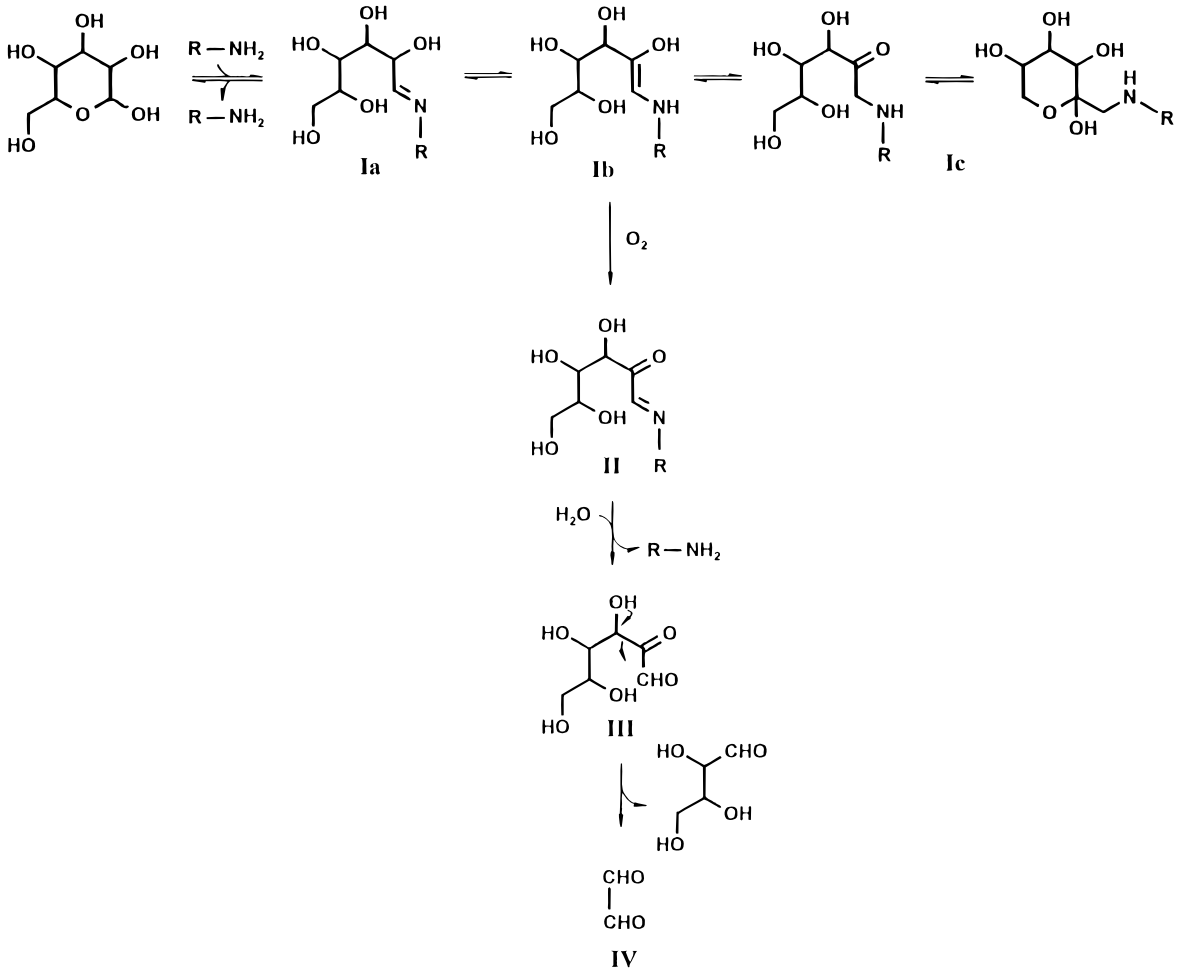

Figure 11. Reaction pathways leading to glyoxal by oxidative breakdown of the Schiff base of carbohydrates and amino acids via glucosone as the intermediate.

however, in an oxygen-dependent side reaction to the corresponding glucosone imine (**II**), which, upon hydrolysis, results in the glucosone (**III**). The formation of glucosone from oxidative degradation of the Amadori compound in the presence of trace amounts of transition metals and oxygen could be very recently demonstrated by model experiments (Kawakishi et al., 1990). C-2/C-3 scission of the glucosone then gives rise to glyoxal (**IV**).

