Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose

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The glycation of proteins by glucose has been linked to the development of diabetic complications and other diseases. Early glycation is thought to involve the reaction of glucose with N-terminal and lysyl side chain amino groups to form Schiff's base and fructosamine adducts. The formation of the α-oxoaldehydes, glyoxal, methylglyoxal and 3-deoxyglucosone, in early glycation was investigated. Glucose (50 mM) degraded slowly at pH 7.4 and 37 °C to form glyoxal, methylglyoxal and 3-deoxyglucosone throughout a 3-week incubation period. Addition of *t*-BOC-lysine and human serum albumin increased the rate of formation of α-oxoaldehydes – except glyoxal and methylglyoxal concentrations were low with albumin, as expected from the high reactivity of glyoxal and methylglyoxal with arginine residues. The degradation of fructosyl-lysine also formed glyoxal, methylglyoxal and 3-deoxyglucosone. α-Oxoaldehyde formation was dependent on the concentration of phosphate buffer and availability of trace metal ions. This suggests that α-oxoaldehydes were formed in early glycation from the degradation of glucose and Schiff's base adduct. Since α-oxoaldehydes are important precursors of advanced glycation adducts, these adducts may be formed from early and advanced glycation processes. Short periods of hyperglycaemia, as occur in impaired glucose tolerance, may be sufficient to increase the concentrations of α-oxoaldehydes *in vivo*.

Key words: advanced glycation endproduct, Amadori rearrangement, autoxidation, fructosamine.

INTRODUCTION

Glycation, non-enzymatic glycosylation, is an endogenous process that contributes to the post-translational modification of proteins [1]. It is slow under physiological conditions, giving rise to the presence of lysine- and arginine-derived glycation adducts in cellular and extracellular proteins. Inside cells, the impact of glycation is countered by high turnover and short half-life of many cellular proteins. Long-lived extracellular proteins, however, accumulate glycation adducts with age [2,3]. Some of these adducts may be removed by enzymatic repair mechanisms, whilst all are removed by degradation of the glycated protein. Degradation of extracellular glycated proteins requires specific recognition by receptors, internalization and proteolytic processing. There are specific receptors, AGE receptors, which fulfil this role [4]. Glycation of proteins has been linked to mechanisms of disease – particularly the development of chronic clinical complications associated with diabetes mellitus – retinopathy, neuropathy and nephropathy [5], non-diabetic nephropathy [6], macrovascular disease [7,8], Alzheimer's disease [9], cataract [3] and ageing [2].

Studies of protein glycation have focused on the reaction of aldoses and ketoses, particularly glucose, with lysine residues of proteins. The acyclic form of the monosaccharide reacts reversibly with the lysyl side chain amino group to form an initial Schiff's base adduct. This exists mainly in the cyclic glycosylamine form [10]. For human serum albumin (HSA) in blood plasma under physiological conditions, the equilibrium between glucose, HSA and the Schiff's base is established with a chemical relaxation time of about 2.5 h. The concentration of the Schiff's base at equilibrium is about 0.3 % of glucose concentration [11]. The acyclic form of the Schiff's base rearranges reversibly to form an N_ϵ-(1-deoxy-D-fructos-1-yl) amino acid residue. This reaction is the Amadori rearrangement and the product is a fructosamine or 'Amadori product'. For HSA in blood plasma, the equilibrium dynamics of the Schiff's base and fructosamine are slower than of glucose and Schiff's base. From experimental data [12], the estimated chemical relaxation time for fructosamine formation is about 38 h. The concentration of fructosamine in blood plasma of normal, healthy human subjects is about 140 μM, and increases 2–3-fold in diabetes mellitus [13]. This is the early glycation process and Schiff's base and fructosamines have been called collectively early glycation adducts [14].

Fructosamine degrades by reversal of the Amadori rearrangement to the Schiff's base, and by further oxidative and non-oxidative reactions to form many stable end-stage adducts, collectively called AGEs [15]. The reactions forming AGEs have been termed advanced glycation [14]. Some AGEs are non-sulphydryl crosslinks, such as pentosidine [16] and imidazolium derivatives [17,18], which confer abnormal stability of glycated proteins to mechanical forces and proteolytic degradation [19]. Some AGEs are also recognition factors for specific receptors of endothelial cells, monocytes, macrophages and other cells [20]. Agonist binding to AGE receptors is associated with cell activation – induction of adhesion molecule expression, cytokine and growth factor synthesis, and in some cases apoptosis [4].

Recent research has shown that physiological glycation processes also involve the modification of proteins by reactive α-oxoaldehydes – particularly glyoxal, methylglyoxal and 3-deoxyglucosone (3-DG), and probably others. There appear to be AGE crosslinks and AGE receptor recognition factors common to proteins modified by glucose and α-oxoaldehydes. One explanation for this may be the formation of glyoxal, methylglyoxal and 3-DG during glycation of proteins by glucose, leading to associated α-oxoaldehydes-derived AGE formation.

In this report, we describe the formation of glyoxal, methyl-

Abbreviations used: AGE, advanced glycation endproduct; DETAPAC, diethylenetriaminepenta-acetic acid; DDB, 1,2-diamino-4,5-dimethoxybenzene dihydrochloride; 3-DG, 3-deoxyglucosone; DEMQ, 6,7-dimethoxy-1-ethyl-2-methylquinoxaline; HSA, human serum albumin; NBT, nitroblue tetrazolium; α-OA, α-oxoaldehyde.

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glyoxal and 3-DG from glucose degradation, and from lysyl residue and protein glycation by glucose *in vitro*. They were formed from the earliest stages of glycation, suggesting that AGEs may be formed in early and advanced glycation. A mechanistic interpretation is given.

