

Identification and Determination of α -Dicarbonyl Compounds Formed in the Degradation of Sugars

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The α -dicarbonyl compounds formed in the degradation of glucose and fructose were analyzed by HPLC using 2,3-diaminonaphthalene as derivatizing reagent, and identified as glucosone (GLUCO), 3-deoxyglucosone (3DG), 3-deoxyxylosone (3DX), tetrosone (TSO), triosone (TRIO), 3-deoxytetrosone (3DT), glyoxal (GO), and methylglyoxal (MGO). The results suggest that α -dicarbonyl compounds were formed from glucose *via* non-oxidative 3-deoxyglucosone formation and oxidative glucosone formation in glucose degradation. In addition, TRIO, GO, and MGO were also formed from glyceraldehyde as intermediate. The α -dicarbonyl compounds might be formed from glucose *via* these pathways in diabetes.

Key words: α -dicarbonyl compounds; 2,3-diaminonaphthalene; Maillard reaction; glycation; glucose degradation products

α -Dicarbonyl compounds such as 3-deoxyglucosone (3DG) are reactive intermediates formed in the Maillard reaction, degradation of sugars, metabolic diseases, and so on.^{1–3} The carbonyls increase in diabetes,^{4,5} and they are a potential precursor of advanced glycation products (AGE) as a post-translational modification of protein.⁶ The carbonyls and AGE are thought to be involved in the progression of chronic diseases such as diabetic microangiopathy.

Various techniques have been reported for the analysis of α -dicarbonyl compounds, and the pre-labeled HPLC method is the most common. Yamada *et al.* reported that 3-deoxyglucosone (3DG) was detected and

identified as the 2,3-diaminonaphthalene (DAN) adduct in diabetic and uremic plasma.⁵ 3-Deoxyglucosone (3DG), glyoxal (GO), and methylglyoxal (MGO) have been identified as benzo[g]quinoxaline derivatives using DAN reagent, but other carbonyls have not been identified. In this study, we identified eight α -dicarbonyl compounds (including 3DG, MGO, and GO) as benzo[g]quinoxaline derivatives, formed in the degradation of glucose and fructose, and quantitatively determined each carbonyl formed from glucose in the physiological pH condition.

Materials and Methods

Chemicals. 2,3-Diaminonaphthalene (DAN) was obtained from Tokyo Chemical Industry (Tokyo). Cyclohexane-1,2-dione was from Nacalai Tesque (Kyoto, Japan). D-Glucose, lysozyme, glyoxal, methylglyoxal, α -cyano-4-hydroxy-cinnamic acid (CHCA), and angiotensin I were from Sigma-Aldrich (St. Louis, MO). D-Xylose, benzaldehyde, acetic acid, CD₃OD, and acetonitrile (HPLC grade) were from Kanto Chemical (Tokyo). *p*-Toluidine and 2,4-dinitrophenylhydrazine (DNP) were from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used in this study were of analytical reagent grade.

Synthesis of glucosone and 3-deoxyglucosone. D-Glucosone (GLUCO) and 3-deoxyglucosone (3DG) were synthesized by the method previously described.^{7–9}

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Abbreviations: AGE, advanced glycation end products; ARA, arabinose; CHCA, α -cyano-4-hydroxy-cinnamic acid; 1,2CHD, cyclohexane-1,2-dione; DA, dihydroxyacetone; DAN, 2,3-diaminonaphthalene; DTPA, diethylenetriaminepentaacetic acid; ERY, erythrose; FFL, furfural; GDPs, glucose degradation products; 3,4DGE, 3,4-dideoxyosone-3-ene; 3,4DXE, 3,4-dideoxyxylosone-3-ene; 3DG, 3-deoxyglucosone; 3DX, 3-deoxyxylosone; GA, glycolaldehyde; GLA, glyceraldehyde; GLUCO, glucosone; HMF, 5-hydroxymethylfurfural; TR, triosereductone; TSO, tetrosone; TRIO, triosone; 3DT, 3-deoxytetrosone; GO, glyoxal; MGO, methylglyoxal

Synthesis of 3-deoxyxylosone. 3-deoxyxylosone (3DX) was synthesized a method similar to that for 3DG. D-xylose (7.6 g) and *p*-toluidine (4.4 g) were refluxed in a mixture of 95% ethanol (180 ml) and acetic acid (8.8 ml) at 110 °C. After 10 min, benzoylhydrazine (13.2 g) was added to the solution, which was refluxed for 2 h. The mixture was cooled at room temperature and then filtered. The residue was washed with methanol (3 × 50 ml), diethylether (3 × 50 ml), and ethanol (3 × 50 ml). It was obtained as 3-deoxyxylosone-bis-benzoylhydrazone (3-DX-bis).

