Amino Acids (2006) 30: 425–433
DOI 10.1007/s00726-006-0269-2

Amino Acids
Printed in Austria

Site specificity of glycation and carboxymethylation of bovine serum albumin by fructose

D. J. S. Hinton[1] and **J. M. Ames**[2]

[1] Hugh Sinclair Unit of Human Nutrition, School of Food Biosciences, University of Reading, Whiteknights, U.K.
[2] School of Biological and Food Sciences, Queen's University Belfast, Belfast, Northern Ireland

Received June 30, 2005
Accepted October 21, 2005
Published online April 4, 2006;

Summary. We report an investigation of the site specificity, extent and nature of modification of bovine serum albumin (BSA) incubated with fructose or glucose at physiological temperature and pH. Sites of early glycation (Heyns rearrangement products (HRP) from fructose; fructoselysine (FL) from glucose) as well as advanced glycation (N^{ε}-(carboxymethyl)lysine; CML) were analyzed by liquid chromatography-mass spectrometry. The major site of modification by fructose, like glucose, is Lysine-524 and this results in, respectively, 31 and 76% loss of the corresponding unmodified tryptic peptide, Gln525-Lys533. In addition, total lysine, HRP, FL, CML and N^{ε}-(carboxyethyl)lysine in the incubations, was quantified. Almost all of the loss of lysine in the fructose-modified BSA was attributed to the formation of CML, with the yield of CML being up to 17-fold higher than glucose-modified BSA. A mechanism for the formation of CML from the HRP is proposed.

Keywords: Fructose – Advanced glycation endproduct – Site specificity – Fructoselysine – Heyns rearrangement products – N^{ε}-(carboxymethyl)lysine

Introduction

Reducing sugars react with the epsilon amino group of lysine residues in proteins to form a Schiff base which spontaneously rearranges to form either the Amadori rearrangement product (ARP), when the sugar is glucose, or Heyns rearrangement products (HRP), when the sugar is fructose (McPherson et al., 1988). In the case of glucose, the ARP is known as fructoselysine (FL). FL and the HRP degrade under physiological conditions by oxidative and nonoxidative fragmentation to form advanced glycation endproducts (AGEs). AGE accumulation on tissue proteins has been associated with many disease disorders including diabetic complications, Alzheimer's disease and atherosclerosis (e.g., Nakamura et al., 1993; Smith et al., 1994; Makino et al., 1996).

The AGE, N^{ε}-(carboxymethyl)lysine (CML), is a frequently used chemical biomarker of AGE formation in tissues (Reddy et al., 1995) and has been detected in, e.g., skin collagen (Dunn et al., 1991) and lens proteins (Dunn et al., 1989). CML is formed via different reaction pathways in glucose-protein systems (Wells-Knecht et al., 1995). It may form either by the oxidative fragmentation of FL, or from glyoxal (GO), a highly reactive compound formed via oxidation of glucose, or from the Schiff base (Thornalley et al., 1999).

The concentration of fructose in human tissues and fluids is generally much lower than that of glucose. The fructose concentration in blood of healthy subjects is about 100-fold lower than that of glucose (Macdonald et al., 1978). Under hyperglycemic conditions (diabetes), sorbitol is formed from the reduction of glucose by aldose reductase (via the polyol pathway) (Hers, 1956). Fructose can be biosynthesized by oxidation of sorbitol in a reaction catalyzed by polyol dehydrogenase (sorbitol pathway) (Hers, 1956). In organs where the sorbitol pathway is active, e.g., ocular lens, fructose accumulates to levels higher than in, e.g., blood plasma (Heaf and Galton, 1975). In diabetes, there is an increased glucose supply and fructose levels in the lens may increase up to 23-fold, becoming at least as high as those of glucose (Gabbay and Kinoshita, 1972). Values $\leq$12 mM fructose have been reported in diabetic ocular lens (Jedziniak et al., 1981). Examination of human ocular lens proteins has shown that a significant proportion (10–20%) of the hexose bound to epsilon amino groups of lysine residues is connected via C_2, indicative of endogenous HRP

(McPherson et al., 1988). In such organs, fructose may react with proteins to give AGEs, such as CML, which has been shown to accumulate in lens proteins with age (Ahmed et al., 1997). However, the importance of fructose as an AGE precursor *in vivo*, is currently unknown.

From previous studies comparing the reactivity of glucose and fructose with proteins at physiological temperature, the following conclusions can be drawn. Firstly, protein-sugar incubations result in a faster accumulation of protein-bound fluorescence and oligomerization (e.g., McPherson et al., 1988; Suárez et al., 1989) from fructose compared to glucose. Secondly, utilization of lysine residues by fructose is more dependent on the presence of oxygen (Yeboah et al., 1999).

Advances in mass spectrometry, especially in the last decade, have led to its establishment as the technique of choice for probing the site specificity of glycation. However, relatively little work has been reported in this area, especially with regard to the site specificity of AGE formation (Brock et al., 2003; Marotta et al., 2003; Cotham et al., 2004). Brock et al. (2003) recently reported the first study of the site specificity of AGE (CML) formation on ribonuclease A (RNase) and Lysine-41 was the main site of carboxymethylation. This was followed by a study reporting that Arginine-39 and Arginine-85 were the main sites of glyoxal-derived dihydroxyimidazolidine and hydroimidazolone formation (Cotham et al., 2004).