MATERIALS AND METHODS

Materials

Human serum albumin (fraction V; HSA), nitroblue tetrazolium (NBT), N_α-*t*-BOC-L-lysine, β-D-glucose and glyoxal were purchased from Sigma. Methylglyoxal, 3-DG, 1,2-diamino-4,5-dimethoxybenzene dihydrochoride (DDB) were prepared as described [21,22]. 6,7-Dimethoxy-1-ethyl-2-methylquinoxaline (DEMQ) was prepared by reaction of DDB (0.2 mmol, 48.2 mg) and pentane-2,3-dione (0.4 mmol, 41.8 μl) in aqueous HCl (0.25 ml of 10.2 M HCl with 1.5 ml of water) under nitrogen for 6 h at room temperature. The product mixture was neutralized to pH 7 with 2 M potassium bicarbonate and the product crystallized. It was recrystallized from water:methanol (19:1, v/v). DEMQ gave ^{1}H-NMR ($CDCl_3$, 270 MHz) δ_H (p.p.m.; *J* Hz): 1.42 (t, 3H, *J* 7.3) 2-CH_2CH_3; 2.83 (s, 3H) 3-methyl; 3.06 (q, 2H, *J* 7.3) 2-CH_2CH_3; 4.06 (s, 6H) 6,7-dimethoxy; 7.52 (s, 1H) 8-H; 7.46 (s, 1H) 5-H; and mass spectrum (EI) with $m/z = 232$. The yield was 24 %.

N_ϵ-(1-Deoxy-D-fructos-1-yl)lysine (fructosyl-lysine) was prepared by modification of published methods [12]. Briefly, N_α-*t*-BOC-lysine (185 mg, 0.75 mmol), D-glucose (1.65 g, 9.15 mmol) and methanol (31 ml) was refluxed for 4 h. The methanol was evaporated, the residue dissolved in 10 mM sodium phosphate buffer (pH 7.4, 2.5 ml) and applied to a solid-phase extraction cartridge (ODS, 500 mg, Waters–Millipore, Watford, U.K.), equilibrated with the same buffer. N_α-*t*-BOC-N_ϵ-(1-deoxy-D-fructos-1-yl)lysine (t-BOC-fructosamine) was eluted with the same buffer, located by analysing eluate fractions by TLC (silica gel 60; propan-1-ol, acetic acid, water 16:1:5 v/v/v) (locating agent ninhydrin 0.2 % in ethanol) and lyophilized. Crude t-BOC-fructosamine was purified by preparative reversed phase HPLC (2.5 cm × 10 cm ODS column) equilibrated with 50 mM acetic acid. The eluate absorbance was monitored at 230 nm, the flow rate was 9 ml/min with a linear gradient of 0–50 % methanol for 25 min. *t*-BOC-fructosamine was de-protected by stirring with 1 M HCl overnight at room temperature. The solution was lyophilized and fructosyl-lysine purified by similar preparative reversed phase HPLC with eluent of 50 mM acetic acid, 9.9 ml/min, and eluate absorbance monitored at 210 nm. The ^{1}H-NMR spectrum (JOEL 270 MHz, D_2O) of fructosyl-lysine gave δ_H (p.p.m.; *J* Hz): lysyl 1.53 2 H (*m*), γ-CH_2; 1.78 2 H (*m*), β-CH_2; 1.95 2 H (*m*), δ-CH_2; 3.12 2 H (t, $J_{5,6} = 7.9$), ϵ-CH_2; 4.00 1 H (t, $J_{2,3} = 5.8$), α-CH; 1-deoxyfructosyl 3.28 2 H (s), H-1; 3.71 (d, $J_{3,4} = 9.7$), H-3; 3.73 (q, $J_{5,6A} = 2.0$, $J_{6A,6B} = 12.0$), H-6A; 3.87 1 H (q, $J_{3,4} = 9.8$, $J_{4,5} = 3.4$), H-4; 3.96–4.00 2 H (*m*), H-5 and H-6B overlapping with lysyl α-CH. The mass spectrum (MALDI II) gave an $(m+1)/z$ value of 309. TLC on silica gel 60 (mobile phase: propan-1-ol, acetic acid, water; 16:1:5, v/v/v) gave an R_f of 0.38. The product yields for each step were: *t*-BOC-fructosamine 53 % and fructosyl-lysine 98 %.

Study of glyoxal, methylglyoxal and 3-DG formation in glucose degradation and glycation

The formation of glyoxal, methylglyoxal and 3-DG was studied in 100 mM sodium phosphate buffer, pH 7.4 and 37 °C over 3 weeks, under aseptic conditions, with incubations containing: (a) 50 mM glucose; (b) 50 mM glucose and 50 mM N_α-*t*-BOC-lysine; (c) 50 mM glucose and 50 mg/ml HSA, and (d) 0.5 mM fructosyl-lysine. The effect of phosphate buffer concentration (10–100 mM) and chelation of trace metal ions with diethylenetriaminepenta-acetic acid (DETAPAC) was also investigated.