The early formation of glyoxal led us to the assumption that glyoxal has to be reduced to glycolaldehyde as a prerequisite for the formation of the radical cation in carbohydrate/amino acid mixtures. This encouraged us to investigate the time course of the formation of reducing substances in the hexose/L-alanine mixture. Thermally treated aqueous solutions of glucose and L-alanine varying in reaction time were, therefore, titrated with a solution of 2,6-dichloroindophenol sodium. Relating the results to a titrated standard solution of ascorbic acid, the concentrations of reducing compounds in the Maillard mixture were calculated as ascorbic acid equivalents (aae). The time course of the formation of reducing Maillard reaction products, displayed in Figure 12, demonstrated that within the first 10 min of heating reducing compounds were lacking. However, after an induction period of ~15 min, the concentration of reducing substances rapidly increased within the next 30 min to ~60 μmol of aae/mmol of glucose. After running through a maximum after ~45 min, the reducing power of the heated hexose/alanine solution decreased again to ~20 μmol of aae/mmol of glucose at 100 min.

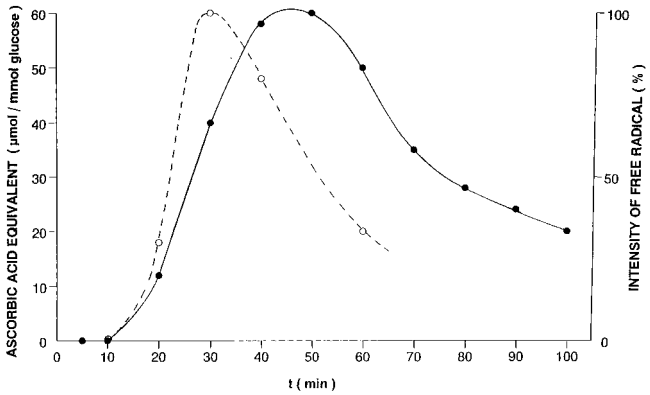

Figure 12. Time course of formation of reducing substances (—) and radical cations (- - -) in a heated aqueous solution of glucose and alanine.

If we assume glycolaldehyde to be the most effective radical precursor and take into account (i) the early formation of glyoxal from hexoses (Figure 9) and (ii) the induction period in the formation of reducing compounds (Figure 12), a delay in radical formation in the glucose/alanine mixture should occur. The data (Figure 12) showed that, after an induction period of ~15 min, the radical cation was observed increasing in intensity with time. After running through a maximum at 30 min, the radical concentration decreased again. These data evi-

Table 4. Reductone-Induced Generation of Free Radicals in the Induction Period of a Glyoxal/L-Alanine Solution[a]

reductone	rel radical intensity (%)
no additive	0
ascorbic acid	100
acetylformoin	94
methylenereductinic acid	69

[a] The model experiment is detailed under Experimental Procedures.

denced that the free radical development was closely correlated with the increase in the formation of reducing substances from glucose and L-alanine.

The experiments showed that glyoxal (Figure 9) is formed during the induction period of the radical formation (Figure 12) in the initial stage of the reaction between hexose and L-alanine. As the concentration of reducing substances such as reductones or aminoreductones begins to increase in the reaction mixture, glyoxal might be reduced into glycolaldehyde, thereby enabling immediate radical formation. If this mechanism works, then the addition of a reductone to a mixture of glyoxal and L-alanine should consequently produce the free radical. This prompted us to study the influence of reductones on the radical formation in more detail. A mixture of glyoxal and L-alanine was heated for 3 min and then analyzed by EPR spectroscopy. As shown in Table 4, the pyrazinium radical cation could not be detected, being well in line with the lack of glycolaldehyde in this mixture. Further incubation of this mixture in the presence of ascorbic acid at room temperature instantaneously produced the radical cation (Table 4). To study whether Maillard-derived reductones were also able to generate the radical cation, comparative experiments were performed by substituting ascorbic acid with acetylformoin or methylenereductinic acid, respectively, which are known as Maillard reaction products formed from hexoses (Ledl et al., 1982; Hofmann, 1998e). As depicted in Table 4, the radical cation was also generated upon addition of acetylformoin as well as methylenereductinic acid, among which the former reductone was found to be as effective as ascorbic acid, whereas the latter reductone showed an effectiveness of ~69% relative to ascorbic acid. These data indicate that glyoxal formed from hexose degradation as well as the respective Schiff bases generated the radical cation upon reduction by Maillard-derived reductones.

