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Kinetic modelling of Amadori *N*-(1-deoxy-D-fructos-1-yl)-glycine degradation pathways. Part I—Reaction mechanism

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Abstract

The fate of the Amadori compound *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) was studied in aqueous model systems as a function of pH and temperature. The samples were heated at 100 and 120 °C with initial reaction pH of 5.5 and 6.8. Special attention was paid to the formation of the free amino acid, glycine; parent sugars, glucose and mannose; organic acids, formic and acetic acid and α-dicarbonyls, 1- and 3-deoxyosone together with methylglyoxal. For the studied conditions decreasing the initial reaction pH with 1.3 units or increasing the temperature with 20 °C has the same effect on the DFG degradation as well as on glycine formation. An increase in pH seems to favour the formation of 1-deoxyosone. The lower amount found comparatively to 3-deoxyosone, in all studied systems, seems to be related with the higher reactivity of 1-deoxyosone. Independently of the taken pathway, enolization or retro-aldolization, DFG degradation is accompanied by amino acid release. Together with glycine, acetic acid was the main end product formed. Values of 83 and 55 mol% were obtained, respectively. The rate of parent sugars formation increased with pH, but the type of sugar formed also changed with pH. Mannose was preferably formed at pH 5.5 whereas at pH 6.8 the opposite was observed, that is, glucose was formed in higher amounts than mannose. Also, independently of the temperature, at higher pH fructose was also detected. pH, more than temperature, had an influence on the reaction products formed. The initial steps for a complete multiresponse kinetic analysis have been discussed. Based on the established reaction network a kinetic model will be proposed and evaluated by multiresponse kinetic modelling in a subsequent paper.

Keywords: Amadori compound; *N*-(1-deoxy-D-fructos-1-yl)-glycine; Maillard reaction; Multiresponse kinetic analysis

1. Introduction

Since Hodge[1] presented the first coherent Maillard reaction scheme in 1953, the Amadori compound *N*-substituted 1-amino-1-deoxy-ketose is believed to be the key intermediate in the early stages of the reaction. The accepted mechanism for the formation of Amadori compounds involves the initial reaction of a reducing sugar with an amino group to give the corresponding glycosylamine, that rearranges to the corresponding ketoseamine, also known as the Amadori rearrangement product (ARP). The set of reactions that occurs thereafter is of great importance in the processing of foods for the production of aroma, taste and colour. Furthermore, evidence strongly suggests that this intricate reaction cascade is involved in the pathology of diabetes and ageing.[2]

Due to the complexity of products that are formed from the degradation of the ARP, recent studies used the Amadori compound as the initial reactant.[3–6] These studies showed that the 1,2- and 2,3-enolizations of Amadori compounds under acid/base catalysis conditions initiate β-elimination reactions, which eventually lead to the formation of reactive intermediates (Scheme 1). The degradation of *N*-(1-deoxy-D-fructos-1-yl)-glycine (DFG) (**1**) by 1,2-enolization (pathway A) and 2,3-

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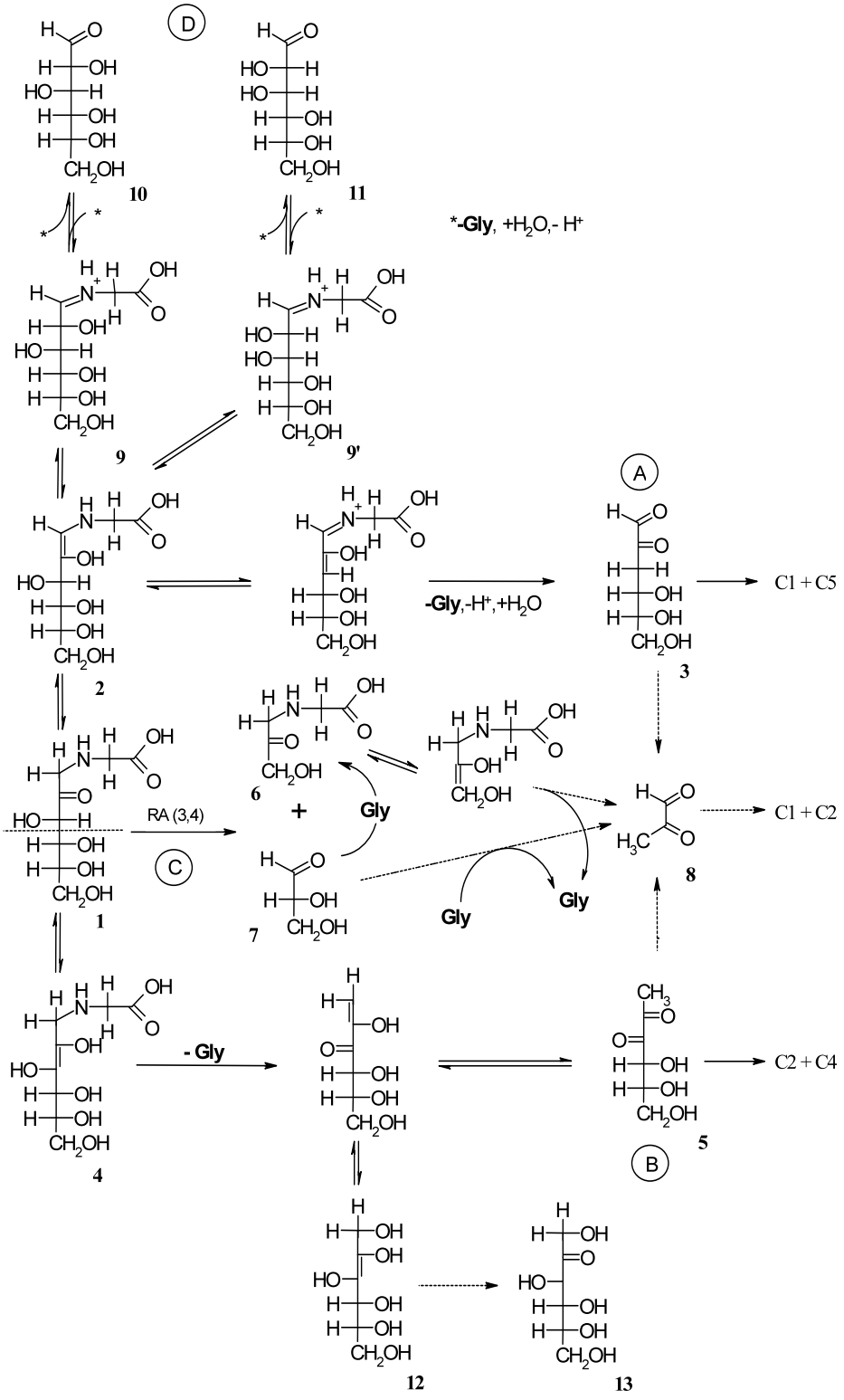


Scheme 1. Amadori compound *N*-(1-deoxy-D-fructos-1-yl)glycine degradation pathways: enolization and retro-aldolization.

enolization (pathway B) leads to the formation of 3-deoxy-2-hexosulose (**3**) and 1-deoxy-2,3-hexodiulose (**5**), respectively. The formation of these intermediates is accompanied by amino acid release. In parallel, other α-dicarbonyls can also be formed from ARP enolization. A recent study[6] presented a detailed scheme of Amadori compound degradation pathways through enolization, such as the formation of glucosones by transition-metal

catalyzed oxidation of 1,2-enaminol (**2**) and 1-amino-1,4-dideoxy-2,3-diulose by elimination of the C-4 OH group of the 2,3-enaminol (**4**).