Assay of glyoxal, methylglyoxal and 3-DG

The concentrations of glyoxal, methylglyoxal and 3-DG were determined by derivatization with DDB and HPLC of the quinoxaline adducts. Several modifications have been made to the method since our first report [23] and other works. Since the avoidance of interferences was critical to this study, the procedure used herein is briefly described. Incubation sample (1 ml) and α-oxoaldehyde standards containing 0.05–5.00 nmol of 3-DG, glyoxal and methylglyoxal (1 ml, in incubation buffer) were placed into glass vials and acidified by addition of ice-cold perchloric acid (0.6 M, 1 ml) and kept on ice for 10 min. Where sample α-oxoaldehyde content exceeded 5 nmol, 0.05–0.50 ml of sample was analysed with addition of 0.95–0.50 ml of phosphate buffer. The internal standard, DEMQ, was added (20 μl of a 5 μM solution in 0.5 M HCl). Samples containing HSA were centrifuged (10000 ***g***, 5 min) at 4 °C. The supernatant was transferred into glass vials and the derivatizing agent, DDB (200 μl of a 1.11 mM solution in 0.5 M HCl, made immediately prior to addition) was added, and the samples were incubated at room temperature in the dark for 4 h. The pH of the samples was adjusted to 2.3 with Na_2HPO_4 (0.5 M, about 1 ml). The samples were applied to solid phase extraction cartridges (ODS, 500 mg) equilibrated with 20 mM ammonium phosphate, pH 2.3, the cartridges were washed with the same buffer (2 × 2.8 ml) and the quinoxaline analytes eluted with methanol (2.8 ml). Methanol was removed at room temperature *in vacuo* with a centrifugal evaporator, the residual solid reconstituted with 200 μl initial mobile phase (20 mM ammonium formate, pH 3.8, with 30 % methanol) and filtered (0.2 μm). Samples were analysed by HPLC: the column was 8 mm × 10 cm NOVA-PAK ODS 4-μm cartridge in a radial compression unit. The flow rate was 2 ml/min with mobile phase of 20 mM ammonium formate, pH 3.8, and solvent programme: 0–40 isocratic 30 % methanol; 40–75 min, a linear gradient of 30–60 % methanol. Detection was by fluorescence ($\lambda_{excitation}$ 352 nm, $\lambda_{emission}$ 385 nm). HPLC was performed with a Waters HPLC system (2 × 510 pumps, Lambda Max 481 LC spectrophotometer and 474 scanning fluorescence detector). For derivatized adducts of 3-DG, glyoxal and methylglyoxal: the retention times were 7.0, 17.3 and 24.0 min (retention time of the internal standard was 60.2 min); the recoveries 52, 70 and 59 %; the limits of detection 21, 11 and 14 pmol; and the interbatch coefficient of variation 15, 11 and 10 % ($n = 9$), respectively. There was no significant interference by glucose (50 mM) or fructosamine (1 mM) in the assay – except there was a small significant amount of 3-DG, 20 nM, detected in 50 mM glucose solution at zero time (this was attributed to a low contamination of glucose with 3-DG rather than degradation of glucose during the assay, since other degradation products of glucose were not present at zero time). Samples were stored at −196 °C for $\leqslant 6$ weeks without significant loss of glyoxal, methylglyoxal or 3-DG.

Assay of fructosamine

Fructosamine content of incubations of glucose with N_α-*t*-BOC-lysine and HSA were determined by the alkaline reduction of NBT using fructosyl-lysine as the calibration standard.

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RESULTS

Glyoxal, methylglyoxal and 3-DG formation in the degradation of glucose under physiological conditions

When glucose (50 mM) was incubated for 3 weeks in 100 mM sodium phosphate buffer, pH 7.4 and 37 °C, glyoxal, methylglyoxal and 3-DG were formed (Figure 1). There were increases in the concentrations of all three α-oxoaldehydes over the incubation period. The final concentrations after 3 weeks were: glyoxal 59.5 ± 3.7 μM, methylglyoxal 2.77 ± 1.22 μM and 3-DG 7.46 ± 1.48 μM ($n = 3$) (Figure 1a). The initial rate of increase in α-oxoaldehyde concentration (determined over the initial 2 days) was: glyoxal 1.72 ± 0.05 μM/day, methylglyoxal 0.098 ± 0.003 μM/day and 3-DG 0.437 ± 0.032 μM/day ($n = 3$).

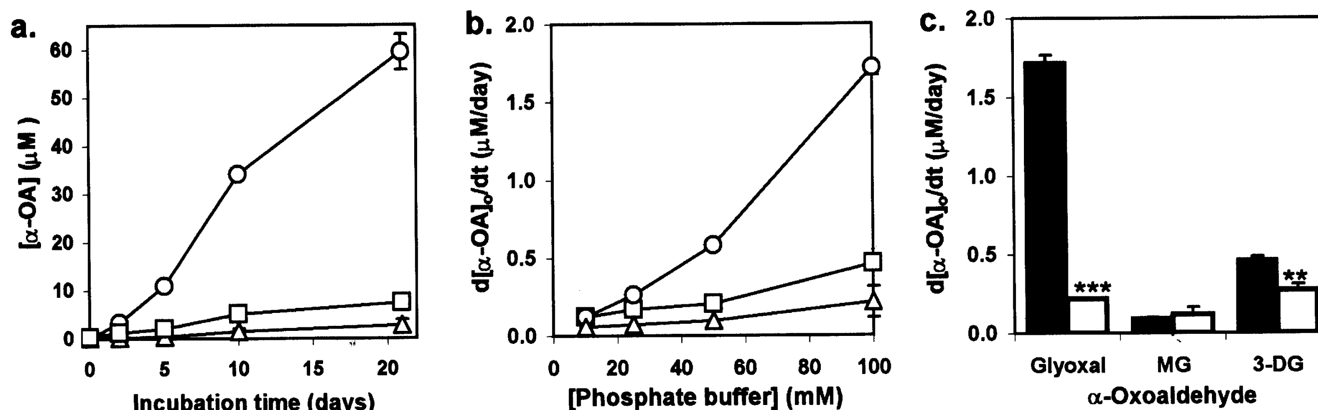
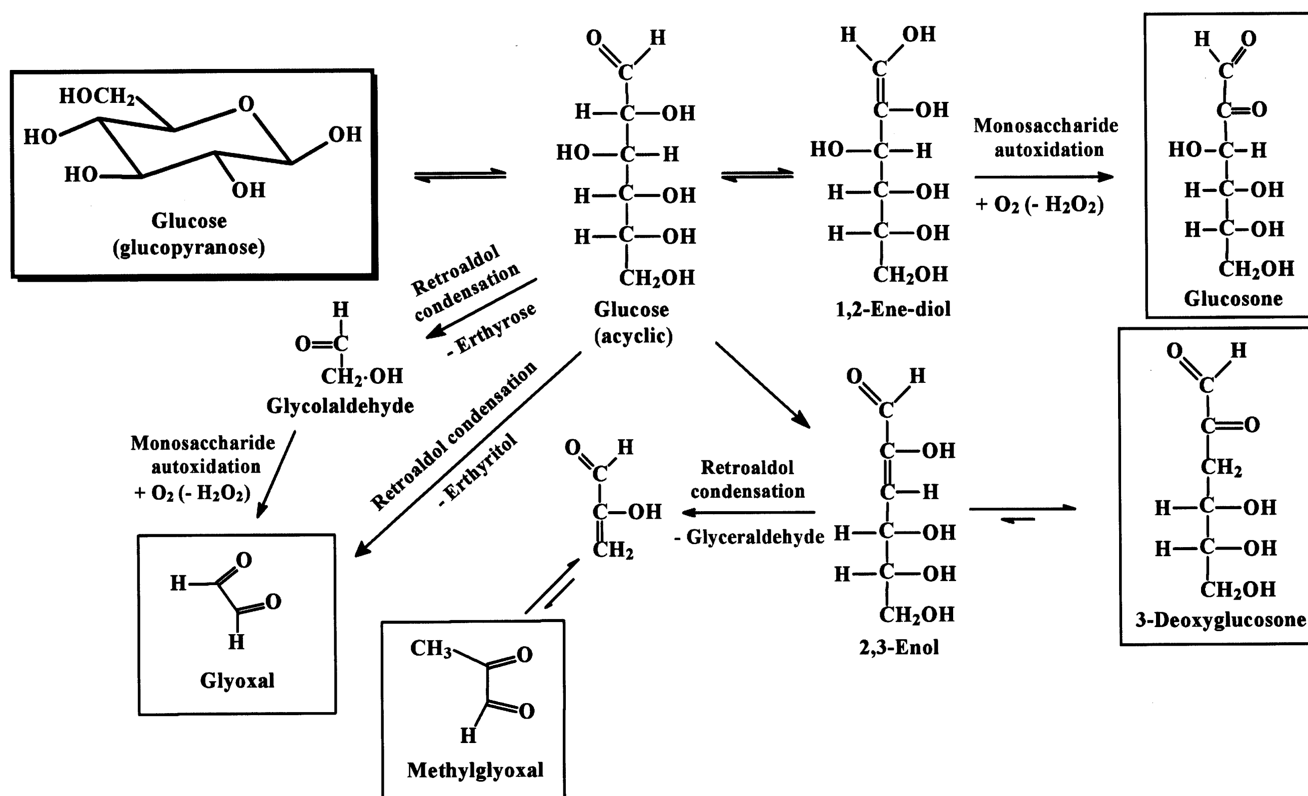