Currently, very few papers (e.g., Ruttkat and Ebersdobler, 1995) have been published on the formation of AGEs (e.g. CML) from fructose, and there are no reports on the site specificity of HRP or AGE formation from fructose. Although Lysine-525 of HSA (equivalent to Lysine-524 of BSA) is known to be the main site of FL formation (Garlick and Mazer, 1983; Iberg and Flückinger, 1986), it is currently unknown if this site is also the main site of lysine-derived AGEs, such as CML. In addition, it is unknown if different sugars share the same specificity for AGE modification of lysine residues of proteins.

Fructose has been shown to be more readily degraded to dicarbonyl compounds in solution, compared to glucose (Sakai et al., 2002). If such dicarbonyls were to be the main source of CML formation from fructose, one might expect a different site specificity for CML formation compared to glucose. In organs such as the lens, where CML may be formed from a variety of sugar sources, this would provide information on which sugars are forming what proportion of CML based on the different lysine residues that are modified. In the work presented here we incubated BSA with either fructose or glucose and compared both the site specificity of modification of lysine residues and the total yield of FL/HRP and CML.

Materials and methods

Chemicals

The following reagents were purchased from Sigma (Gillingham, UK): BSA (fraction 5, >98%), D-(+)-glucose (ACS grade), D-(−)-fructose (ACS grade), trypsin (sequencing grade). Sodium eluants for ion-exchange chromatography were obtained from Pickering Labs, Mountain View, CA, USA. CML, [$^2H_4$]-CML, N^{ε}-(carboxyethyl)lysine (CEL), [$^2H_8$]-CEL, [$^2H_8$]-Lysine and formyl-FL (fFL) standards were kindly donated by Dr. John Baynes.

Sample preparation

BSA (1 mM) was incubated with either D-(+)-glucose or D-(−)-fructose (0.4 M) in phosphate buffer (0.2 M; pH 7.50) at 37 °C for 0, 3, 7 and 14 d. BSA incubated in the absence of sugar served as the control. Incubations were prepared in duplicate.

Tryptic digestion

Protein was separated from buffer salts and residual sugar, reduced with dithiothreitol, derivatized with 4-vinylpyridine and digested with trypsin at an enzyme:substrate ratio of 5:100 (w/w), at 37 °C for 5.5 h (Brock et al., 2003). The digest was separated into low molecular mass (LMM, <5 kDa) and high molecular mass (HMM, >5 kDa) fractions by ultrafiltration. This involved placing sample (300 µl), in a 5 kDa molecular mass cut-off ultrafiltration device (Millipore, Watford, UK) and centrifuging at 3,500 *g* for 60 min. The LMM fractions were analyzed by LC-MS.

Liquid chromatography-mass spectrometry (LC-MS)

Samples were fractionated on an Agilent (Palo Alto, CA) series 1100 liquid chromatograph, coupled to a Micromass, (Manchester, UK) Quattro mass spectrometer. Separations were conducted using a reverse phase column packed with ACE® C_{18}, 5 µm particle size, 300 Å pore size (15 cm × 2.1 mm id, Hichrom Ltd., Theale, UK). Solvent B was 0.1% aqueous TFA and solvent B was acetonitrile. Tryptic digests were diluted 50:50 with water and 40 µl aliquots (2.4 nmol, 160.8 µg) were injected. A linear gradient was applied running from 2% solvent A to 50% solvent B over 92 min. Modified peptides were located and RAs were estimated as described previously (Brock et al., 2003). Briefly, ion chromatograms were extracted from the full scan data and the sum of the peak areas of all detected charge states of each peptide was divided by the sum of the peak areas of all the detected charge states of the C-terminal peptide ^{574}LeuValValSerThrGlnThrAlaLeuAla^{583} (Leu574-Ala583), to give the RA value. Modification of reactive amino acid residues, e.g., Lysine-524, was estimated by calculating the % decrease of the RA of the unmodified peptide of interest, e.g., peptide ^{525}GlnThrAlaLeuValGluLeuLeuLys^{533} (Gln525-Lys533) for Lysine-524, in the incubated systems, compared to native BSA. Where both unmodified peptides were detected, the longer one was selected for integral ratio determination. Preliminary experiments showed that the range of the RA values was $<\pm 18\%$ depending on whether the longer or shorter of the two unmodified peptides was used to estimate

amounts. Based on data for replicate experiments, losses of >20% of the unmodified peptides of interest were quantified.