Another possible mechanism of degradation of Amadori compounds involves a retro-aldol reaction, as shown in pathway C. Previous studies[4,7] have indicated that **1** generates 1-glycine-1-deoxy-D-glyceraldehyde (**6**) and glyceraldehyde (**7**) through a retro-aldol cleavage at C-3 to C-4. Compound **7** can react with free glycine and produce more of compound **6** that subsequently undergoes a β-elimination to form methylglyoxal (**8**) and release glycine. Moreover, **8** can also be produced from **7** through the catalytic action of the amino acid.

In spite of all the work done in Maillard reaction, the reversibility of ARP is still a controversial issue. Theoretically, as a series of equilibrium reactions, the Amadori rearrangement is expected to be a reversible process, that is to undergo a non-enzymatic reversal into enolamines and subsequently into free sugars and amino acid, as shown in pathway D. However, other pathways as side reactions from ARP enolization or aldol-type condensations between smaller sugar fragments generated from the decomposition of ARPs can also give rise to the original aldose. Under physiological conditions (pH 7.4 and 37 °C), Glc and Man were identified as major products formed from protein-bound Amadori compound fructoselysine, along with tetroses, pentoses and 3-deoxyglucosone.[8] Up until recently the formation of parent sugars had only been reported for physiological conditions. However, quantitative data concerning the formation of parent sugars, Glc (**10**) and Man (**11**), from DFG at the early stage of the reaction have now been reported for conditions relevant to food processing.[6] Besides **10** and **11**, formic and acetic acid were also detected, together with the free amino acid. Formic and acetic acid have also been identified as two major degradation products by heating the isolated Amadori compound from a glucose/casein system.[9] The proposed mechanism suggests the C-1 to C-2 cleavage of **3** for the formic acid formation and C-2 to C-3 cleavage of **5** or a cleavage reaction of trioses intermediates, like **8**, for the formation of acetic acid. However, no attempt was made to identify and quantify any α-dicarbonyl compound to support such mechanism.

The aim of the present study was to establish the main thermal degradation pathways of DFG by applying multiresponse kinetic modelling. The multiresponse kinetics analysis considers reaction pathways in more detail. It provides extra information about the reaction mechanism since the reactants degradation is analyzed simultaneously with the intermediates formation. First a reaction mechanism is proposed and then the multiresponse kinetic analysis is applied.[10] The following steps should be taken into account: (i) identification and quantification of the reactants and main products formed; (ii) identification of reaction pathways based on reaction conditions; (iii) differentiate between primary and secondary reaction routes; (iv) propose a kinetic model based on the established reaction network; (v) test the hypothesized mechanism; (vi) estimate the rate constants. The present paper (Part I) deals with the first three steps. Special attention was paid to the formation of the free amino acid, glycine; parent sugars, Glc and Man; organic acids, formic and acetic acid and α-dicarbonyls, 1- and 3-deoxyosone together with methylglyoxal. A subsequent paper, Part II,[11] deals with the remaining steps with a focus on the kinetics of the hypothesized mechanism.

2. Experimental

2.1. Chemicals

The following compounds were obtained commercially: Glycine, D-Glc, D-fructose (D-Fru), D-Man, formic acid, ammonium acetate (Merck, Darmstadt, Germany); AcOH, methylglyoxal, 2-methylquinoxaline, 5-(hydroxymethyl)-furan-2-carboxaldehyde (Sigma–Aldrich, Germany); 1,2-diaminobenzene (78410, Fluka, Switzerland).

2.2. *N*-(1-Deoxy-D-fructos-1-yl)-glycine (1)[12]

Glycine (5.25 g, 0.07 mol), D-Glc (50.3 g, 0.28 mol) and $Na_2S_2O_5$-sodium disulphite (7.6 g, 0.04 mol) were added to a 1:1 MeOH–water solution (80 mL). The mixture was then stirred for 20 min at room temperature (rt) and subsequently refluxed for 8 h. In the end a 1:1 EtOH–water solution (250 mL) was added. The final mixture was applied onto an ion-exchange resin column (Dowex 50W*8, 3 × 30 cm, H^+). The column chromatography of the residue was performed with 1:1 EtOH–water solution (750 mL), water (250 mL) and elution with ammonia solution (2.5 L, 0.1 mol/L). Fractions of 100 mL were collected. Biochrom 20 Amino Acid Analyzer, Pharmacia, was used to check the presence of **1**. The fractions containing only **1** were pooled and concentrated until dryness at 40 °C in a rotavapor. The resulting syrup was dissolved in MeOH (using ultrasonic bath) and kept in the fridge overnight. A white precipitate was collected by filtration through a glass-sintered funnel (G4). The final yield was 19% based on the amount of glycine used. Anal. Calcd for $C_8H_{15}NO_7$: C, 40.50; H, 6.37; N, 5.91. Found: C, 40.13; H, 6.4; N, 5.89. mp: 160–163 °C. On the Biochrom 20 Amino Acid Analyser the synthesised compound showed only one peak. In addition, DFG [3] was also kindly offered by Drs. Blank and Davidek, from Nestlé Research Center Lausanne, P.O. Box 44, Vers-Chez-les-Blanc, CH-1000 Lausanne 26, Switzerland.

2.3. 2-Methyl-3-(1,2,3-trihydroxypropyl)quinoxaline (5′)[13]

A mixture of D-Glc (196 mmol), L-alanine (183 mmol) and 1,2-diaminobenzene (183 mmol) in phosphate buffer (800 mL, 0.5 M, pH 6.8) was refluxed for 12 h. A very black mixture was obtained, with a crude type precipitate at the bottom. After cooling to rt, the reaction mixture was extracted with methylene chloride (5 × 200 mL). The organic layers were then combined and dried over Na_2SO_4. After concentration till dryness, the residual was dissolved in ethyl acetate (EtOAc) and the separation was performed by column chromatography (35 × 400 mm) on silica gel (200 g, silica gel 60, Merck) conditioned with EtOAc. Elution was performed first with EtOAc (1.5 L) followed by a 99:1 EtOAc–MeOH (1 L). Fractions of 30 mL were collected and analysed by thin layer chromatography (TLC) on silica gel (20 × 20 cm, 0.5 mm, Merck) using EtOAc–MeOH (99:1) as the mobile phase. The target compound was found at $R_f = 0.3$–0.4. It was visualized by UV and spraying with a $KMnO_4$ solution. Pure fractions were combined and concentrated till dryness and then recrystalised from MeOH. In the end we obtained fine, white yellowish crystals (0.02% in yield). ^{1}H NMR (200 MHz; CD_3OD) δ 2.86 (s, 3 H, $-CH_3$), 3.81–3.92 (m, 2 H, $-CH_2OH$), 4.08 (m, 1 H, –CH(OH)–), 5.10 (d, 1 H, –CCH(OH)–), 7.77 (m, 2 H, =CH–), 7.98 (m, 1 H, =CH–), 8.096 (m, 1 H, =CH–) which is in agreement with the reference used. Also the high performance liquid chromatography (HPLC) detection method revealed only one peak.