Figure 1 Glyoxal, methylglyoxal and 3-DG formation in glucose degradation

α-Oxoaldehyde (α-OA) concentration (**a**), and dependence of d[α-OA]$_0$/dt on phosphate buffer concentration (**b**) and effect of DETAPAC (**c**). Key: curves (○—○) glyoxal, (□—□) 3-DG, and (△—△) methylglyoxal; bars — DETAPAC (■), + 100 μM DETAPAC (□). Incubations contain 50 mM glucose in 100 mM sodium phosphate buffer, pH 7.4 and 37 °C, except (**b**) 10–100 mM sodium phosphate buffer, pH 7.4 and 37 °C.



Scheme 1. Mechanistic interpretation of glyoxal, methylglyoxal, 3-deoxyglucosone and glucosone formation in glucose degradation

Unhydrated and acyclic forms of α-oxoaldehyde products are shown although glyoxal and methylglyoxal exist in solution mainly as monohydrates and dihydrates and 3-DG and glucosone as cyclic hemiacetal and hemiketal hydrates.

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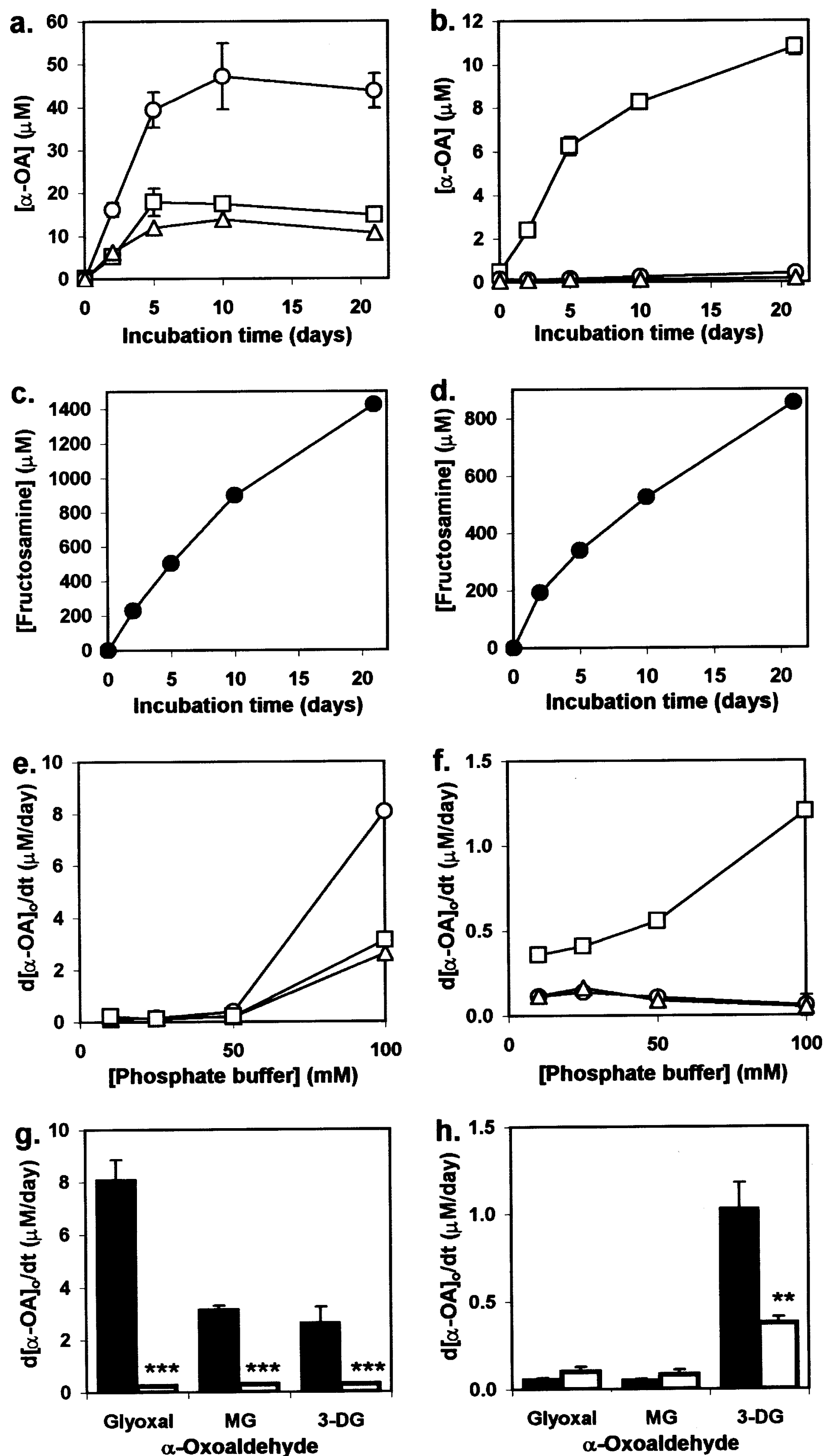