Quantification of lysine, arginine, FL and HRP by ion-exchange chromatography

Incubation (300 µl) was ultrafiltrated using a 5 kDa molecular mass cut-off ultrafiltration device at 3,500 *g* for 30 min to remove LMM reaction products and residual sugar. Water (200 µl) was added to the high molecular mass (HMM, ≥5 kDa) fraction and the sample was centrifuged again at 3,500 *g* for 30 min. This washing procedure was repeated twice. The resulting HMM fraction, containing the protein, was diluted to 5 mg/ml BSA and 20 µl (containing 0.15 nmol BSA) was reduced with $NaBH_4$ (1 M in 0.1 M NaOH; 200 µl) in sodium borate buffer (0.5 M, pH 9.2; 150 µl) at 4 °C overnight. The sample was washed by ultrafiltration (as described above) and hydrolyzed in HCl (6 M; 1 ml) at 110 °C for 24 h. Separations were performed on a Shimadzu (Kyoto, Japan) system comprising of LC-10Ai and LC-10AS pumps, a SIL-10Ai autosampler and a SCL-10A system controller, using a divinylbenzene cation-exchange column (3 × 250 mm; Pickering Labs, Mountain View, CA). Acid hydrolyzates were reconstituted in solvent A (Na328; 357 µl) and 60 µl aliquots (containing 34 µg BSA) were injected. Separations were conducted using a gradient from solvent A to solvent B (Na740). Solvent A (100%) was maintained for 15 min; then the solvent composition was modified linearly over 24 min to 100% solvent B. Finally, the solvent composition was returned to 100% solvent A linearly over 21 min. Samples were post-column derivatized with *o*-phthalaldehyde and separations were monitored using a Shimadzu (Kyoto, Japan) RF-10AXL fluorimeter using an λ_{ex} of 375 nm and an λ_{em} of 425 nm. Standard amino acid and FL solutions (5 nmol in a 60 µl injection) were run every five runs and used to quantify components in the sample. FL standard was used to quantify both FL and the HRP. Valine was used as an internal standard to calculate the amount of BSA (nmol).

Quantification of total CML and CEL by gas chromatography-mass spectrometry (GC-MS)

Incubations were derivatized as described previously (Dunn et al., 1991). Briefly, sample (equivalent to 2 mg BSA) was reduced with $NaBH_4$ (2 M in 0.1 M NaOH; 100 µl) in borate buffer (0.2 M, pH 9.1; 2 ml) at room temperature for 4 h. Lysine, CML, CEL, [$^2H_8$]-lysine, [$^2H_4$]-CML and [$^2H_8$]-CEL standards were also reduced (Dunn et al., 1991). The reduced samples and standards were hydrolyzed in HCl (110 °C; 24 h) and the resulting amino acids converted to their *N*-trifluoroacetyl methyl esters (Dunn et al., 1991). GC-MS was performed on a Hewlett-Packard (HP) now Agilent (Palo Alto, CA) model 6890 gas chromatograph linked to a HP model 6890 mass selective detector, using a 30 m Rtx-5 (5% diphenyl-95% dimethyl polysineiloxane) capillary column (Restek, Bellefonte, PA). Sample and standards were analyzed in single ion monitoring mode. The following ions were monitored: m/z 180 (lysine), 187 ([$^2H_8$]-lysine), 392 (CML), 396 ([$^2H_4$]-CML), 379 (CEL) and 387 ([$^2H_8$]-CEL). The injection volume was 1 µl. The initial column temperature (130 °C) was held for 3 min and then increased to 180 °C over 12.5 min, further increased to 240 °C over 12 min, followed by a final increase to 290 °C over 8.33 min, giving a total run time of 35.83 min. Amounts of CML and CEL were normalized to the lysine content of the standards and samples by external standardization using calibration curves generated from solutions of constant lysine, but variable CML or CEL concentration. Thus, data were expressed initially as mol CML or CEL per mol lysine and finally as mol CML or CEL per mol BSA. This was achieved by dividing the amount of CML or CEL per mol Lysine obtained by GC-MS by the amount of lysine per mol BSA obtained for the same sample analyzed by ion-exchange chromatography.

Results

Site specificity of HRP and CML formation in fructose-modified BSA (BSA-F)

The reaction of fructose with the epsilon amino group of lysine residues results in HRP isomers, i.e., N^{ϵ}-(2-deoxyhexos-2-yl)lysine and N^{ϵ}-(1-deoxyhexos-1-yl)lysine (McPherson et al., 1988). The former accounts for ∼85% of the HRP (McPherson et al., 1988). A single modification was detected for each peptide, corresponding to a fructose adduct to the peptide, derived from N^{ϵ}-(2-deoxyhexos-2-yl)lysine or its isomers. We detected a single peak corresponding to the HRP peptide adduct of BSA in the incubations and this is most likely due to the predominant N^{ϵ}-(2-deoxyhexos-2-yl)lysine compound or a mixture of the isomers.

We detected the HRP at sixteen of the fifty-nine lysine residues of BSA incubated with fructose (BSA-F) (see BSA sequence in Fig. 1 and Table 1). This compares to fourteen sites of FL formation in glucose-modified BSA (BSA-G). Ten of the 16 sites of HRP formation in BSA-F were also sites of FL formation in BSA-G. Only Lysine-524 gave losses of its corresponding unmodified peptide of >20%, i.e., 31% for BSA-F at 14 d of incubation, compared to 76% for BSA-G. In addition, in BSA-G, modification at Lysine-275 gave ∼50% loss of peptide ^{276}GluCysCysAsp LysProLeuLeuGluLys^{285} (Glu276-Lys285) by 3 d of incubation, with no further loss between 3 and 14 d.