2.4. Preparation of the reaction mixtures

Compound **1** (0.237 g, 10 mmol) was dissolved in phosphate buffer (100 mL, 0.1 M K_2HPO_4/KH_2PO_4, pH 6.8 and 5.5) and heated at 100 °C for a maximum of 4 h and at 120 °C for a maximum of 3 h, in an oil bath, in screw-capped glass tubes (Schott, 16 × 160 mm). At predetermined heating times, samples were taken and immediately cooled in ice water, prior to analyses. Each reaction mixture was prepared, heated and analysed in at least duplicate.

2.5. Quantification of DFG (1) and glycine

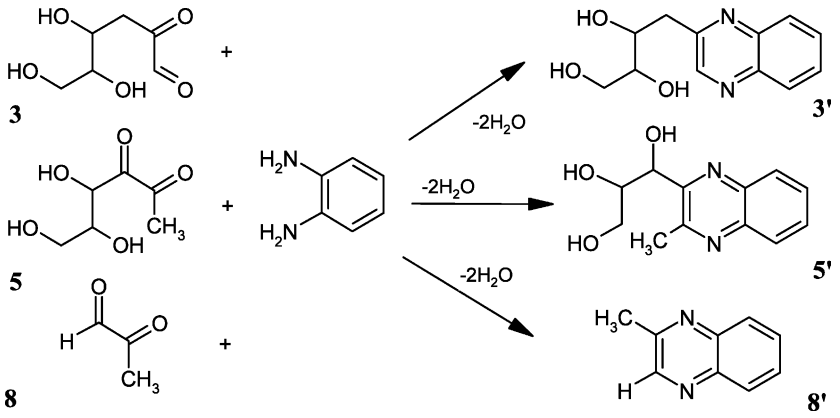
The reaction mixtures were diluted with water (1:10) and the pH adjusted with loading buffer (LB, Biochrom, England) to 2.2 in the proportion 100 μL of diluted sample to 900 μL of LB. The capsules were then loaded with 20 μL of sample, 20 μL of internal standard (glutamic acid; 25 μmol/mL) and 20 μL of LB. The analysis was performed on a Biochrom 20 Amino Acid Analyser, Pharmacia, England. The separation was done using the following program: lithium citrate buffer pH 2.8 for 16 min and column temperature 80 °C; lithium citrate buffer pH 3.0 for 21 min and column temperature 75 °C; lithium citrate buffer pH 3.15 for 12 min and column temperature 75 °C; lithium citrate buffer pH 3.55 for 21 min and column temperature 75 °C; lithium hydroxide solution for 6 min and column temperature 85 °C; lithium citrate buffer pH 2.8 for 8 min and column temperature 85 °C; lithium citrate buffer pH 2.8 for 20 min and column temperature 75 °C; lithium citrate buffer pH 2.8 for 5 min and column temperature 80 °C. All buffers were provided by Biochrom, England. After postcolumn derivatization with ninhydrine, the reaction products were determined photometrically at 570 nm: **1** (t_r 9.5 min); glycine (t_r 38.8 min). Quantification was done using external standards.

2.6. Quantification of 1-deoxy-2,3-hexodiulose (5), 3-deoxy-2-hexosulose (3) and methylglyoxal (8)[14]

At each reaction time, 1 mL of sample was withdrawn and put into 1 mL of water and 2 mL of a methanolic solution of 1,2-diaminobenzene (1 mol/L). The mixtures were maintained overnight at rt (25 °C). With previous experimental tests it was established that this treatment ensured a complete derivatization of the target compounds into their quinoxaline form (Scheme 2). The mixtures were analysed by RP-HPLC using a solvent gradient starting with a mixture (10/90; v/v) of acetonitrile and ammonium acetate buffer (pH 3.5; 20 mmol/L) and increasing the acetonitrile content to 30% within 50 min. The flow rate was 0.4 mL/min. By monitoring the effluent at a wavelength of 320 nm a similar chromatogram to the used reference was obtained. The peaks were identified by direct comparison of their retention times with that of authentic samples and by spiking the samples with standards. By comparison with **5′** the 2-(2,3,4-trihydroxybutyl)quinoxaline (**3′**) was identified. The quantification was done using **5′** and methylquinoxaline (**10′**) as external standards. Both quinoxalines have the same extinction coefficient. The same extinction coefficient was found for 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline and 2-(1,2,3,4-tetrahydroxybutyl)quinoxaline.[15] Increasing the column temperature to 40 °C optimised the peak separation. Compound **5**, **3** and **8** were detected as their corresponding quinoxaline derivatives at the retention times (t_r) given in parenthesis: 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline (t_r 11.3 min); 2-(2,3,4-trihydroxybutyl)quinoxaline (t_r 12.4 min); 2-methylquinoxaline (t_r 38.8 min).

2.7. Quantification of sugars (D-Glc (10) and D-Man (11)) and organic acids (formic and acetic acid)

Diluted samples (1:10) were analysed by HPLC, using an ion-exchange column (ION-300, Interaction Chromatography Inc., San Jose, CA). Eluent 2.5 mmol/L



Scheme 2. α-Dicarbonyl derivatization with 1,2-diaminobenzene: 3-deoxy-2-hexosulose (**3**); 1-deoxy-2,3-hexodiulose (**5**); methylglyoxal (**8**) and the respective quinoxaline derivatives: 2-(2,3,4-trihydroxybutyl)quinoxaline (**3′**); 2-methyl-3-(1,2,3-trihydroxypropyl)quinoxaline (**5′**); 2-methylquinoxaline (**8′**).

H_2SO_4 in milipore water, flow 0.4 mL/min, column temperature 85 °C. Sugars were detected by monitoring the refractive index: Glc (t_r 15.1 min); Man (t_r 16.4 min); and organic acids by their absorbance at 210 nm: formic acid (t_r 21.6 min); and AcOH (t_r 23.7 min). All compounds were quantified by external standards.

2.8. Quantification and separation of D-Glc (10), D-Man (11) and D-Fru (13)

After heating, diluted samples (1:100) were analysed by a Dionex system (Sunnyvale, CA) using a CarboPack PA100 column. The separation was done using the following gradient: starting mixture (16/0/84, v/v) of NaOH (0.1 M), sodium acetate (NaOAc) (1 M in NaOH 0.1 M) and water for 20 min. After NaOAc increased to (0/100/0) and was kept isocratic for 10 min, after which the gradient changed to (100/0/0) in 1 min and was kept isocratic for 5 min. The gradient was then brought to the initial starting mixture and kept for 25 min, before the next injection. The flow rate was 1 mL/min. Detection was performed by an electrochemical detector (model ED-40): D-Glc (t_r 12.95 min); D-Man (t_r 13.9 min); and D-Fru (t_r 16.59 min). All compounds were quantified by external standards.

2.9. Determination of heterocyclic compounds[16]

5-(Hydroxymethyl)furan-2-carboxaldehyde (HMF), 4-hydroxy-2-(hydroxymethyl)-5-methyl-3(2*H*)-furanone (HHMF) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) were determined by RP-HPLC (Lichrosorb RP-18, Merck). The eluent was 7.5:100 MeOH–water solution, flow 0.6 mL/min, UV detection at 280 nm. Only HMF was quantified by external calibration line with 5-(hydroxymethyl)furan-2-carboxaldehyde (Sigma–Aldrich, Germany).