To prove that glyoxal is also a radical precursor in the heated glucose/L-alanine solutions, an additional experiment was performed in which the radical formation was measured after addition of a reductone at different times within the induction period. A glucose/L-alanine solution was, therefore, heated, and after 5, 10, and 20 min, aliquots were spiked with ascorbic acid. After incubation for 5 min at room temperature, the ESR signal was measured. The data (Figure 13) showed that, after addition of a reductone, the radical cation is formed already after 5 min of heating. After running through a maximum at 10 min, the radical concentration decreased again. Comparison of these data with the time course of radical cation formation in the absence of ascorbic acid, which is also displayed in Figure 13, indicates that during heating of the glucose/L-alanine mixture a radical precursor, which can form the radical upon reduction, is formed in a very early stage. The time course of the radical precursor (Figure 13) fits well into the time course found for the glyoxal formation (Figure 9), corroborating our proposal that glyoxal (**Ia**), formed

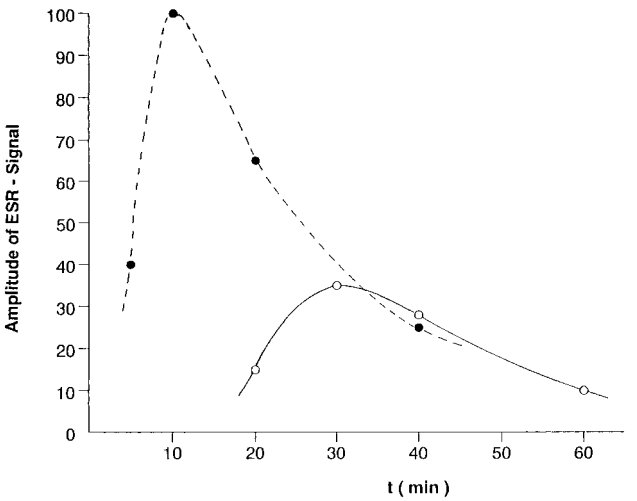

Figure 13. Time course of radical precursor concentration (- - -) and free radical formation (—) in a heated aqueous solution of glucose and alanine.

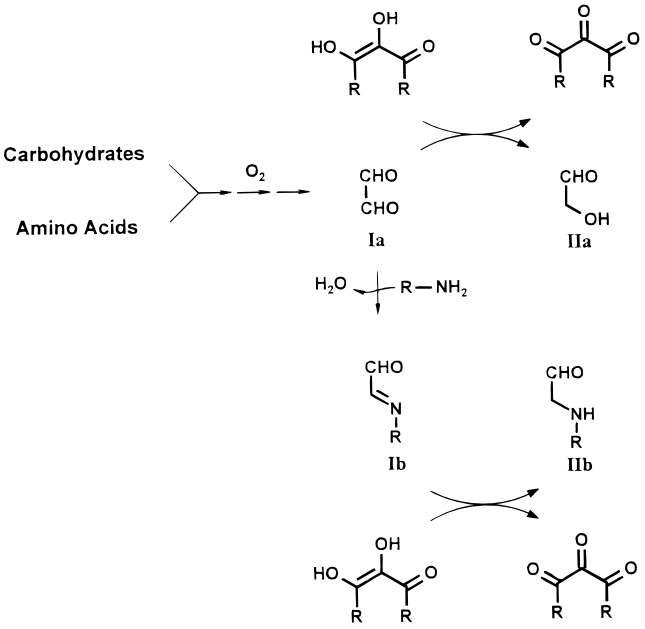

Figure 14. Formation of glycolaldehyde and alkylaminoacetaldehyde upon redox reaction of glyoxal and glyoxal imine with reductones.

as primary reaction product from carbohydrates and amino acids, and/or the corresponding glyoxal imine (**Ib**) yields glycolaldehyde (**IIa**) and/or alkylaminoacetaldehyde (**IIb**) by a redox reaction with a reductone as displayed in Figure 14.

Conclusions. The data presented demonstrate that the determination of the time course of the formation of certain Maillard intermediates is very helpful to gain detailed information on the complex reaction pathways of the nonenzymatic browning. Additional studies on certain reaction steps of the Maillard reaction have to be undertaken to increase the knowledge on the complex network of reactions occurring during thermal processing of foods.

ACKNOWLEDGMENT

We thank the Institute of Organic Chemistry, Technical University of Munich, for the NMR measurements and the Institute of Food Chemistry, Technical Univer-

sity of Munich, for the LC/MS analyses. We are grateful to Mr. J. Stein for his excellent technical assistance.

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Received for review June 9, 1998. Revised manuscript received November 3, 1998. Accepted November 12, 1998.

JF980626X