Figure 2 Glyoxal, methylglyoxal and 3-DG formation in early glycation

α-OA concentration (**a** and **b**), fructosamine concentration (**c** and **d**), and dependence of d[α-OA]$_0$/dt on phosphate buffer concentration (**e** and **f**) and effect of DETAPAC (**g** and **h**). Key: curves (○—○) glyoxal, (□—□) 3-DG, (△—△) methylglyoxal, and (●—●) fructosamine; bars — DETAPAC (■) and + 100 μM DETAPAC (□). Incubations contain 50 mM glucose and 50 mM N_{α}-t-BOC-lysine (**a**, **c**, **e** and **g**) or 50 mg/ml HSA (**b**, **d**, **f** and **h**) in 100 mM sodium phosphate buffer, pH 7.4 and 37 °C, except **e** and **f** 10–100 mM sodium phosphate buffer, pH 7.4 and 37 °C.

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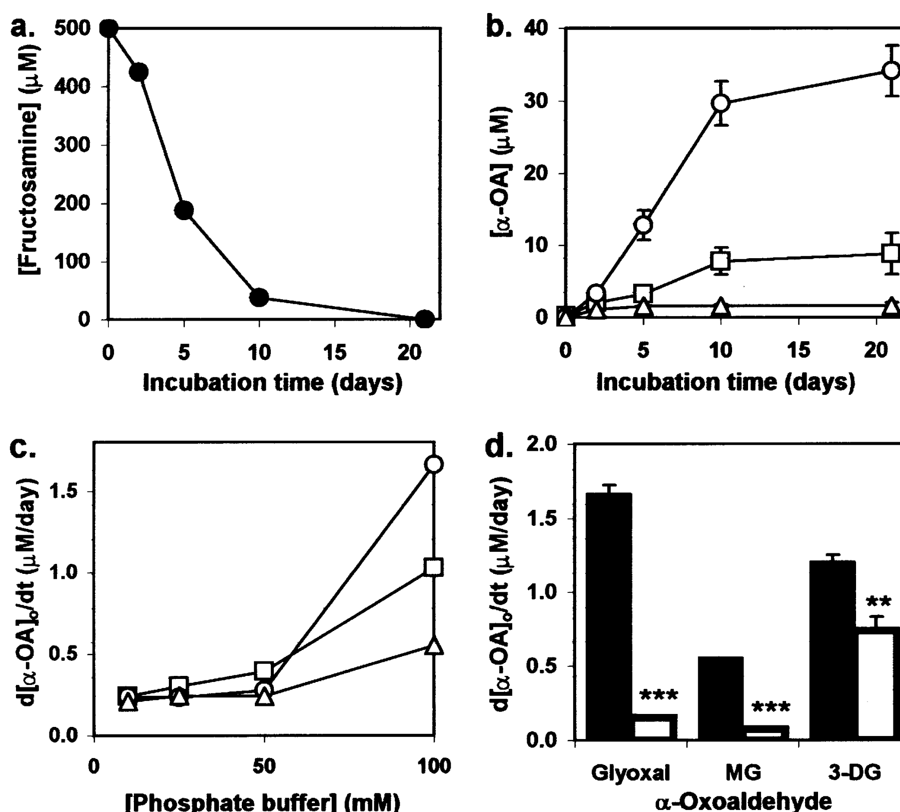

Figure 3 Glyoxal, methylglyoxal and 3-DG formation in the degradation of fructosyl-lysine

Fructosamine concentration (**a**), α-OA concentration (**b**), and dependence of $d[\alpha\text{-OA}]_0/dt$ on phosphate buffer concentration (**c**) and effect of DETAPAC (**d**). Key: curves (●—●) fructosamine, (○—○) glyoxal, (□—□) 3-DG, (△—△) methylglyoxal, and; bars — DETAPAC (■) and + 100 μM DETAPAC (□). Incubations contain 500 μM fructosyl-lysine in 100 mM sodium phosphate buffer, pH 7.4 and 37 °C, except **c** 10–100 mM sodium phosphate buffer, pH 7.4 and 37 °C.

The initial rate of glyoxal formation was markedly dependent on the concentration of phosphate buffer and was markedly decreased in the presence of the metal ion chelator DETAPAC. The initial rates of formation of methylglyoxal and 3-DG were less dependent on phosphate buffer concentration and presence of DETAPAC (Figures 1b and 1c).

A potential source of formation of methylglyoxal in glucose degradation and glycation reactions is from the fragmentation of 3-DG by a retroaldol condensation reaction (Scheme 1). To investigate this, 3-DG (50 μM) was incubated in 100 mM phosphate buffer, pH 7.4 and 37 °C, for 2 days. Methylglyoxal was indeed formed. Final α-oxoaldehyde concentrations were: 3-DG 43.1 ± 2.4 μM, and methylglyoxal 0.09 ± 0.03 μM ($n = 3$).