2.10. Melanoidins quantification

The browning intensity of the heated reaction mixtures was determined by measuring the absorbance at 470 nm with a spectrophotometer (Pharmacia Biotech, Upsala, Sweden). When necessary the samples were diluted with demi water. The absorbance was then recalculated to the concentration of the melanoidins by using the equation of Lambert–Beer. The extinction coefficient formed in Glc-glycine was measured to be 0.64 ± 0.03 l/mmol cm at 470 nm.[17] The concentration of melanoidins is thus expressed as moles of sugar incorporated in the brown polymers.

3. Results and discussion

The effect of the combination of pH and temperature on the decomposition of **1** was studied at 100 and 120 °C, with initial pH values of 5.5 and 6.8. By analysing the reactant degradation and the formation of the main products, under different reaction conditions, the reaction routes could be established. The following steps were taken.

3.1. Identification and quantification of the reactant 1 (DFG) and main products formed

3.1.1. Degradation of DFG. The rate of degradation of **1** increased with temperature and pH (Fig. 1). After heating for 30 min at 120 °C and pH 6.8, **1** was completely degraded whereas after 180 min at 100 °C and pH 5.5, only approximately 57% of the initial concentration had reacted. Moreover, when comparing the system at 100 °C and pH 6.8 with the system at 120 °C and pH 5.5, a similar degradation rate was observed. These results indicate that decreasing the initial reaction pH with 1.3 units or increasing the

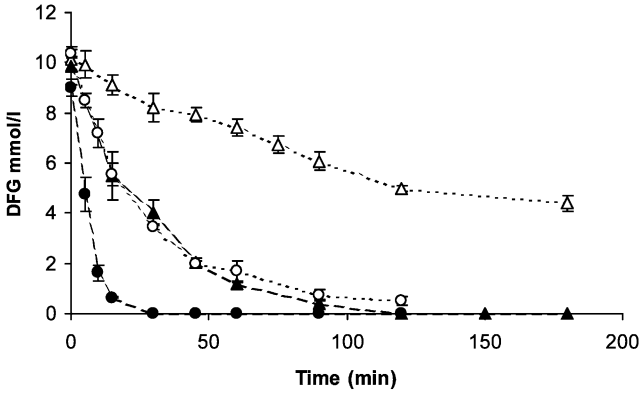


Fig. 1. DFG thermal degradation at 100 (△, pH 5.5;▲, pH 6.8) and at 120 °C (○, pH 5.5; ●, pH 6.8).

temperature with 20 °C has the same effect on the DFG degradation. However, this observation is only valid in the pH range between 5 and 7.[6]

3.1.2. Main products formed. Special attention was paid to the formation of the free amino acid, glycine; α-dicarbonyls, 1- and 3-deoxyosone together with methylglyoxal, parent sugars, Glc and Man; and organic acids, formic and acetic acid.

3.1.3. Free amino acid formation. Similar to DFG degradation, glycine formation increased with pH and temperature (Fig. 2). Also a decrease in the initial reaction pH of 1.3 units had a similar effect in glycine formation as increasing the temperature with 20 °C. Moreover, when comparing the yield of glycine (amount of glycine formed/amount of DFG that reacted), we observed that at the early stage of the reaction the yield of glycine increased with time (Table 1). However, as the reaction proceeded a decrease was observed. Moreover, in all the studied systems the decrease in the yield of glycine was followed by an increase again. Also, the yield of glycine increased with increasing pH, independently of the temperature. In a recently published study[6]

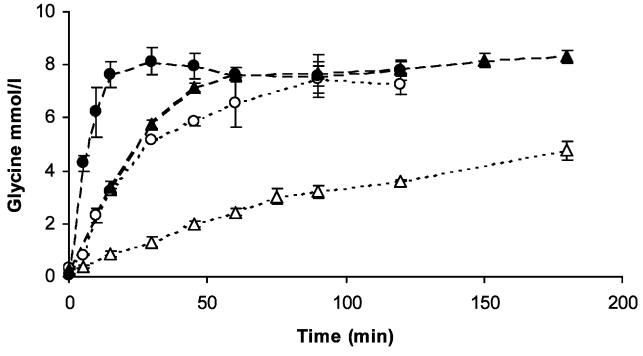


Fig. 2. Glycine formation during thermal degradation of DFG at 100 (△, pH 5.5;▲, pH 6.8) and 120 °C (○, pH 5.5; ●, pH 6.8).

the thermal degradation of **1** was compared at different pH, either in water or in phosphate buffer solution. It was observed that the yield of glycine in water increased with increasing pH, whereas in phosphate buffer solution the opposite was observed. Also NaOH was added to keep the pH constant throughout the reaction period. The authors suggested that phosphate slowed down the degradation of glycine at lower pH, mainly pH 5 and 6. However, it has been reported[18] that phosphate ions act as a catalyst leading to enhanced degradation of Amadori compounds with an optimum in the pH range between 5 and 7, which contradicts their suggestion. A possible reason for the observed decrease of the glycine yield with increasing pH in phosphate buffer solution can be the increasing addition of NaOH with increasing pH. It may enhance the formation of alkyl(1*H*)pyrazinones, which can retain more than one glycine molecule.[7] We will come back to glycine degradation pathways in the following step.

3.1.4. α-Dicarbonyls formation. (a) The deoxyosones, **3** (3-DG) and **5** (1-DG) were identified and quantified in their quinoxaline form. As shown in Fig. 3, at both studied pH values and temperatures, both deoxyosones were formed. Compared to the amount of DFG reacted, a quite high concentration of 1-DG and 3-DG was already present after 5 min at pH 5.5 and temperature 100 °C (34%). Independently of the temperature, at lower pH 3-DG was present in larger amounts relatively to 1-DG. At pH 6.8 that difference decreased. An increase in pH seems to favour the 1-DG formation. These findings are in line with what has been reported.[19] Under more alkaline conditions, relative to the pK_a of the ARP (DFG pK_a is 8.2), Amadori compound is believed to undergo 2,3-enolization, as well, with 1-DG formation. Also, independently of the pH and temperature, as the reaction proceeded the amount of 1-DG decreased much faster than the one of 3-DG. (b) Another identified and quantified α-dicarbonyl was **8** (MG) as its quinoxaline derivative. Contrary to the temperature increase, the increase of pH had almost no influence on the MG formation (Fig. 4). A very low increase in the peak area of methylquinoxaline, when the pH increased from five to seven, was also observed in a glucose/β-alanine reaction mixture heated in phosphate buffer for 12 h.[20] However, the influence of temperature is quite significant. An increase from 100 to 120 °C more than doubled the formation of MG. No decrease in its amount was observed with time.