Formation of peptide ^{524}LysGlnThrAlaLeuValGlu LeuLeuLys^{533}, with Lysine-524 modified to the HRP ($Lys524_{HRP}$-Lys533) in BSA-F, increased between 3 and 7 d, followed by a decrease to 14 d (Fig. 2A). In BSA-G,

D_1TH**K**SEIAHRF$_{11}$**K**DLGEEHF**K**G$_{21}$LVLIAFSQYL$_{31}$QQCPFDEHV**K**$_{41}$LVNELTEFA**K**$_{51}$TC

VADESHAG$_{61}$CE**K**SLHTLFG$_{71}$DELC**K**VASLR$_{81}$ETYGDMADCC$_{91}$E**K**QEPERNEC$_{101}$FLSH**K**

DDSPD$_{111}$LP**K**L**K**PDPNT$_{121}$LCDEF**K**ADE**K**$_{131}$**K**FWG**K**YLYEI$_{141}$ARRHPYFYAP$_{151}$ELLYY

AN**K**YN$_{161}$GVFQECCQAE$_{171}$D**K**GACLLP**K**I$_{181}$ETMRE**K**VLAS$_{191}$SARQRLRCAS$_{201}$IQ**K**FG

ERAL**K**$_{211}$AWSVARLSQ**K**$_{221}$FP**K**AEFVEVT$_{231}$**K**LVTDLT**K**VH$_{241}$**K**ECCHGDLLE$_{251}$CADDR

ADLA**K**$_{261}$YICDNQDTIS$_{271}$S**K**L**K**ECCD**K**P$_{281}$LLE**K**SHCIAE$_{291}$VE**K**DAIPENL$_{301}$PPLTA

DFAED$_{311}$**K**DVC**K**NYQEA$_{321}$**K**DAFLGSFLY$_{331}$EYSRRHPEYA$_{341}$VSVLLRLA**K**E$_{351}$YEATL

EECCA$_{361}$**K**DDPHACYST$_{371}$VFD**K**L**K**HLVD$_{381}$EPQNLI**K**QNC$_{391}$DQFE**K**LGEYG$_{401}$FQNAL

IVRYT$_{411}$R**K**VPQVSTPT$_{421}$LVEVSRSLG**K**$_{431}$VGTRCCT**K**PE$_{441}$SERMPCTEDY$_{451}$LSLIL

NRLCV$_{461}$LHE**K**TPVSE**K**$_{471}$VT**K**CCTESLV$_{481}$NRRPCFSALT$_{491}$PDETYVP**K**AF$_{501}$DE**K**LF

TFHAD$_{511}$ICTLPDTE**K**Q$_{521}$I**KK**QTALVEL$_{531}$L**K**H**K**P**K**ATEE$_{541}$QL**K**TVMENFV$_{551}$AFVD**K**

CCAAD$_{561}$D**K**EACFAVEG$_{571}$P**K**LVVSTQTA$_{581}$LA

Fig. 1. Amino acid sequence of BSA. Lysine residues are shown in bold

Table 1. Summary of the modified lysine residues, following incubation with glucose or fructose for 14 d

Peptide sequence	Modified residue	BSA-F		BSA-G	
		HRP	CML	FL	CML
^{1}DTHKSEIAHR^{10}	Lysine-4		*		
^{11}FKDLGEEHFK^{20}	Lysine-12	*	*	*	
^{42}LVNELTEFAKTCVADESHAGCEK^{64}	Lysine-51	*			
^{52}TCVADESHAGCEKSLHTLFGDELCK^{76}	Lysine-64	*	*	*	
^{99}NECFLSHKDDSPDLPK^{114}	Lysine-106	*			
^{115}LKPDPNTLCDEFKADEK^{131}	Lysine-127	*	*	*	
^{174}GACLLPKIETMR^{185}	Lysine-180	*			
^{233}LVTDLTKVHK^{242}	Lysine-239	*	*		
^{262}YICDNQDISSKLK^{275}	Lysine-273	*	*	*	
^{274}LKECCDKPLLEK^{285}	Lysine-275	*	*	***	
^{363}DDPHACYSTVFDKLK^{377}	Lysine-375	*		*	
^{378}HLVDEPQNLIKQNLDQFEK^{396}	Lysine-388	*		*	
^{389}QNLDQFEKLGEYGFQNALIVR^{409}	Lysine-396	*	*	*	
^{413}KVPQVSTPTLVEVSR^{427}	Lysine-413			*	
^{428}SLGKVGTR^{435}	Lysine-431		*		
^{459}LCVLHEKTPVSEK^{471}	Lysine-465			*	
^{466}TPVSEKVTK^{474}	Lysine-471		*		
^{472}VTKCCTESLVNR^{483}	Lysine-474	*			
^{484}RPCFSALTPDETYVPKAFDEK^{504}	Lysine-499	*	*		
^{500}AFDEKLFTFHADICTLPDTEK^{520}	Lysine-504		*		
^{522}QIKK^{525}	Lysine-524	**	**	****	****
^{534}HKPKATEEQLK^{544}	Lysine-537	*		*	
^{538}ATEEQLKTVMENFVAFVDK^{556}	Lysine-544			*	
^{557}CCAADDKEACFAVEGPK^{573}	Lysine-563			*	