3-DX-bis (6.5 g) and distilled benzaldehyde (10.2 ml) were refluxed in a mixture of 95% ethanol (200 ml) and acetic acid (8.8 ml) at 110 °C for 2 h. The mixture was cooled to room temperature, and water (50 ml) was added to the solution. The solution was evaporated to about 40 ml. The concentrate was cooled to 4 °C overnight. The solution was filtered, and the filtrate was washed with diethylether (10 × 50 ml). The aqueous layer was put on an Amberlite IR-120(H⁺) (Rohm and Haas, Philadelphia, PA) column, and fractionated. The 3DX-containing fractions were detected with DNP reagent (0.4% 2,4-dinitrophenylhydrazine in 2 N HCl). Then they were lyophilized as crude-3DX.

Crude-3DX (0.4 g) was dissolved in a small amount of the solvent as ethylacetate-methanol-water (6:1:1). The solution was put on a silica gel 60 (Merck KGaA, Darmstadt, Germany) column equilibrated with the same solvent. The 3DX-containing fractions were concentrated under reduced pressure. The residue was dissolved in water, and the solution was lyophilized as 3DX.

Sample preparation. Glucose (200 mM) or fructose (200 mM) was dissolved in 200 mM sodium phosphate buffered solution (pH 7.4), and then incubated with or without diethylenetriaminepentaacetic acid (DTPA, 5.5 mM) at 37 °C or 50 °C for one week. Glucosone, 3-deoxyglucosone, 3-deoxyxylosone and glyceraldehyde were incubated in the same manner.

HPLC analyses of α -dicarbonyls. α -Dicarbonyl compounds were analyzed as 2,3-diaminonaphthalene (DAN)-adduct on reversed phase HPLC. The method, originally reported by Yamada *et al.*⁵⁾ was modified. A sample was added to an equal volume of DTPA (5.5 mM) containing 1,2-cyclohexanedione (1 mM) as the internal standard. The mixture was combined with an equal volume of 2 mM DAN (dissolved in hot water at 80 °C, and then cooled to room temperature), and incubated at 50 °C for 1 h. The reaction mixture was filtered with a hydrophilic LCR (PTFE) membrane filter unit (Millex-LH, Millipore, Billerica, MA), and then put on a Puresil C₁₈ column (150 × 4.6 mm I.D., Waters, Milford, MA). The column temperature was controlled at 40 °C. The sample was eluted with a linear gradient of 14.5–31.0% acetonitrile from 0 to 70 min at a flow rate of 1 ml/min, and absorbance was monitored at 268 nm.

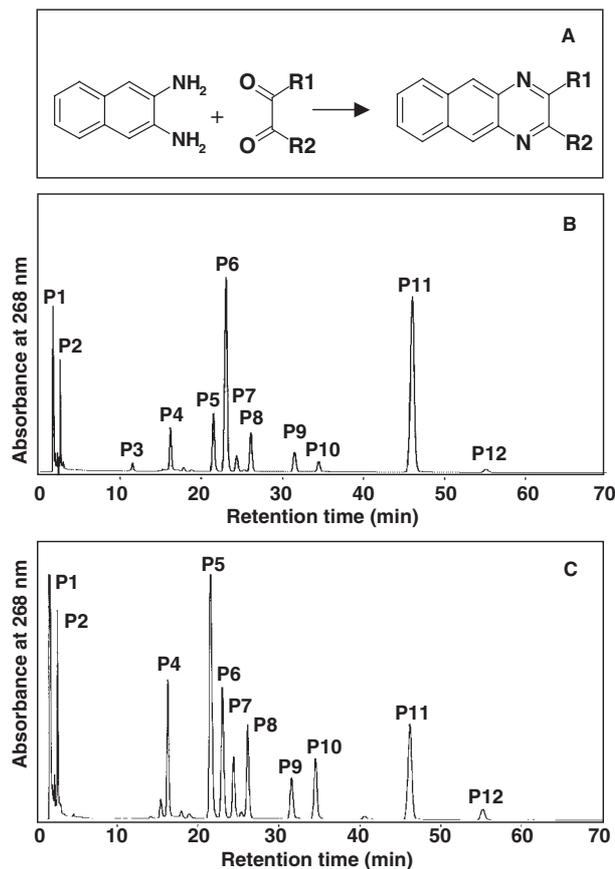


Fig. 1. Detection of α -Dicarbonyl Compounds Formed in the Degradation of Glucose and Fructose.

Glucose and fructose solutions were incubated at 50 °C for 1 week. The α -dicarbonyl compounds were detected as benzo[g]quinoxaline derivatives using 2,3-diaminonaphthalene as derivatizing reagent. The reaction scheme is shown in A. Typical HPLC profiles of α -dicarbonyl compounds are shown in B (glucose) and C (fructose). Major peaks are named P1–P12.

Identification of α -dicarbonyls. Benzo[g]quinoxaline derivatives as the carbonyl-DAN adduct were isolated by preparative reversed phase HPLC. The sample was put on a μ Bondasphere C₁₈ column (150 × 19 mm I.D., Waters), and eluted with an isocratic of 20% acetonitrile at a flow rate of 7 ml/min. Absorbance was monitored at 268 nm, and eight benzo[g]quinoxaline derivatives were collected. After lyophilization, they were identified by MS and NMR.