3.1.5. Parent sugars formation. Compound **10** and **11** have recently been reported as the DFG parent sugars.[6] No other sugar was mentioned in that study, even though Fru (**13**) can also be formed by isomerisation of Glc and Man.[21] Based on those results,[6] initial sugar analyses were carried out using an Ion exchange HPLC

Table 1
Glycine yield from DFG heated in phosphate buffer (0.1 M) at different reaction conditions

Heating time (min)	Glycine yield (%) [a]			
	A (T 100 °C pH 5.5)	B (T100 °C pH 6.8)	C (T120 °C pH 5.5)	D (T120 °C pH 6.8)
5	43.40	n.a.	25.87	n.a.
10	n.a.	n.a.	62.05	83.92
15	57.89	71.02	59.56	94.72
30	53.61	93.85	69.79	95.82
45	78.23	87.44	66.20	92.36
60	79.69	84.91	71.95	84.41
75	81.46	n.a.	n.a.	n.a.
90	72.39	78.42	74.20	84.05
120	65.03	76.59	70.18	86.39
150	n.a.	80.14	n.a.	n.a.
180	78.31	81.45	n.a.	n.a.

n.a.: not analyzed.
[a] The yield of glycine was calculated based on the amounts of DFG reacted.

column, knowing that with this method Man and Fru have the same retention time, but providing a good separation of these sugars with Glc. It was assumed that the Fru/Man peak was mainly Man. However, to check the assumption that no Fru was formed, samples from all the systems studied taken at 120 min were also analysed using the Dionex method, with which it was possible to separate Fru from Man, besides Glc. Contrary to our expectations, Fru was also observed in addition to Glc and Man, in samples heated at pH initial value 6.8. To gain insight whether Fru would be an isomerisation product from Glc and Man, systems at pH 6.8 heated at 100 and 120 °C were analysed with the Dionex method. As shown in Fig. 5 the formation of Fru showed no lag phase, indicating that it can be formed directly from DFG rather than from isomerisation of Glc and Man. Moreover, independently of the temperature, at pH 5.5, Man was formed in higher amounts than Glc (Fig. 5 A and C). Also, as the reaction proceeded at 120 °C (Fig. 5C) Man reached a maximum after 30 min, decreasing afterwards, whereas Glc did not decrease after 120 min of heating. At pH 6.8 the opposite was observed, that is, Glc not only was formed in higher amounts than Man but also seemed to be more reactive, showed by the fast decrease at 120 °C (Fig. 5 B and D). Fru, even though formed in considerable

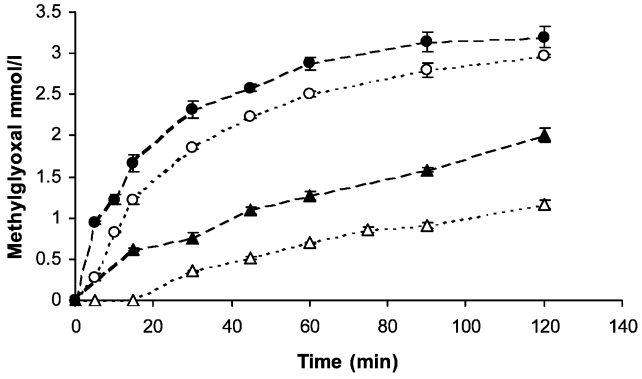


Fig. 4. Methylglyoxal formation during thermal degradation of DFG at 100 (△, pH 5.5; ▲, pH 6.8) and 120 °C (○, pH 5.5; ●, pH 6.8).

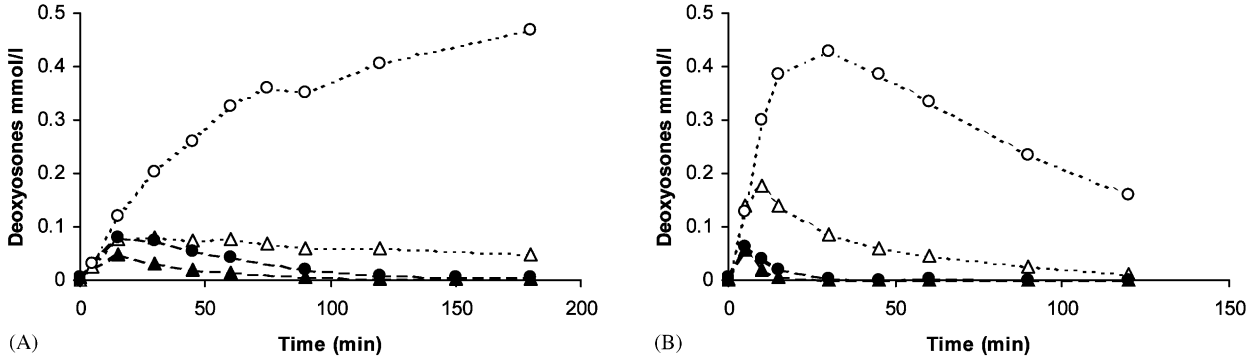


Fig. 3. Deoxyosones formation during thermal degradation of DFG at 100 (A) and 120 °C (B): 1-DG (△, pH 5.5; ▲, pH 6.8) and 3-DG (○, pH 5.5; ●, pH 6.8).