Modification resulted in losses of <20% (*), ca 30% (**), ca 50% (***) or ca 75% (****) loss of the corresponding unmodified peptide at 14 d of incubation

we observed that peptide ^{524}LysGlnThrAlaLeuValGluLeuLeuLys^{533} with Lysine-524 modified to FL ($Lys524_{FL}$-Lys533) formed rapidly up to 3 d (RA = 0.87), after which it increased at a slower rate resulting in an RA of 1.96 at 14 d (Fig. 2A). In addition, peptide ^{274}LeuLysGluCysCysAspLysProLeuLeuGluLys^{285} with Lysine-275 modified to FL (Leu274-$Lys275_{FL}$-Lys285) increased linearly to 7 d, followed by a small decrease to 14 d in BSA-G.

CML was formed in detectable amounts at thirteen of the fifty-nine lysine residues in BSA-F (Fig. 1 and Table 1). In comparison, only one of these residues (Lysine-524) was modified to CML in BSA-G. BSA-F, peptide ^{524}LysGlnThrAlaLeuValGluLeuLeuLys^{533} with Lysine-524 modified to CML ($Lys524_{CML}$-Lys533) shows a linear increase between 0 and 14 d of incubation, with no initial lag phase (Fig. 2B), in contrast to the kinetics of formation of peptide $Lys524_{CML}$-Lys533 in BSA-G (Fig. 2C). After 14 d of incubation, the yield of $Lys524_{CML}$-Lys533 was 16-fold greater in BSA-F (RA = 1.4), compared to BSA-G (RA = 0.09). The ratio, RA of $Lys524_{HRP}$-Lys533:RA of $Lys524_{CML}$-Lys533 was similar up to 7 d of incubation, i.e., 0.775:1 and 0.747:1 for 3 and 7 d, respectively. However, a decrease in the RA of peptide $Lys524_{HRP}$-Lys533, coupled with an increase in the RA of peptide $Lys524_{CML}$-Lys533 between 7 and 14 d, resulted in a 10-fold lower ratio, i.e., 0.07:1, at 14 d of incubation. Throughout incubation, the ratio $Lys524_{HRP}$-Lys533:$Lys524_{CML}$-Lys533 in BSA-F was much lower (<1:1) than the ratio for $Lys524_{FL}$-Lys533:$Lys524_{CML}$-Lys533 in BSA-G (>20:1).

There are two possible explanations for the decrease in peptide $Lys524_{HRP}$-Lys533 at 14 d in BSA-F. The first is that, after 7 d, peptide $Lys524_{HRP}$-Lys533 degrades to other

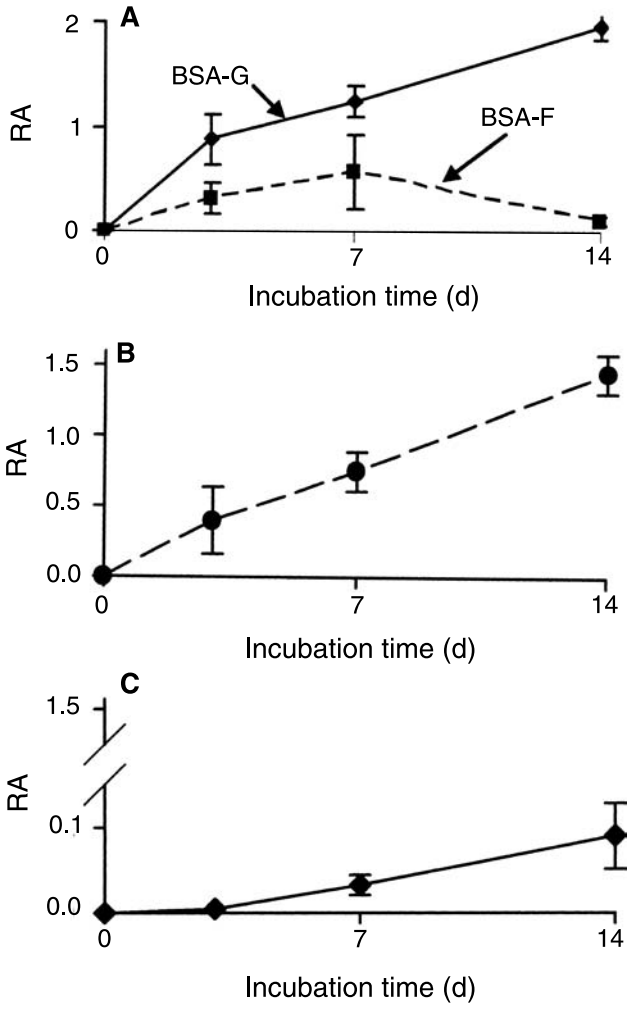

Fig. 2. Kinetics of change of FL, HRP and CML peptides at the major site of modification in BSA-fructose (BSA-F) and BSA-glucose (BSA-G) incubations. **A** HRP formation at Lysine-524 (peptide $Lys524_{HRP}$-Lys533) in BSA-F and FL formation at Lysine-524 (peptide $Lys524_{FL}$-Lys533) in BSA-G. **B** CML formation at Lysine-524 (peptide $Lys524_{CML}$-Lys533) in BSA-F. **C** CML formation at Lysine-524 (peptide $Lys524_{CML}$-Lys533) in BSA-G. Amounts are expressed as the relative amount (see Materials and methods). Data points are the means of duplicate incubations. Error bars represent the range