Apparatus. HPLC Systems (Shimadzu, Kyoto, Japan) consisted of HPLC Pump LC-10AS, UV-VIS Spectrophotometric Detector SPD-10AV, Column Oven CTO-10A, and a C-R6A Chromatopac. FAB-MS was recorded with tandem mass spectrometer SX102 (JEOL, Tokyo) with glycerol as the matrix and polyethylene-glycol 400 as the mass standard. MALDI-TOF-MS was recorded with a Voyager DE Pro (Applied Biosystems, Foster City, CA) with CHCA as the matrix and angiotensin I as the mass standard. NMR were recorded by the ECP-500 system (500 MHz, JEOL) in CD₃OD.

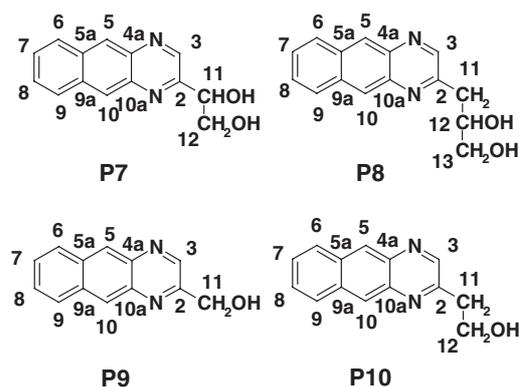


Fig. 2. Chemical Structure of P7, P8, P9, and P10.

P7, P8, P9, and P10 were identified as 2-(1,2-dihydroxyethyl)-benzo[g]quinoxaline formed from tetosone (TSO), 2-(2,3-dihydroxypropyl)-benzo[g]quinoxaline from 3-deoxyxylosone (3DX), 2-hydroxymethyl-benzo[g]quinoxaline from triosone (TRIO), and 2-(2-hydroxyethyl)-benzo[g]quinoxaline from 3-deoxytetosone (3DT) respectively.

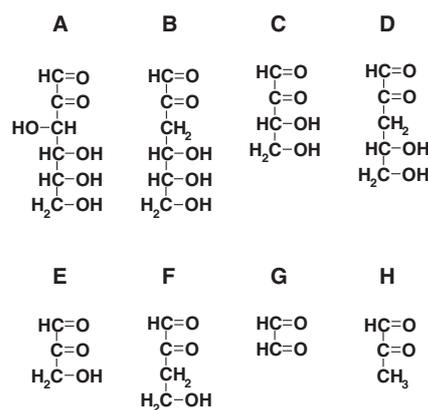


Fig. 3. Chemical Structures of α -Dicarbonyl Compounds Formed in the Degradation of Glucose and Fructose.

A, glucosone; B, 3-deoxyglucosone; C, tetosone; D, 3-deoxyxylosone; E, triosone; F, 3-deoxytetosone; G, glyoxal; H, methylglyoxal.

Table 1. NMR Chemical Shift (ppm) of Benzo[g]-Quinoxaline Derivatives Formed from Carbonyls

P7			P8			P9			P10		
No. ^a	¹³ C- δ	¹ H- δ	No. ^a	¹³ C- δ	¹ H- δ	No. ^a	¹³ C- δ	¹ H- δ	No. ^a	¹³ C- δ	¹ H- δ
12	65.55	3.97–4.02 (m)	11	40.15	3.13–3.18 (m)	11	64.62	4.89 (s)	11	39.92	3.18–3.20 (t)
11	74.27	5.02–5.04 (m)	13	65.82	3.66 (d)	—	—	—	12	61.19	4.01–4.03 (t)
—	—	—	12	71.52	4.21–4.23 (m)	—	—	—	—	—	—
5	126.88	8.68 (s)	5	126.4	8.60 (s)	5	127.5	8.53 (s)	5	127.2	8.53 (s)
7	127.01	7.62–7.64 (m)	7	126.7	7.60–7.62 (m)	7	127.7	7.52–7.54 (m)	7	127.5	7.52–7.52 (m)
10	126.88	8.68 (s)	10	126.9	8.64 (s)	10	127.8	8.57 (s)	10	127.70	7.52–7.53 (s)
8	127.01	7.62–7.64 (m)	8	127	7.60–7.62 (m)	8	127.9	7.52–7.54 (m)	8	127.8	7.52–7.54 (m)
6	128.10	8.17 (m)	6	128.1	8.14–8.16 (m)	6	128.9	8.06 (m)	6	128.9	8.06 (m)
9	128.17	8.17 (m)	9	128.1	8.14–8.16 (m)	9	129	8.06 (m)	9	129	8.06 (m)
5a	133.87	—	5a	133.59	—	5a	134.6	—	5a	134.4	—
9a	134.20	—	9a	134.12	—	9a	135	—	9a	135	—
4a	137.79	—	4a	137.46	—	4a	138.5	—	4a	138.3	—
10a	138.04	—	10a	138.24	—	10a	138.7	—	10a	139.1	—
3	145.37	9.11 (s)	3	147.82	8.88 (s)	3	146.2	8.94 (s)	3	148.3	8.79 (s)
2	157.53	—	2	156.34	—	2	157.6	—	2	157.3	—

^aNumbers represent assigned positions as shown in Fig. 2.