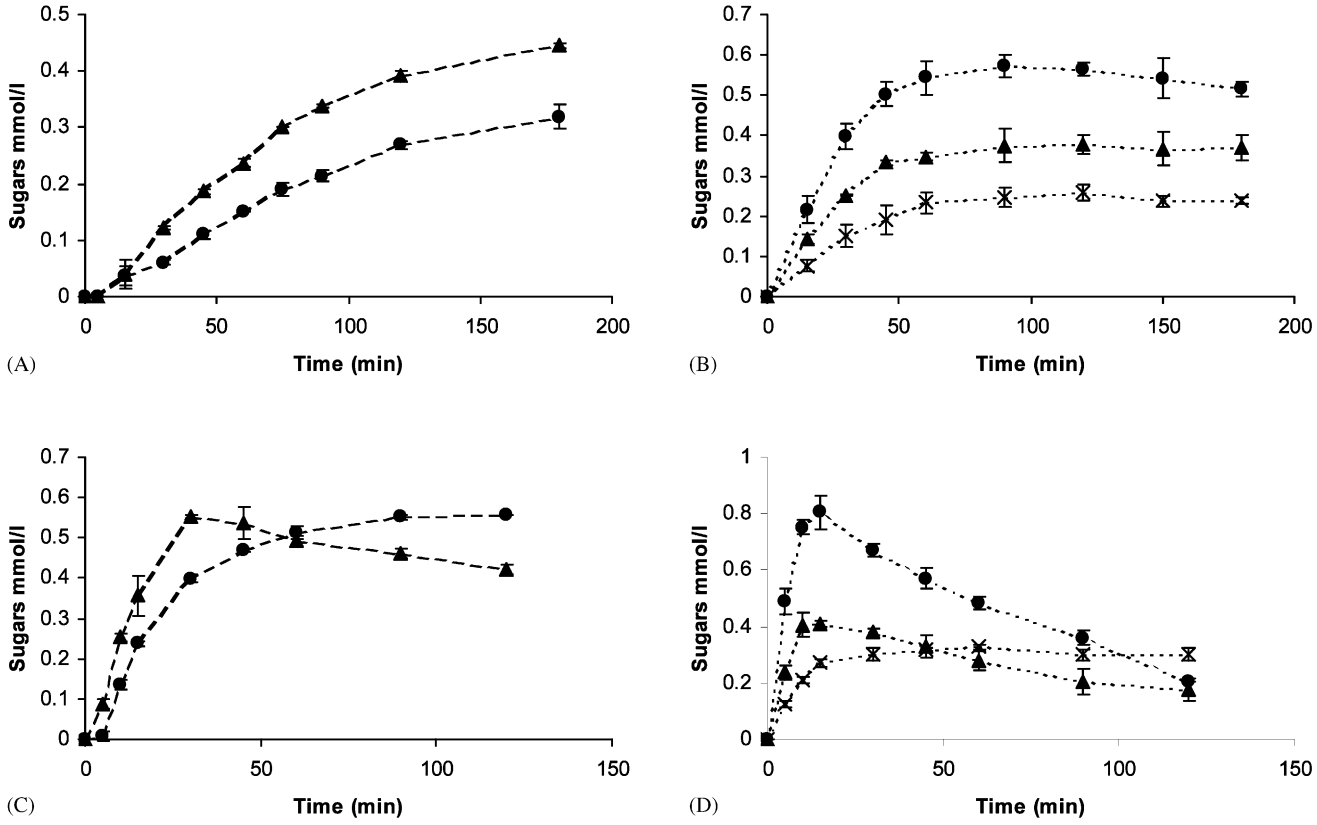


Fig. 5. Sugars formation during thermal degradation of DFG at pH 5.5 (---) and pH 6.8 (···). A and B heated at 100 °C; C and D heated at 120 °C. D-Glc (●); D-Man (▲); D-Fru (×).

amounts was always lower in concentration than the other two sugars. Also independently of the reaction conditions no decrease in the Fru amount was observed.

3.1.6. Organic acids formation. The formation of formic acid and acetic acid was particularly favoured with increasing pH (Fig. 6). At pH 5.5 the yield of formic and acetic acid was considerably lower relative to pH 6.8. Also an increase of temperature enhanced formation of both acids. The sum of the yields of both acids reached up to 0.8 mmol/mmol of DFG, which indicates that organic acids, in particular acetic acid, are important end products of the Maillard reaction. Independently of the reaction conditions, acetic acid was always formed in higher amounts than formic acid. The difference was more significant at pH 6.8.

In the present study the reaction pH was not kept constant. As the reaction proceeded a pH drop was observed at both studied initial pHs. In Fig. 7 the results are shown for initial pH 6.8. After 120 min at 120 °C the pH drop was more significant than at 100 °C after 180 min, 0.21 and 0.19, respectively. Due to the low buffer

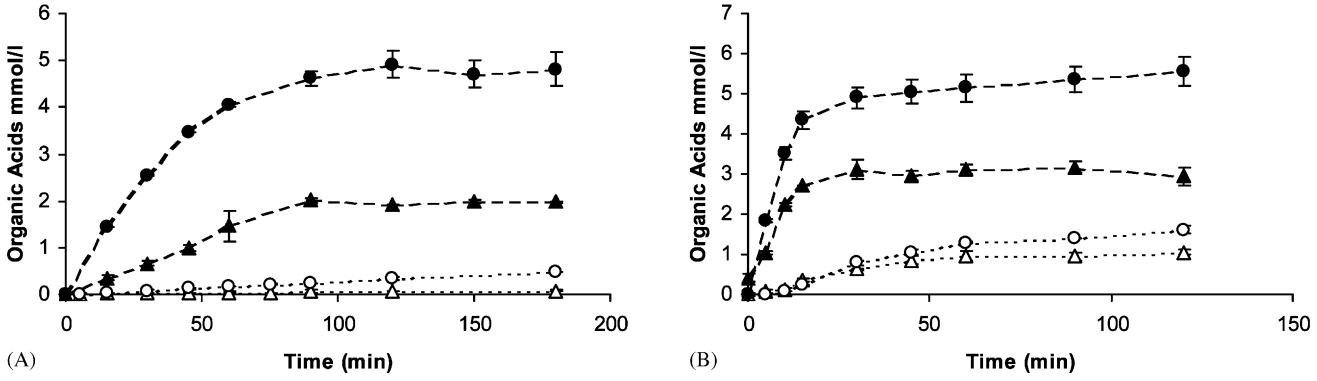


Fig. 6. Organic acids formation during thermal degradation of DFG at 100 (A) and 120 °C (B): acetic acid (○, pH 5.5; ●, pH6.8); Formic acid (△, pH 5.5;▲, pH 6.8).

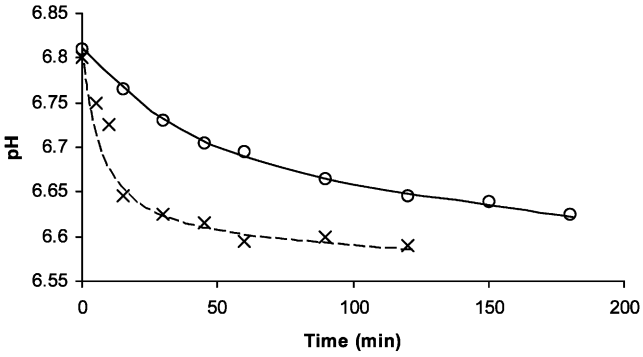


Fig. 7. pH drop during thermal degradation of DFG at initial reaction pH 6.8. (○, 100 °C; ×, 120 °C).

capacity at initial pH of 5.5 the pH drop for the same reaction conditions was 0.8 and 0.9, respectively, even though the sum of acids formed was lower. Davidek and co-workers[6] observed in their study that the total amounts of acetic and formic acid were generally lower than the amounts of NaOH added to keep the pH constant, suggesting that other acids might be formed. It would be interesting though to study the reaction mass balance to see how much is missing. One of the consequences of allowing the pH to fall is the inhibition of the reaction by the pH drop. However, the level-off observed on organic acids formation (Fig. 6B) matched the complete degradation of DFG, which implies direct involvement of DFG or its early degradation product in the formation of organic acids.

3.1.7. Melanoidins formation. The concentration of melanoidins (nitrogenous brown polymers) was calculated from the absorbance data through the extinction coefficient. As observed in Fig. 8, pH more than temperature had a strong influence in melanoidins formation from DFG. Moreover, the observed colour formation is much less than when one starts with Glc/glycine system,[22] even though the amount of DFG formed then is approximately the same as we used as reactant in the present study.

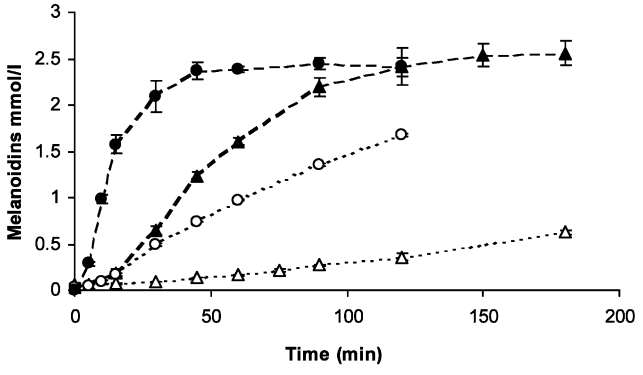


Fig. 8. Melanoidins formation during thermal degradation of DFG at 100 (△, pH 5.5; ▲, pH 6.8) and 120 °C (○, pH 5.5; ●, pH 6.8).