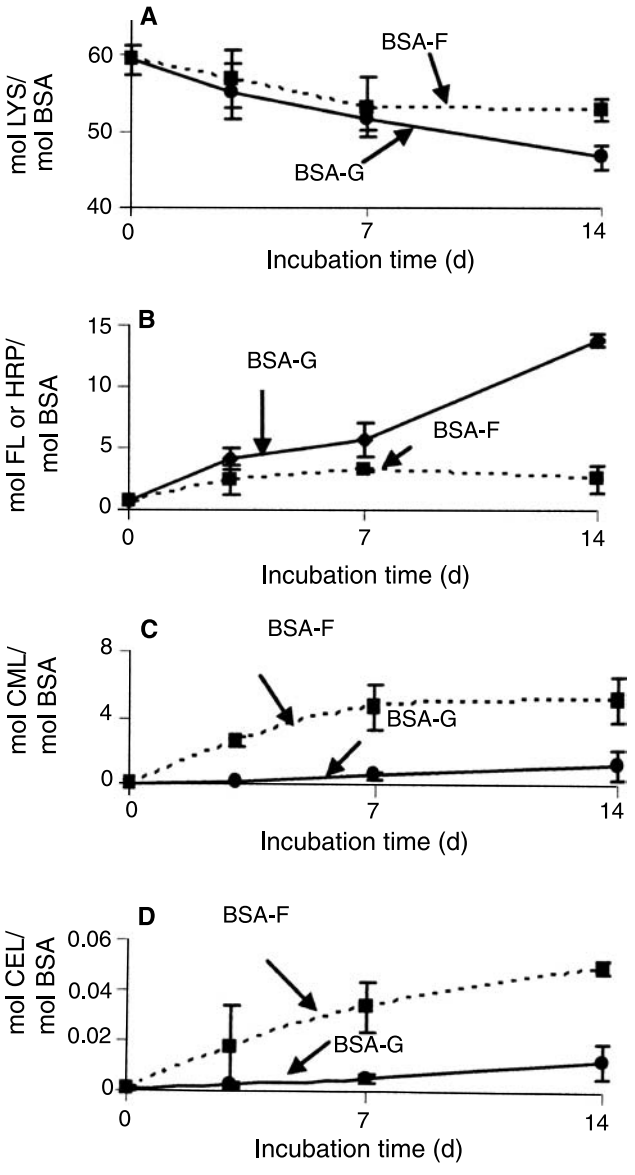

Fig. 3. Kinetics of change of total lysine, HRP, FL, CML and CEL in BSA-fructose (BSA-F) and BSA-glucose (BSA-G) incubations. **A** Total lysine loss. **B** Total HRP and FL formation. **C** Total CML formation. **D** Total CEL formation. Lysine, FL and HRP were quantified by ion-exchange chromatography and CML and CEL were quantified by GC-MS (see Materials and methods). Amounts are expressed as mol/mol BSA. Data points are the means of duplicate incubations. Error bars represent the range

products, e.g., oxidation to peptide $Lys524_{CML}$-Lys533. The second is that peptide $Lys524_{HRP}$-Lys533 is further modified, e.g., by glycation at Lys523 to form the peptide ^{523}LysLysGlnThrAlaLeuValGluLeuLeuLys^{533}, with Lysine-523 and Lysine-524 modified to HRP ($Lys523_{HRP}$-$Lys524_{HRP}$-Lys533). The former explanation is the more likely since neither peptide $Lys523_{HRP}$-$Lys524_{HRP}$-Lys533 nor peptide ^{523}LysLys^{524}, with Lysine-523 modified to HRP, could be detected. Neither HRP-nor CML-modified peptides were detected in the native or control samples.

Kinetics of loss of total lysine and formation of FL, HRP, CML and CEL

Figure 3A confirms that on average, all fifty-nine lysine residues were unmodified in the native protein. Losses for BSA-F were 2, 6 and 6 mol Lysine/mol BSA after 3, 7 and 14 d, respectively. In contrast, for BSA-G, the loss of lysine was 4, 7 and 14 mol lysine/mol BSA at the same timepoints.

The formation of the HRP was only 2.5–3 mol/mol BSA at each timepoint (Fig. 3B), suggesting that up to 58% of the lysine lost in BSA-F after 7 d is due to modifications other than the HRP. In comparison, the formation of FL in BSA-G was 4, 6 and 14 mol FL/mol BSA after 3, 7 and 14 d, respectively (Fig. 3B) and therefore accounts for virtually all of the loss of lysine.