α-Oxoaldehyde and fructosamine formation in the glycation of N_α-*t*-BOC-lysine and human serum albumin

Glycation of proteins by glucose initially occurs by interaction of glucose with N-terminal and lysyl side chain amino groups. To model this reaction, glucose (50 mM) was incubated with N_α-*t*-BOC-lysine (50 mM) in 100 mM phosphate buffer, pH 7.4 and 37 °C. Glyoxal, 3-DG and methylglyoxal were formed. The concentrations of these α-oxoaldehydes increased rapidly in the initial 2–5 days; thereafter, the concentrations remained at a maximum value and tended to slowly decrease at 21 days (Figure 2a). After 21 days, the α-oxoaldehyde concentrations were: glyoxal 43.7 ± 4.0 μM, methylglyoxal 10.7 ± 2.0 μM and 3-DG 14.8 ± 0.5 μM. In contrast, the formation of fructosamine increased throughout the incubation period to 1424 ± 8 μM after 21 days (Figure 2b). The initial rates of α-oxoaldehyde formation were: glyoxal 8.09 ± 0.78 μM/day, methylglyoxal 3.13 ± 0.15 μM/day, 3-DG 2.62 ± 0.62 μM/day. They were increased significantly with respect to glucose degradation ($P < 0.001$). The initial rate of fructosamine formation (116 ± 4 μM/day) was much higher than α-oxoaldehyde formation. The initial rates of α-oxoaldehyde formation were dependent on phosphate buffer concentration – particularly at phosphate concentration > 50 mM, and decreased markedly in the presence of DETAPAC (Figures 2e and 2g). In the presence of DETAPAC, the initial rates of glyoxal and 3-DG formation in the glycation of N_α-*t*-BOC-lysine by glucose were not significantly different from those of glucose degradation ($P > 0.05$). The initial rate of methylglyoxal formation in the glycation of N_α-*t*-BOC-lysine by glucose was increased significantly, however, with respect to that of glucose degradation ($P < 0.01$). A potential source of methylglyoxal formation in glucose glycation reactions is the fragmentation of 3-DG-N_α-*t*-BOC-lysine aldimine Schiff's base adduct by a retroaldol condensation reaction. To investigate this, 3-DG (50 μM) was incubated in 100 mM phosphate buffer, pH 7.4 and 37 °C, in the presence of 50 mM N_α-*t*-BOC-lysine for 2 days. Methylglyoxal was formed in this period. α-Oxoaldehyde concentrations were: 3-DG 30.3 ± 2.0 μM and 0.55 ± 0.08 μM ($n = 3$). Methylglyoxal formation was increased significantly by the presence of N_α-*t*-BOC-lysine ($P < 0.001$).

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When a similar investigation was performed of 50 mM glucose incubated with HSA (50 mg/ml), there were also increases in the concentrations of 3-DG (Figure 2b) and fructosamine (Figure 2d) with time. The concentrations of glyoxal and methylglyoxal, however, were very low ($< 0.5\ \mu$M) throughout the incubation period (Figure 2b). The initial rate of formation of 3-DG was dependent on phosphate buffer concentration and was decreased in the presence of DETAPAC but the initial rates of formation of glyoxal and methylglyoxal were not (Figure 2f and 2h).

α-Oxoaldehyde formation in the degradation of fructosyl-lysine

Fructosyl-lysine (500 μM) degraded completely during the 21-day incubation period (Figure 3a). This was associated with the formation of glyoxal, methylglyoxal and 3-DG. The concentrations of these α-oxoaldehydes increased rapidly in the initial 2–5 days, and thereafter approached maximum values (Figure 3b). After 21 days, the α-oxoaldehyde concentrations were: glyoxal $34.1 \pm 3.5\ \mu$M, methylglyoxal $1.61 \pm 0.45\ \mu$M and 3-DG $8.80 \pm 2.86\ \mu$M. The initial rates of α-oxoaldehyde formation were: glyoxal $1.66 \pm 0.60\ \mu$M/day, methylglyoxal $0.55 \pm 0.01\ \mu$M/day, 3-DG $1.03 \pm 0.15\ \mu$M/day; cf. the initial rate of fructosamine degradation which was $37.5 \pm 10.8\ \mu$M/day ($n = 3$). The initial rates of α-oxoaldehyde formation were dependent on phosphate buffer concentration, particularly for phosphate concentration > 50 mM, and were markedly decreased in the presence of DETAPAC (Figures 3c and 3d).

DISCUSSION

α-Oxoaldehyde formation in the degradation of glucose

Our initial study of glucose degradation in 1984 indicated that it was an oxidative process forming glucosone. We called this monosaccharide autoxidation [24]. From data presented herein and other reports [25,26], it can be seen that the spontaneous degradation of glucose under physiological conditions also forms glyoxal, methylglyoxal and 3-DG.

Glyoxal may be formed in the degradation of glucose by retroaldol condensation reactions activated by deprotonation of the 2- or 3-hydroxy groups. Concurrent oxidative processes forming hydrogen peroxide (autoxidation of glycoaldehyde to glyoxal, and glucose to glucosone) also stimulate glyoxal formation by hydroxyl radical-mediated acetal proton abstraction from glucopyranose and β-elimination reactions [24,27]. 3-DG and glucosone formation occur by an initial common activation step, deprotonation of carbon-2: re-distribution of the electron density between carbon-1 and carbon-2 leads to the formation of the ene-diol (enolization) [28]; alternatively, re-distribution of the electron density between carbon-2 and carbon-3 leads to dehydration forming the 2,3-enol and thereby 3-DG. Methylglyoxal may be formed by fragmentation of 3-DG (Scheme 1). The initial rates of formation of glyoxal, methylglyoxal and 3-DG were dependent on phosphate buffer. This may be due to phosphate dianion HPO_4^{2-} catalysing the activating deprotonation of glucose [24]. The marked inhibition of glyoxal formation by DETAPAC is consistent with redox active metal ions (ferric Fe^{3+} and cupric Cu^{2+}) catalysing the autoxidation of glycoaldehyde and hydroxyl radical formation implicated in glyoxal formation [24]. The decrease of 3-DG formation by DETAPAC was unexpected but a similar effect has been observed previously in glucose glycation reactions [29]. Trace metal ion-phosphate complexes may be involved in the activation of glucose for 3-DG formation.