3.2. Identification of the reaction pathways based on reaction conditions

3.2.1. Degradation of DFG. Two possible degradation pathways of DFG are the enolization pathway and the retro-aldolization pathway. As mentioned before, through enolization the aminoenol intermediates can lead to either **3** or **5**. Although only small amounts may be present at a given time, large amounts of ARP may degrade via one or both of these pathways. The reaction conditions, especially pH, are believed to determine the degradation pathway taken.[23] At lower pH, 1,2-enolization is believed to be favoured and therefore a higher yield of 3-DG is expected, which is in agreement with the results obtained in the present study. On the other hand as the pH increases to neutral/alkaline values, 2,3-enolization is believed to be favoured and therefore 1-DG should be present in higher amounts. In literature the results are however contradictory. By heating 1-deoxy-1-propylamino-D-fructose in phosphate buffer for 10 h under reflux the 1-DG/3-DG ratio was 8:5 at pH 4.5 and 20:1 at pH 7.[24] For quantification of deoxyosones several researchers reacted Maillard mixtures in the presence of excess amounts of 1,2-diaminobenzene.[15,20,24] This in situ trapping technique leads to an accumulation of the quinoxalines, but does not offer insights into the relative changes in the concentration of the deoxyosones or any other dicarbonyl compound.[25] We, therefore, derivatized the deoxyosones produced with 1,2-diaminobenzene after rapid cooling of the thermally treated DFG. From the results presented in the previous section, independently of the pH, 3-DG was always present in higher concentration than 1-DG. Hofmann and co-workers[13,14] in a heated aqueous solution of Glc and l-alanine under reflux at pH 7 found 3-DG in four times higher amounts than 1-DG. In the present study at 100 °C and pH 6.8 a ratio of 3.2 was found.

To gain insight whether the amount of 1-DG is lower because its formation is not favoured or because it is more reactive than 3-DG, compounds formed by cyclization/condensation of the deoxyosones were also analysed. They include HHMF, 2,3-dihydro-3,5-dihydroxy-6-methyl-4(*H*)-pyran-4-one (DDMP) and 5-(hydroxymethyl)-2-furfural (HMF). Both the furanone (HHMF) and the dihydropyranone (DDMP) derive from 1-DG and are regarded as the 2,3-enolization indicator.[16] In the present study both HHMF and DDMP were identified at both pHs, which indicates that 2,3-enolization occurs not only at pH 6.8 but also at pH 5.5. Unfortunately, their quantification was not possible since no reference material was available. However, judging by the response factor of HMF they

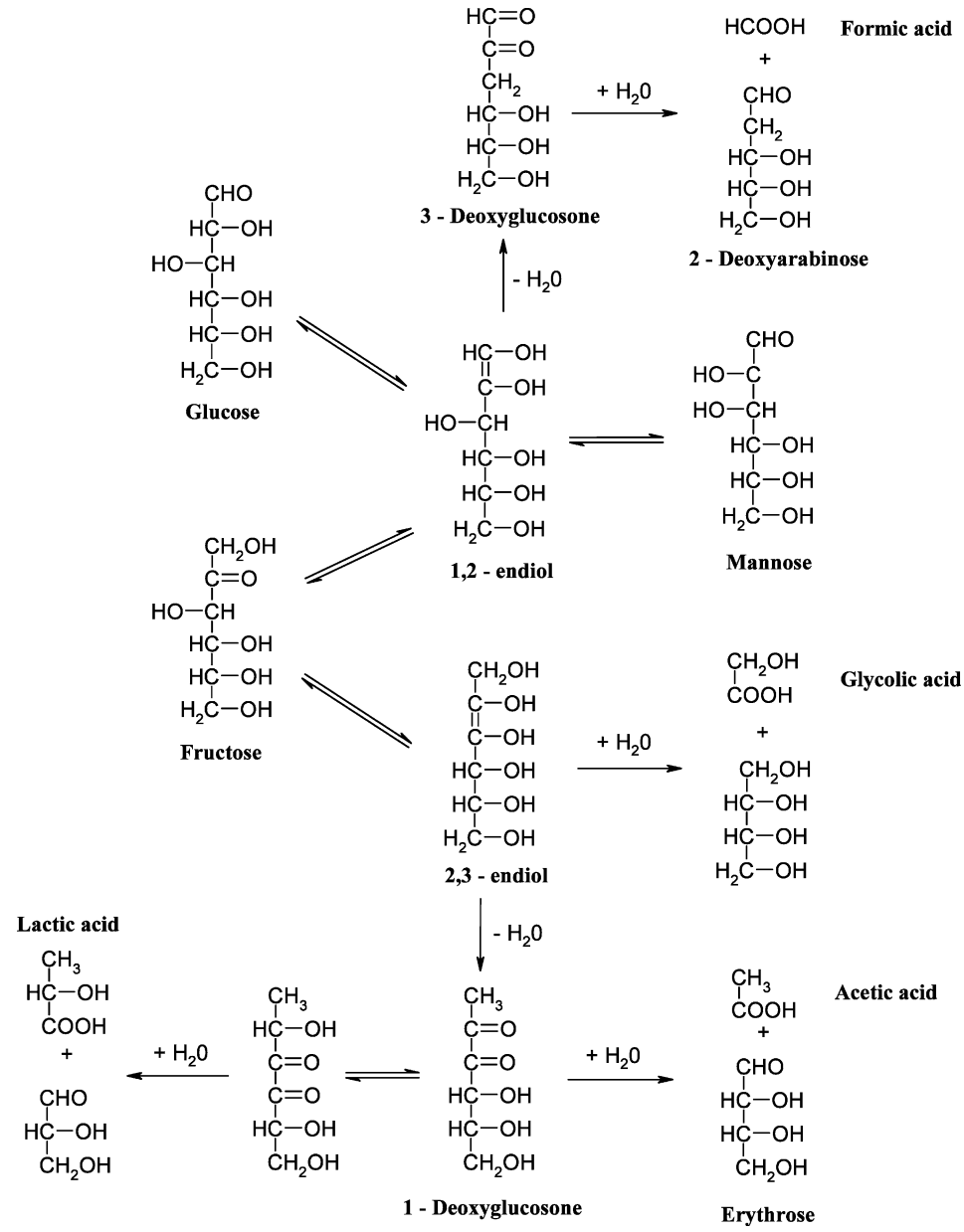
were formed in the order of magnitude of mmoles. On the other hand, HMF was not formed at pH 6.8 and only μmole amounts at pH 5.5. The relatively low reactivity of 3-deoxyglucosone as well as its relatively poor methylated pyrazine precursor has already been observed in previous studies.[26,27] Also HMF formation has only been reported in more acidic conditions, such as pH 3.5.[28] Besides enolization, intact Amadori products can also undergo retro-aldol reactions or acid/base-catalyzed thermal degradations without deoxyosone formation, to produce a variety of other reactive intermediates that retain the amino acid, such as 1-amino-1,4-dideoxy-2,3-dicarbonyl compounds.[29] These compounds can release the free amino acid later by β-elimination, which can justify the oscillation observed in the yield of glycine.