The yield of CML in BSA-F was up to 17-fold higher than that in BSA-G (Fig. 3C). A lag phase is apparent for BSA-G but not for BSA-F. After 3 d, the amount of CML in BSA-F was 2.59 mol/mol BSA, compared to 0.15 mol/mol BSA for BSA-G. These values increased to 4.71 and 0.59 mol CML/mol BSA, respectively, for BSA-F and BSA-G, between 3 and 7 d. At 14 d, the amount of CML had begun to plateau for BSA-F but increased to 1.26 mol/mol BSA, for BSA-G. The sum of total HRP and total CML formed in BSA-F accounted for the observed loss of lysine at each time point.

The yield of total CEL increased throughout the incubation period for both BSA-F and BSA-G (Fig. 3D). Total CEL was up to 5-fold higher in BSA-F compared to BSA-G at every time point. For example, at 14 d, CEL formation is ~4.5-fold greater in BSA-F, compared to BSA-G. This is in line with fructose or fructose-lysine adducts (e.g., the Schiff base) being more readily degraded to dicarbonyl compounds, compared to glucose and glucose- lysine adducts (Heyns et al., 1967). However, the values obtained for CEL are about 100-fold lower than those obtained for CML.

Discussion

Quantification of peptide modification by LC-MS

In order to make valid comparisons of the amounts of different native tryptic peptides formed on digestion of a protein, each peptide would have to produce a similar response in the mass spectrometer. Many factors influence mass spectrometer response, including degree of peptide ionization. The degree of ionization within an electrospray source depends on several factors including the extent to which the analyte exists as an ion in solution, the extent to which the analyte is present on the surface of the droplet formed on the ESI needle (where it is more easily desorbed from the droplet), and the mechanism by which a condensed ion is converted to a gas phase ion (Pam and McLuckey, 2003). The coupling of LC to ESI-MS contributes additional variables that affect the final MS response. These include chromatographic elution time when operating a solvent gradient, since this affects the solvent composition in which the analyte is introduced into the ESI. Therefore, a high MS response for one peptide does not necessarily mean that it is more abundant than another peptide and a comparison of RA values obtained for different peptides may be misleading.

To overcome these issues, modification at the various lysine residues was estimated by calculating the RA value (to account for the differences in amount of protein between samples) and monitoring the percentage loss of each relevant unmodified peptide. The disadvantage of this approach is that it provides no information about the type of modification on a specific amino acid residue of the protein. The C-terminal peptide of BSA, (Leu574-Ala583), was chosen as an internal standard for calculation of RA values as it contains no reactive lysine or arginine groups and, therefore, loss of the native C-terminal peptide will only occur following modification at the preceding lysine (Lysine-573). Preliminary experiments resulted in no detectable modification at Lysine-573. We have used this approach in a related study (Cotham et al., 2004).

Early glycation

For the first time using any protein, the site specificity of the HRP formed from fructose has been shown to share the site specificity of FL from glucose, with both preferentially modifying Lysine-524 of BSA. The ease of glycation by glucose at Lysine-525 of HSA has been hypothesized to be a result of its location in a sequence of basic amino acids (Iberg and Flückinger, 1986). Some of the other lysine residues of BSA are also located in a basic microenvironment e.g., Lysine-535, in a LysHisLys sequence, but are not preferentially modified. Therefore, additional factors may also play a role in the preferential modification of Lysine-524 in BSA, including accessibility of Lysine-524, effects of bound ligands and location on the surface of the protein molecule.

Reduction of the two N^{ϵ}-(2-deoxyhexos-2-yl)lysine forms of the HRP, i.e., N^{ϵ}-(2-deoxyglucos-2-yl)lysine and N^{ϵ}-(2-deoxymannos-2-yl)lysine, gives 2-(mannitol)-lysine and 2-(glucitol)lysine (McPherson et al., 1988), that were found to possess the same retention times as the reduced forms of FL when analyzed by ion-exchange chromatography. McPherson et al. (1988) have demonstrated that a small amount of FL is formed from the incubation of fructose with BSA. This would be measured as HRP in our analyses.

Formation of the HRP in BSA-F and FL in BSA-G shared similar site specificity, with ten of the sixteen sites of HRP formation in BSA-F also being sites of FL formation in BSA-G (Table 1). Previous studies conducted on the

site specificity of fructose modification of proteins have only reported on Schiff base formation (e.g., Pennington and Harding, 1994; Zhao et al., 1996). The N-terminal amino group of bovine γ-II-crystallin has been described as the main site of Schiff base formation, following incubation with both glucose (Casey et al., 1995) and fructose (Pennington and Harding, 1994). Zhao et al. (1996), reported that Schiff base formation occurred at 8 of the 13 lysine residues of βB_2-crystallin following modification by either glucose or fructose, but that Schiff base formation was detected at an additional three residues in the fructose incubations. Thus, it seems that glucose and fructose have similar site specificity for Schiff base formation.