Statistical analysis. The concentrations of glucosone (GLUCO), 3-deoxyglucosone (3DG), 3-deoxyxylosone (3DX), glyoxal (GO), and methylglyoxal (MGO) were calculated by regression analysis using standards. The tetosone (TSO), triosone (TRIO), and 3-deoxytetosone (3DT) concentrations were calculated by regression analysis using 3-deoxyglucosone as standard.

Results and Discussion

Detection of α -dicarbonyls

Benzo[g]quinoxaline derivatives of α -dicarbonyl compounds were detected by HPLC analysis. As shown in Fig. 1A, benzo[g]quinoxaline derivatives were formed from α -dicarbonyl compounds and 2,3-diaminonaphthalene (DAN) derivatizing reagent. In this study, metal chelator DTPA was used in the derivatization procedure. Since the trace metal (included in phosphate salt)

promotes oxidation, DTPA suppresses oxidation.

Figure 1B and C show the α -dicarbonyl formed by the incubation of glucose and fructose. Twelve major compounds were generated from glucose (Fig. 1B) and fructose (Fig. 1C) in the physiological pH condition, and named P1–P12. The UV spectrum of benzo[g]quinoxaline showed a maximum peak at 268 nm. The UV spectra of P3, P5, P7, P8, P9, P10, P11, and P12 proved to be benzo[g]-quinoxaline derivatives (the UV spectra are not shown), and P6 was identified as DAN.

Identification of P3, P5, P11, and P12

P3, P5, P11, and P12 were compared with synthesized carbonyls (as standard) on HPLC and FAB-MS analysis. The FAB-MS measured for P3, P5, P11, and P12 showed the $[M + H]^+$ ion at m/z 301, 285, 181, and 195 respectively. The carbonyls were identified as 2-(1,2,3,4-tetrahydroxybutyl)-benzo[g]quinoxaline (P3)

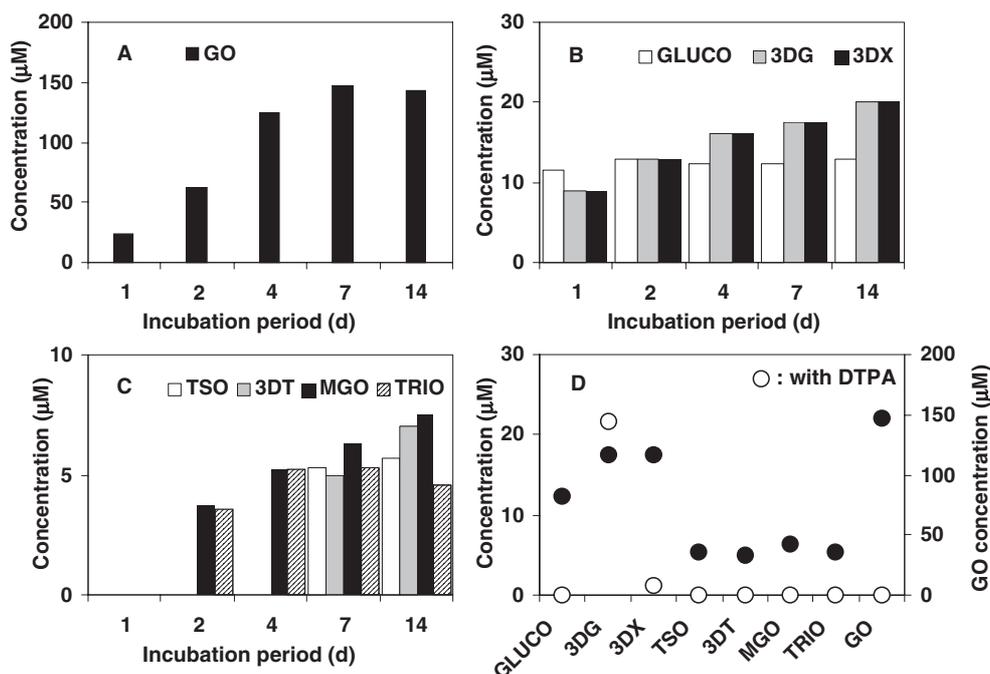


Fig. 4. Determination of α -Dicarbonyl Compounds Generated in the Degradation of Glucose.