α-Oxoaldehyde formation and early glycation of proteins by glucose

The formation of fructosamine residues is the major pathway of early glycation by glucose, but not the only one. In the model glycation of N_α-*t*-BOC-lysine by glucose, the initial rate of formation of fructosamine was 8-fold higher than the initial rate of glyoxal, methylglyoxal and 3-DG formation combined (and about 16 % of this may be attributed to α-oxoaldehyde formation from glucose autoxidation). The formation of α-oxoaldehydes in early glycation, therefore, can be easily overlooked. α-Oxoaldehyde precursors of AGEs, such as 3-DG, and AGEs, were initially considered to be formed from fructosamine only. Recent research [30,31], mechanistic considerations [32] and this study suggest this is not so.

Glyoxal, methylglyoxal and 3-DG were formed in glucose glycation of N_α-*t*-BOC-lysine early and throughout the incubation period. The change in α-oxoaldehyde concentration did not follow that of the fructosamine. α-Oxoaldehydes rather may be formed from other sources: from glucose degradation and from the Schiff's base. Mechanistically, we propose that α-oxoaldehyde formation occurs similarly to that envisaged in glucose degradation except for the presence of the aldimine moiety throughout; the product is the aldimine Schiff's base of the α-oxoaldehyde (which hydrolyses to the free α-oxoaldehyde and amine) (Scheme 2). The presence of the aldimine group accelerates the formation of α-oxoaldehydes: the initial rates of formation of glyoxal, methylglyoxal and 3-DG were increased 5-fold, 32-fold and 6-fold, respectively, by the presence of N_α-*t*-BOC-lysine, although there was only a significant increase in the rate of methylglyoxal formation in the presence of DETAPAC. The Amadori rearrangement is analogous to ene-diol formation in monosaccharide enolization: the ene-aminol moiety (NH–CH=COH) being isoelectronic with the ene-diol moiety (O–CH=COH). The formation of 3-DG and fructosamine are parallel reaction pathways from a common activating step. The critical point in the formation of both fructosamine and 3-DG is the deprotonation of carbon-2 of the Schiff's base. Fructosamine formation is facilitated by the polarizing effect of the imine bond of the Schiff's base that favours the ene-aminol formation. The formation of glyoxal from the Schiff's base was proposed by Namiki and co-workers [30]. Glyoxal formation may occur by retroaldol condensation or free radical mechanisms (see above). Indeed, superoxide radical formation from the Schiff's base in early glycation was detected [33], supporting evidence for the involvement of oxidative processes in early glycation.

The concentrations of the α-oxoaldehydes increased in the incubations to a maximum value where the rate of α-oxoaldehyde formation equals the rate of α-oxoaldehyde removal; the latter is expected to occur by reaction with N_α-*t*-BOC-lysine to form AGEs and spontaneous degradation. In the glycation of HSA by glucose, α-oxoaldehydes may also be consumed by reaction with arginine residues to form hydroimidazolone derivatives [34–36]. This accounts for the lack of accumulation of glyoxal and methylglyoxal in the glucose glycation incubation with HSA. 3-DG, however, still accumulated but it has about 200-fold lower reactivity with arginine residues than glyoxal and methylglyoxal [37,38].

The initial rates of formation of the glyoxal, methylglyoxal and 3-DG by glucose glycation were dependent on phosphate buffer concentration and availability of trace metal ions. The exception was the initial increase in concentration of glyoxal and methylglyoxal with HSA but here only very low steady state concentrations of these α-oxoaldehydes were found. An initial activating deprotonation is implicated in the formation of all of

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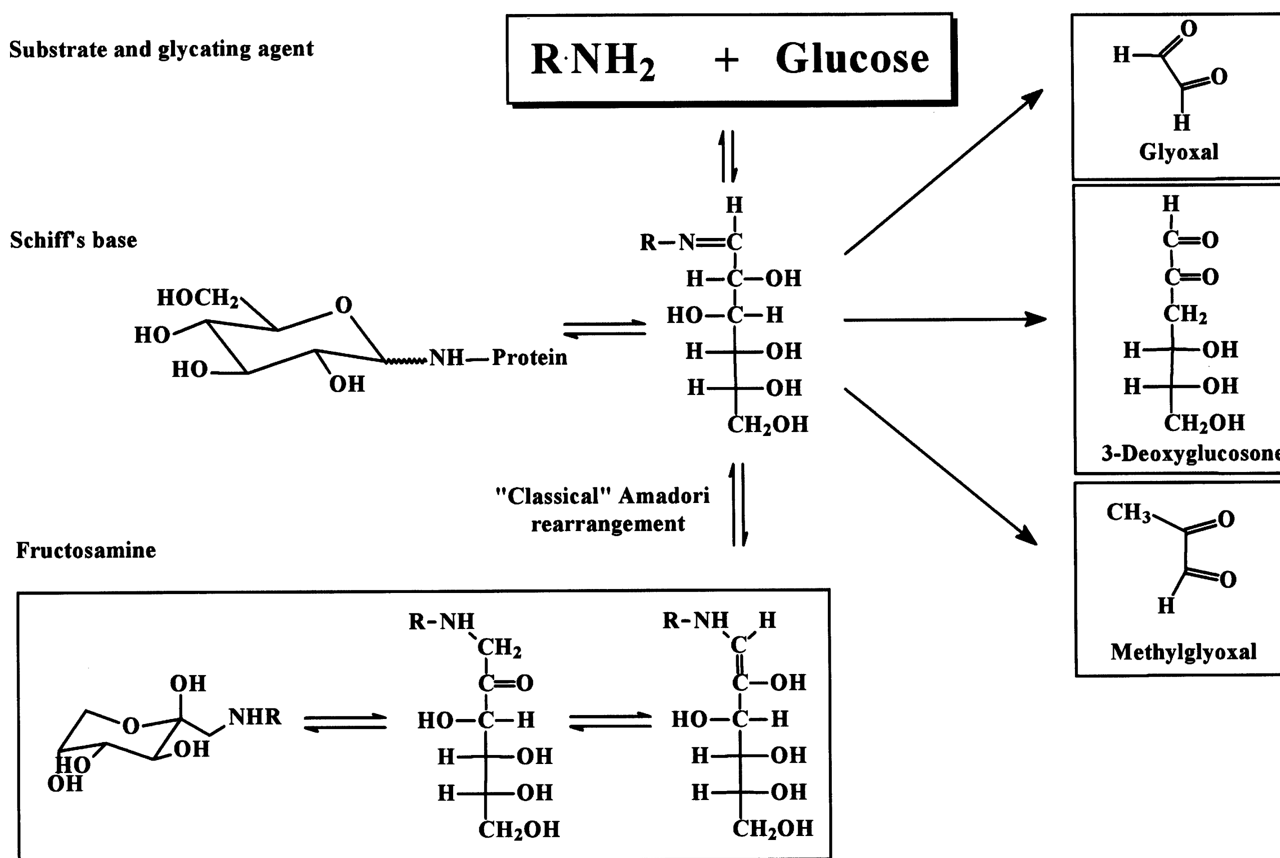