3.2.2. Formation of glycine. Under the studied conditions the formation of glycine from DFG can occur via both 1,2- and 2,3-enolization. In theory, 1 mol of DFG should yield 1 mol of glycine. The results obtained in the present study show that the yield of glycine increased in the beginning of the reaction and decreased as the reaction proceeded. However, the decrease in the yield of glycine was followed by an increase again. This indicates that glycine is first liberated and then reacts with other compounds present in the reaction mixture (e.g., α-dicarbonyls and hydroxycarbonyls). These reactions may include: (i) the formed α-dicarbonyls can further react with glycine through Strecker degradation to produce the corresponding amines, carboxylic acids and Strecker aldehydes; (ii) formation of pyrazinones and pyrazines;[30] (iii) chain elongation of reactive C-2 and C-3 α-dicarbonyls by one carbon unit originating from C-2 atom of glycine;[31] (iv) incorporation of glycine into melanoidins. However, because after a decrease an increase was observed, glycine is suggested to react with other compounds present in the reaction mixture and subsequently released again. These reactions may include not only the conversion of carbohydrate fragments by catalytic action of glycine, but also glycine can be formed directly from DFG with the formation of **6** that subsequently undergoes β-elimination to produce MG and release free glycine.[4,7]

3.2.3. Methylglyoxal formation. In a previous study[27] a mechanism for the MG formation was proposed through retro-aldolization of both 1- and 3-deoxyosones. This would justify the absence of pH influence observed in MG formation. Another possibility would be the direct cleavage of DFG into MG as presented in Scheme 1. This process of conversion of glyceraldehyde into MG by catalytic action of amino acid was also observed with Glc/glycine model systems.[32] Literature results thus give us some indication that DFG degrades also by retro-aldolization into MG with release of glycine. Moreover, MG is also believed to degrade further into formic and acetic acid.[4]

3.2.4. Parent sugars formation. The formation of Glc and Man together with the free amino acid, at early stages of the reaction could be considered as sufficient evidence for the reversibility of the rearrangement, as shown in pathway D in Scheme 1. However, this pathway does not explain the formation of Fru at the early stage of the reaction. The fact that no lag phase was observed made us exclude the isomerisation possibility from Glc and Man. According to the Lobry–de-Bruyn–Alberda–van-Eckenstein-rearrangement all three sugars, Glc, Man and Fru are in equilibrium with the same intermediate, the 1,2-endiol. However, Fru is also in equilibrium with the 2,3-endiol.[21] The formation of 1,2-endiol from the respective enaminol (**2**) in Scheme 1 is not so likely to happen, however, by release of the amino acid the enaminol **4** can originate its endiol, 2,3-endiol (**12**), which through enolization can lead to sugars formation,[19] in particular Fru (**13**). This indicates that Fru can be formed from DFG by its 2,3-enolization step, whereas Man and Glc can only be formed via the 1,2-enaminol, through the schiff base (**9** and **9′**). Moreover, it could also be argued that the sugars might arise by aldol-type condensations between smaller sugar fragments generated from the decomposition of DFG. This hypothesis has, however been excluded by labelling studies.[33] To get a better insight in the sugars enolization pathways two additional experiments were performed. Glc and Fru were heated alone in an aqueous phosphate buffer (0.1 mol/L) solution at 120 °C and pH 6.8. It was observed (results not shown) that Glc isomerised preferably into Fru, whereas Man was only formed in small amounts. On the other hand, the amount of Glc and Man formed from Fru was very low relatively to its decrease. Fru degraded preferably into acids. It is suggested that not only 2,3-enolization is favoured under these conditions with Fru preferably formed from Glc, but also that the reversibility of 2,3-endiol into 1,2-endiol occurs in a quite low extent. These findings are in agreement with what we have observed before, namely that 2,3-enolization gains importance in the reaction mechanism as pH and temperature increased.

3.2.5. Organic acids formation. Labelling experiments[34] suggest that Glc, Man and Fru together with 1-DG and 3-DG can be regarded as aliphatic acid precursors (Scheme 3). From the experiments where Glc and Fru were heated alone we observed that the main acid formed in the beginning of the reaction was formic acid. Acetic acid formation showed a lag phase, especially in the Glc system. These results were compared with the ones obtained from heating Glc and Fru, separately with glycine. When the amino acid was



Scheme 3. Reaction scheme for organic acids formation from primary thermal degradation products of DFG (adapted from Ginz and co-workers[34]).

present, the formic acid amount decreased considerably and acetic acid became the dominant acid (results not shown). It is worth noting that no glycolic acid or lactic acid were detected. These results suggest that formic and acetic acid can also be formed by sugars enolization. However, in a study[35] where Glc was heated alone at 100 °C (pH 10), 3-DG was estimated to be only 14% of the total α-dicarbonyls detected. In the present study we have therefore considered 1-DG and 3-DG to be mainly formed by DFG degradation. Through dicarbonyl cleavage the deoxyosones become the main precursors of organic acids formation. The fact that no MG degradation was observed made us assume that a C-1 to C-2 cleavage of MG would be a secondary route in organic acids formation.

3.3. Differentiate between primary and secondary reaction routes

Based on the discussion above we suggest the following primary and secondary routes.

3.3.1. Primary routes.

- DFG degradation through enolization occurs through two intermediates, designed as E_1 and E_2,

which can be the Schiff's base, the cation form of the Schiff's base, the 1,2-enaminol or the 2,3-enaminol. These intermediates have not been isolated yet from the Maillard reaction due to their reactivity, however, according to previous studies[36,37] ARPs undergo 1,2- or 2,3-enolizations while the amino acid moiety is still attached. E_1 is favoured at lower pH whereas E_2 is favoured at higher pH.

- The intermediates E_1 and E_2 by release of the amino acid lead to the formation of 3-DG and 1-DG, respectively.
- Glc, Man and 3-DG are formed through the same intermediate (E_1) whereas Fru is formed by E_2. Moreover, Man and Glc can isomerise into each other as well as degrade into C_n ($n \leq 6$) carbonyl compounds.
- DFG degradation through retro-aldol cleavage leads to MG formation, with amino acid release.
- DFG, 3-DG and 1-DG due to their reactive functional group can easily degrade to produce reactive C_n carbonyl compounds.
- Formic acid and acetic acid are formed from DFG degradation pathways through E_1 by 3-DG dicarbonyl cleavage and through E_2 by 1-DG dicarbonyl cleavage, respectively.
- Melanoidins formation results from the interaction of C_n carbonyl compounds with glycine.

3.3.2. Secondary routes.

- Products formed by cyclization/condensation of the deoxyosones that include HMF, HHMF and DDMP.
- Incorporation of glycine in pyrazinones and pyrazines, as well as in chain elongation of α-dicarbonyls. Also degradation of glycine through Strecker degradation to produce the corresponding amines, carboxylic acids and Strecker aldedehydes, which can be included in the melanoidins formation.
- MG formation from deoxyosones retro-aldol reaction and its degradation into carboxylic acids.
- Isomerisation of Glc and Man into Fru, as well as direct degradation of Glc into 3-DG.
- Fru formation from E_1 as well as its degradation into reactive C_n carbonyl compounds.
- Carboxylic acids formation from sugars enolization or scission products from C_n carbonyl compounds.

4. Conclusions

The main degradation pathways for DFG have been established. pH, more than temperature, has an influence on the reaction products formed. An increase in pH seems to favour the formation of 1-deoxyosone. The lower amount found comparatively to 3-deoxyosone, in all studied systems, seems to be related with the higher reactivity of 1-deoxyosone. Independently of the pathway taken, enolization or retro-aldolization, DFG degradation is accompanied by amino acid release. Together with glycine, acetic acid was the main end product formed. At this stage we are able to propose a kinetic model based on the established reaction network. In Part II[11] the following steps for a complete multiresponse kinetic analysis will be taken.

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