A, GO concentration; B, GLUCO, 3DG, and 3DX concentrations; C, TSO, 3DT, MGO, and TRIO concentrations; D, concentrations of carbonyls formed with DTPA (open circle) and without DTPA (closed circle).

formed from glucosone (GLUCO), 2-(2,3,4-trihydroxybutyl)-benzo[g]quinoxaline (P5) from 3-deoxyglucosone (3DG), benzo[g]quinoxaline (P11) from glyoxal (GO), and 2-methyl-benzo[g]quinoxaline (P12) from methylglyoxal (MGO).

Identification of P7

P7 was analyzed by FAB-MS and NMR (including ^1H - ^1H COSY and ^{13}C DEPT spectra). The carbonyl compound was identified as 2-(1,2-dihydroxyethyl)-benzo[g]quinoxaline, formed from tetosone (TSO). The chemical structure of P7 is shown in Fig. 2. The spectral data were as follows: FAB-MS (m/z): 241 ($[\text{M} + \text{H}]^+$). ^1H -NMR (500 MHz, CD_3OD) δ and ^{13}C -NMR (CD_3OD) δ were shown in Table 1.

Identification of P8

P8 was identified as 2-(2,3-dihydropropyl)-benzo[g]quinoxaline, formed from 3-deoxyxylosone (3DX). Figure 2 shows the chemical structure of P8. The FAB-MS for P8 showed an $[\text{M} + \text{H}]^+$ ion at m/z 255. The ^1H -NMR (500 MHz, CD_3OD) δ and ^{13}C -NMR (CD_3OD) δ are shown in Table 1. These assignments were verified by ^1H - ^1H COSY and ^{13}C DEPT spectra. Furthermore, the carbonyl-DAN adduct from synthesized 3DX showed the same data on HPLC analysis.

Identification of P9

P9 was identified as 2-hydroxymethyl-benzo[g]quinoxaline, formed from triosone (TRIO). The chemical structure of P9 is shown in Fig. 2. MALDI-TOF-MS for

P9 showed an $[\text{M} + \text{H}]^+$ ion at m/z 211. The ^1H -NMR (500 MHz, CD_3OD) δ and ^{13}C -NMR (CD_3OD) δ are shown in Table 1. These assignments were also verified by ^1H -detected ^1H - ^{13}C COSY, ^1H - ^1H COSY, and ^{13}C DEPT spectra.

Identification of P10

P10 was identified as 2-(2-hydroxyethyl)-benzo[g]quinoxaline, formed from 3-deoxytetosone (3DT), by NMR (including ^1H - ^1H COSY, ^1H - ^{13}C -COSY, and the ^{13}C -DEPT method). The chemical structure of P10 was shown in Fig. 2. MALDI-TOF-MS for P10 showed an $[\text{M} + \text{H}]^+$ ion at m/z 225. ^1H -NMR (500 MHz, CD_3OD) δ and ^{13}C -NMR (CD_3OD) δ are shown in Table 1.

Determination of α -dicarbonyls

In this study, eight carbonyls (glucosone, GLUCO; 3-deoxyglucosone, 3DG; 3-deoxyxylosone, 3DX; tetosone, TSO; triosone, TRIO; 3-deoxytetosone, 3DT; glyoxal, GO; methylglyoxal, MGO) were identified as glucose and fructose degradation products. The chemical structures of the carbonyls are shown in Fig. 3. Subsequently, eight carbonyls formed with or without DTPA in the glucose degradation in the physiological pH condition were determined to be benzo[g]quinoxaline derivatives. Since the trace metal promotes oxidation, the presence of the metal chelator DTPA indicates a non-oxidative condition and absence of DTPA indicates an oxidative condition.

As shown in Fig. 4A and B, GLUCO, 3DG, 3DX, and especially GO were generated at an early stage of