Scheme 2. Mechanistic interpretation of glyoxal, methylglyoxal and 3-deoxyglucosone formation in early glycation

RNH_2 represents lysyl side chain and N-terminal amino groups.

the α-oxoaldehydes and phosphate dianion HPO_4^{2-} may fulfil this role. Phosphate buffer has been found previously to promote glucose glycation [39]. There are also trace redox active metal ions in phosphate buffer that promote oxidative steps involved in glyoxal formation [24]. This explains why glyoxal formation was particularly sensitive to the presence of DETAPAC. The formation of methylglyoxal and 3-DG, however, does not involve oxidation. Trace metal ion–phosphate complexes may facilitate activating deprotonation of the Schiff's base. A similar effect was observed previously [29]. In the presence of the DETAPAC, the initial rates of formation of glyoxal and 3-DG by glucose and N_α-*t*-BOC-lysine were not significantly different from those of glucose degradation alone (Figures 1c and 2g). In early glycation processes under anti-oxidative conditions, glucose degradation may be a significant source of glyoxal, methylglyoxal and 3-DG.

The fragmentation of the hexose moiety during α-oxoaldehyde formation forms other monosaccharides: erythrose and glyceraldehyde. Evidence of 3 and 4 carbon products was reported [40]. These also participate in oxidation and dehydration reactions forming α-oxoaldehydes [24]. This work has focused on glyoxal, methylglyoxal and 3-DG because of their importance in AGE formation. Ribose and other saccharides may also undergo similar fragmentation reactions forming glyoxal and methylglyoxal. The detection of glyoxal and methylglyoxal-derived hydroimidazolones in collagen glycated by ribose supports this [36].

α-Oxoaldehyde formation from the degradation of fructosyl-lysine

The degradation of fructosyl-lysine also formed glyoxal, methylglyoxal and 3-DG. The rates of formation paralleled the rate of loss of fructosamine and were increased by phosphate buffer and trace metal ions, as found for the rate of fructosamine degradation [41]. Mechanistically, the formation of α-oxoaldehydes may occur via reversal of the Amadori rearrangement and formation from the Schiff's base [29,32]. 3-DG is formed from fructosamine without reversal of the Amadori rearrangement *in vivo* by phosphorylation to fructosamine-3-phosphate and spontaneous fragmentation of this to 3-DG [42]. Other α,β-dicarbonyl compounds are also formed from fructosamine without reversal of the Amadori rearrangement [12,43].

Consequences of α-oxoaldehyde formation in early glycation

Fragmentation of glucose in early glycation processes establishes many parallel glycation pathways that lead to the subsequent formation of AGEs. Analogous oxidation and dehydration reactions have been found in glycation by other hexoses and pentose derivatives. α-Oxoaldehydes react with lysine and arginine residues in proteins to form AGEs: glyoxal forms N_ϵ-carboxymethyl-lysine, the glyoxal-derived bis(lysyl)imidazolium crosslink GOLD and hydroimidazolone; methylglyoxal forms N_ϵ-carboxyethyl-lysine, methylglyoxal-derived bis(lysyl)-

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imidazolium crosslink MOLD, hydroimidazolone and argpyrimidine; and 3-DG forms pyrraline, the 3-DG-derived bis-(lysyl)imidazolium crosslink DOLD and hydroimidazolone – reviewed in [14]. Glyoxal, methylglyoxal and 3-DG derived AGEs may be present in proteins glycated by glucose: we have recently detected N_ϵ-carboxyethyl-lysine in albumin glycated by glucose [44]. Methylglyoxal-derived AGEs are common to proteins modified by glucose and by the authentic α-oxoaldehyde. Indeed, similar binding to AGE receptors has been found for these proteins [20]. The formation of α-oxoaldehydes from monosaccharides, Schiff's bases and fructosamines suggests that AGEs may be formed at all stages of glycation.

The increased formation of α-oxoaldehydes in early glycation implicates relatively short-term excursions to hyperglycaemia, as occurs in impaired glucose tolerance, in the increased formation of α-oxoaldehydes and associated AGEs. There are, however, other important sources of these α-oxoaldehydes *in vivo*: glyoxal is formed in lipid peroxidation; methylglyoxal is formed by the fragmentation of triosephosphates, and the catabolism of ketone bodies and threonine; and 3-DG is formed from fructose-3-phosphate. AGEs are risk markers and risk predictors of diabetic complications. A wealth of experimental evidence supports the hypothesis that AGEs formed from glyoxal, methylglyoxal and 3-DG have an aetiological role in the development of diabetic complications and other diseases [14,45]. It is important, therefore, to characterize further the sources of these α-oxoaldehyde AGE precursors. Glyoxal, methylglyoxal and 3-DG can now be seen to play a part in all stages of glycation by glucose.

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