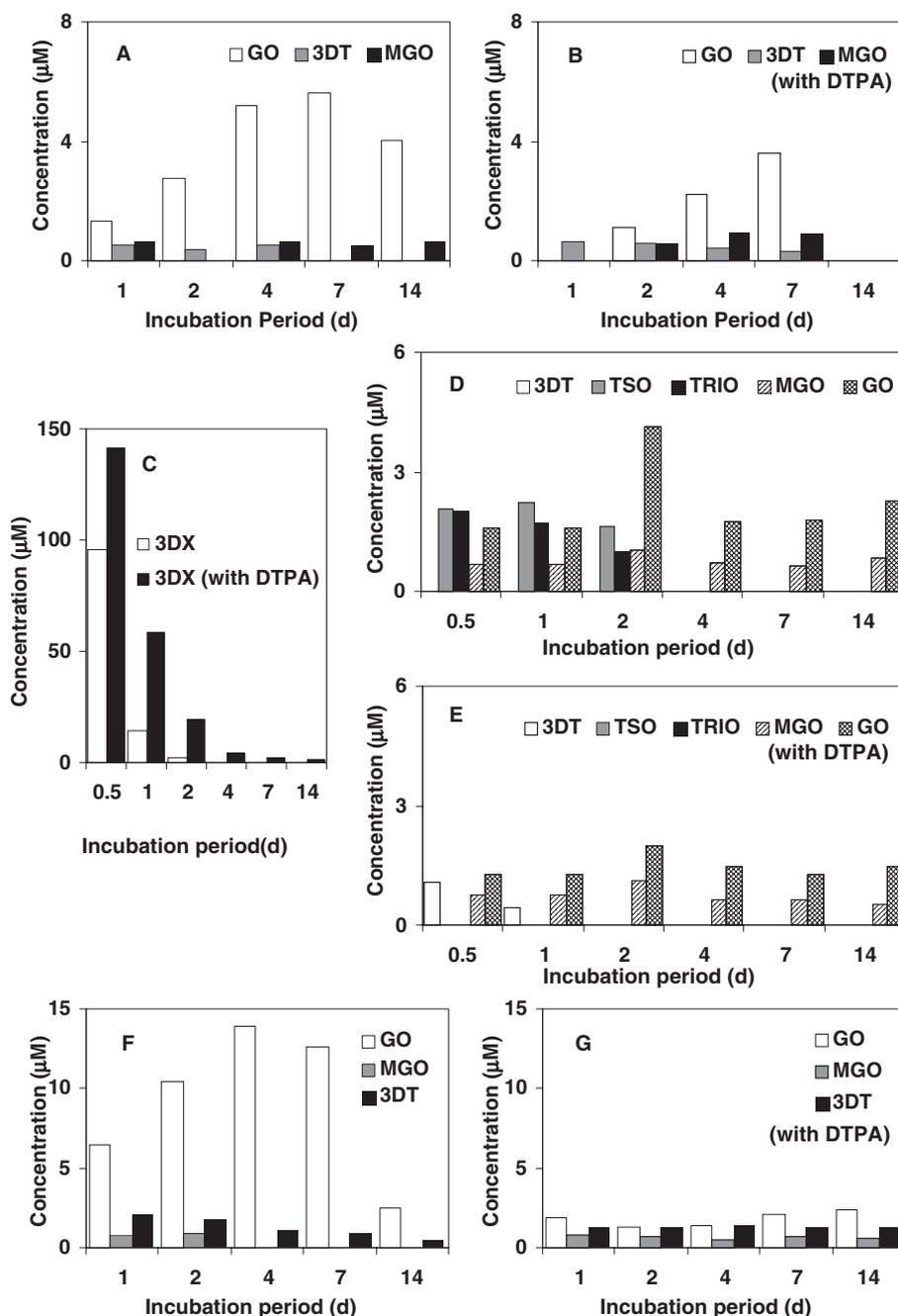


Fig. 5. Determination of α -Dicarbonyl Compounds Formed from 3-Deoxyglucosone, Glucosone, and 3-Deoxyxylosone.

Each carbonyl was formed from 3DG without DTPA (A) and with DTPA (B). 3DX was formed from GLUCO with or without DTPA, and carbonyls were formed from GLUCO without DTPA (D) and with DTPA (E). Carbonyls were formed from 3DX without DTPA (F) and with DTPA (G).

glucose degradation. Subsequently, MGO and TRIO were formed in the reaction, and then TSO and 3DT were generated in the reaction mixture (Fig. 4C). MGO, TRIO, TSO, and 3DT might have been formed from the intermediates. The metal chelator DTPA inhibited GLUCO, 3DX, TSO, TRIO, 3DT, GO, and MGO formation, but did not inhibit the 3DG formation (Fig. 4D). 3DG might be formed under non-oxidative conditions.

Moreover, α -dicarbonyls were analyzed in 3DG,

GLUCO, and 3DX degradation. 3DT, MGO, and especially GO were formed from 3DG (Fig. 5A). The formation of GO was suppressed by DTPA, but DTPA hardly affected the generation of 3DT and MGO (Fig. 5B).

3DX, MGO, GO, TRIO, and TSO were formed from GLUCO (Fig. 5C and D). 3DX especially was generated in the GLUCO degradation. Since 3DX formation increased with addition of DTPA (Fig. 5C), 3DX might have been formed in the non-oxidative condition. In

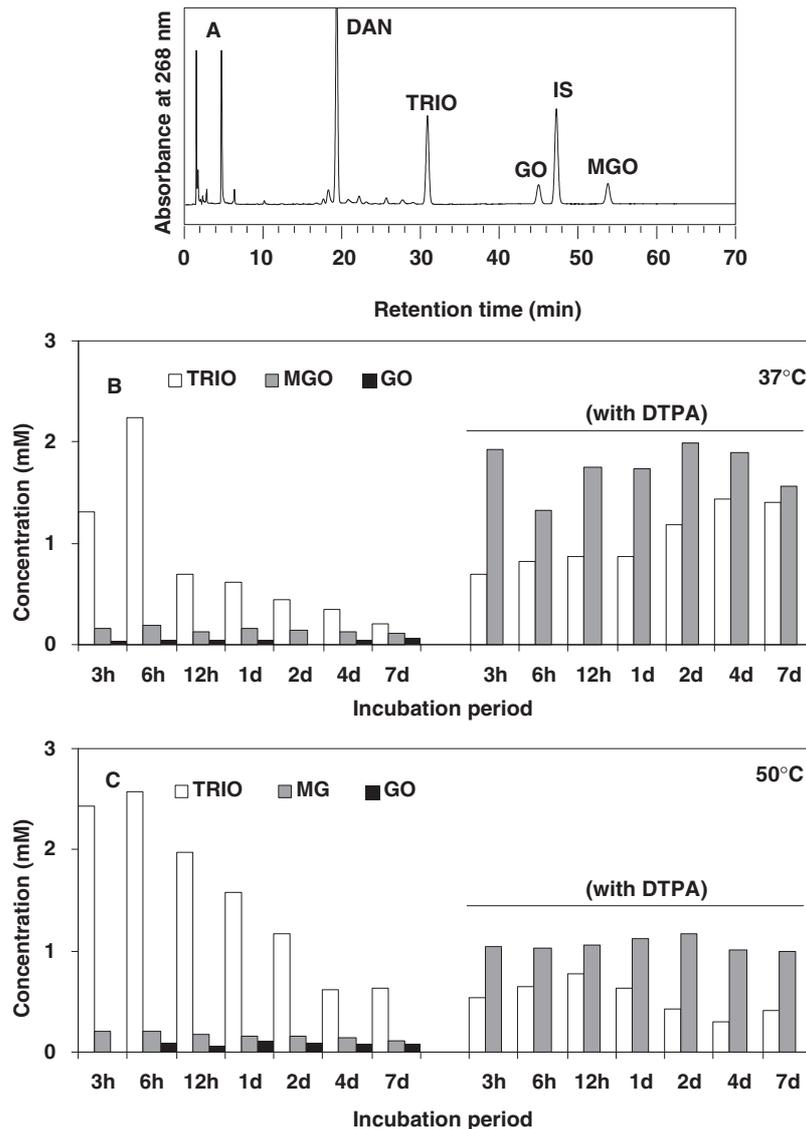


Fig. 6. Determination of α -Dicarbonyl Compounds Formed from Glyceraldehyde.

A, typical HPLC profile (IS, internal standard); B, carbonyls from GLA with and without DTPA at 37°C; C, carbonyls from GLA with and without DTPA at 50°C.

addition, 3DT was newly formed with the addition of DTPA.

3DT, MGO, and especially GO were formed from 3DX (Fig. 5F), and the formation of GO was suppressed by DTPA (Fig. 5G). In addition, the time-dependent loss of MGO was repressed by DTPA. DTPA might suppress the oxidation of MGO.

Figure 6 shows the concentration of α -dicarbonyls generated from GLA. A typical chromatogram is shown in Fig. 6A. TRIO, MGO, and GO were detected as GLA-derived α -dicarbonyls. As shown in Fig. 6B, the TRIO level increased rapidly in GLA degradation, and then TRIO gradually decreased. The formation of TRIO was affected by DTPA. TRIO might have been formed from triose reductone (TR), which is formed by the oxidation of glyceraldehyde. The TRIO level (without DTPA) at 50°C was higher than at 37°C, but the TRIO

level (with DTPA) at 37°C was higher than at 50°C (Fig. 6C). TRIO might be converted to glycolaldehyde (GA). GO and MGO were also formed from GLA. The formation of GO was repressed by DTPA. Perhaps GO is formed by oxidation. MGO was generated from GLA and from TR by dehydration. MGO was increased by the metal chlator, because MGO was transformed to the oxidant of MGO by oxidation.

Formation of α -dicarbonyls

Recent study suggests that α -dicarbonyl compounds induce a progression of diabetic complications. In this study, we investigated α -dicarbonyl formation in glucose degradation. The proposed pathway of α -dicarbonyl formation formed in glucose degradation is summarized in Fig. 7.

3DG is generated from glucose by dehydration. It has

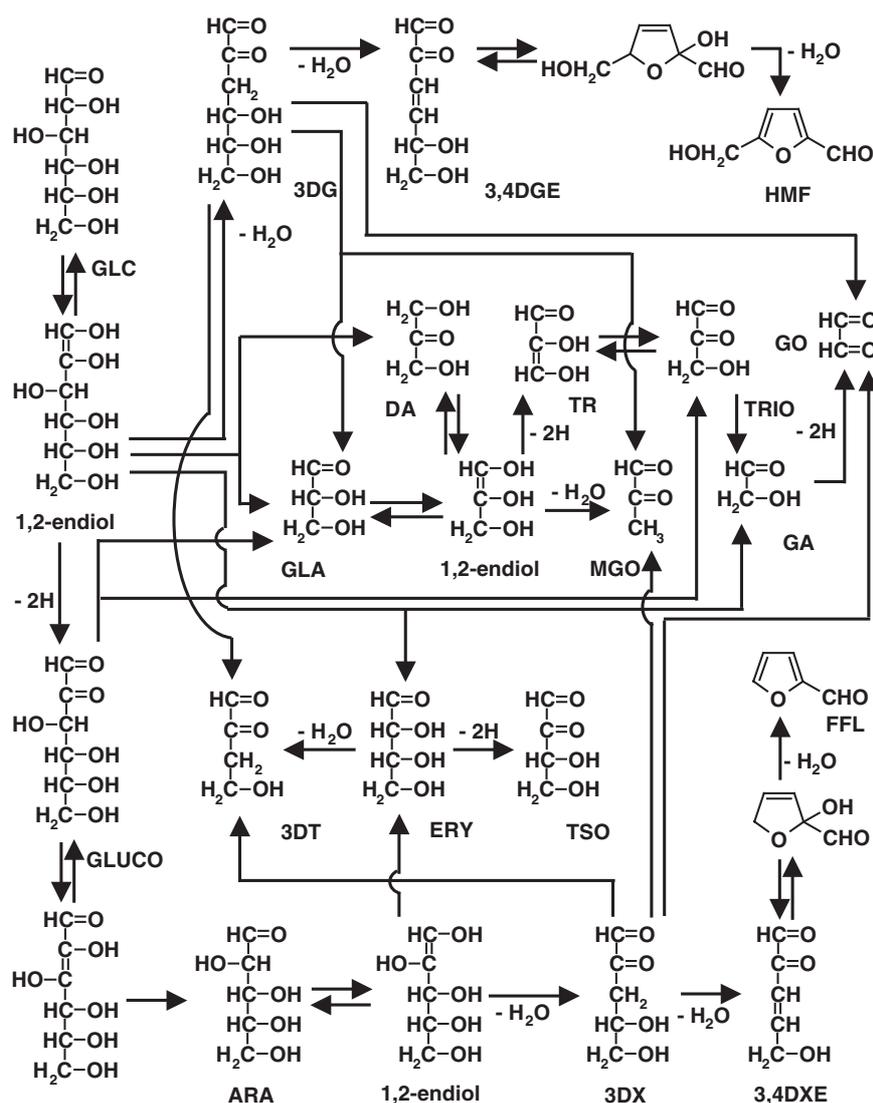


Fig. 7. Proposed Formation Pathway of α -Dicarbonyl Compounds Generated in the Degradation of Glucose.

been reported that 3DG is related to diabetic microangiopathy,⁵⁾ inhibits cell proliferation,¹⁰⁾ and induces apoptosis in cells.^{11,12)} 3,4-Dideoxyxosone-3-ene (3,4DGE) and 5-hydroxymethylfurfural (HMF) as intermediates were formed from 3DG by dehydration. The intermediates are known as cytotoxic substances. The 3,4DGE intermediate has been reported to be a major toxic product in peritoneal dialysis solution as a glucose-containing medicines.¹³⁾ HMF is also known as a cytotoxic substance in glucose-containing medicines such as parental solution.¹⁴⁾ It is utilized as a glucose degradation marker for the product quality of each medicine. It is formed from 3,4DGE by dehydration. The intermediates are formed under the non-oxidative condition, and the formation shows temperature dependence in sterilization and storage. Moreover, the carbonyls in glucose-containing medicines are often called glucose degradation products (GDPs).¹⁵⁾ GDPs are quality markers in medicines and are thought to affect cell function.

On the other hand, GLUCO was generated from glucose by oxidation. Arabinose (ARA) was formed from GLUCO by α -dicarbonyl cleavage, and then 3DX was formed from ARA by dehydration. Then 3,4-dideoxyxylosone-3-ene (3,4DXE) was formed from 3DX by dehydration, and furfural (FFL) was formed from 3,4DXE by dehydration. In addition, 3DT and TSO might have been formed from ERY in GLUCO degradation. 3DT was also formed from 3DG and 3DX.

Furthermore, we have reported that GLA is generated in glucose degradation.⁹⁾ GLA might be formed from glucose, 3DG, and GLUCO by the retro-aldol reaction. In this case, TRIO, GO, and MGO were formed from GLA intermediate. MGO was also formed from 3DG and 3DX, and GO were formed from 3DG, 3DX, and GA respectively. GA was generated from GLC and TRIO. The GLA-related pathway is one of major pathways in α -dicarbonyl formation.

In this study, we investigated whether α -dicarbonyl compounds would be generated from glucose *via non-*

oxidative 3DG formation, oxidative GLUCO formation, GLA formation, and other pathways. It is suggested that α -dicarbonyl compounds are formed in hyperglycemia from diabetes, and by the infusion of glucose-containing medicines such as peritoneal dialysis solution. Carbonyls might cause the progression of metabolic diseases.

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