Advanced glycation

The kinetics of formation of CML at Lysine-524 involved an initial lag phase for BSA-G but not for BSA-F. This suggests that the step prior to CML formation, i.e., oxidation of the early glycation product FL or HRP, or dicarbonyl formation, occurs at a faster rate in BSA-F, compared to BSA-G. This is supported by the HRP having a faster rate of degradation compared to FL (Heyns et al., 1967).

The HRP and CML were formed at nine common sites and thus CML could be formed by oxidation of the HRP at these residues. CML formation at the four sites, i.e., Lysine-4, Lysine-431, Lysine-471 and Lysine-504, at which no HRP could be detected may be due to either oxidation of the HRP to CML resulting in the HRP being present at below the limit of detection or GO formed from fructose/Schiff base oxidation reacting directly with the lysine residues (Glomb and Monnier, 1995).

After 14 d incubation of BSA-F, peptide $Lys524_{CML}$-Lys533 produced ~140-fold greater response in the mass spectrometer than peptide $Lys524_{HRP}$-Lys533. Peptides $Lys524_{CML}$-Lys533 and $Lys524_{HRP}$-Lys533 differ only in the nature of the adduct at Lysine-524 and this may result in different mass spectrometer response for equivalent amounts of these peptides. However, it would be unlikely that such a small change in structure would cause a >100-fold difference in response. The more likely reason is that peptide $Lys524_{CML}$-Lys533 is far more abundant than peptide $Lys524_{HRP}$-Lys533, in BSA-F and the loss of the corresponding unmodified peptide Gln525-Lys533, resulting from modification at Lysine-524, is largely the result of CML formation, rather than formation of the HRP. To our knowledge, this study reports the first comparison of sugar-dependent site specificity of an AGE (CML) on any protein.

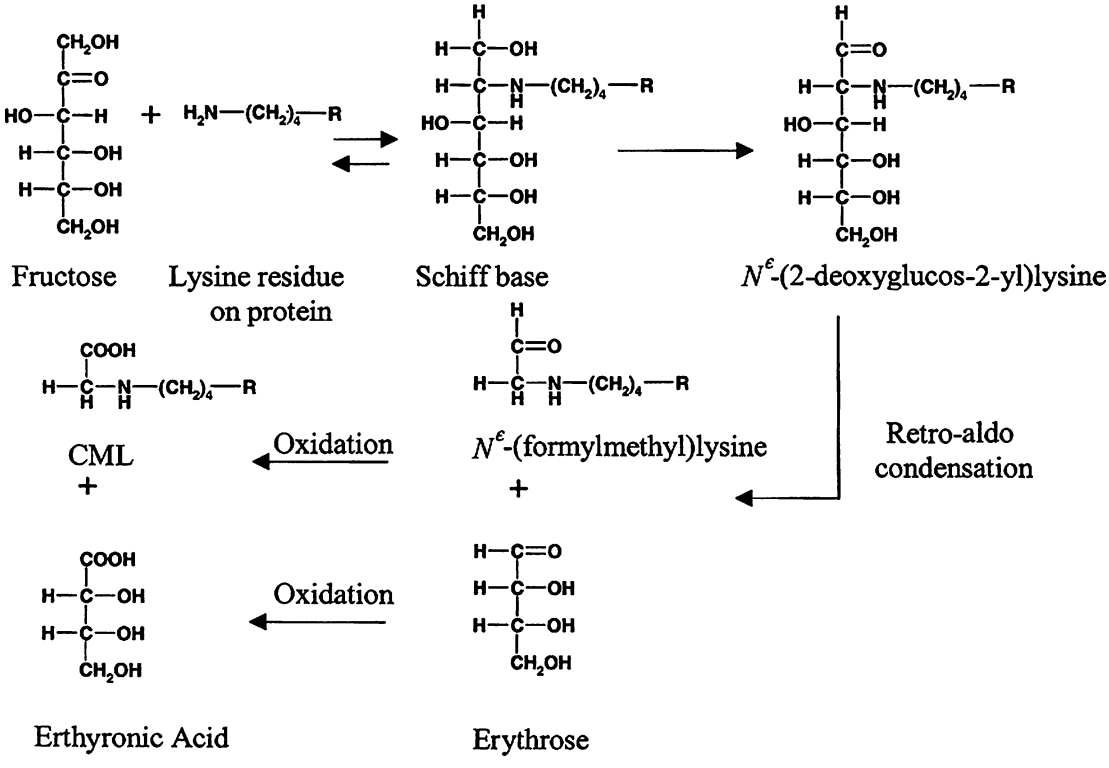

Fig. 4. Proposed reaction pathway for the formation of CML from the HRP

No mechanism has previously been reported for the formation of CML from the HRP. We propose that CML formation can occur by retroaldol condensation between C_1 and C_2 of the N^{ε}-(2-deoxyglucos-2-yl)lysine form of the HRP, followed by oxidation of the N^{ε}-(formylmethyl)lysine (Fig. 4). Erythronic acid is produced as a side product. We suggest that the formation of CML from the HRP is favored over formation of CML via oxidation of FL. This would explain the higher yields of CML from fructose compared to glucose. The plateauing of free lysine loss in BSA-F observed between 7 and 14 d could be due to conversion of HRP to AGEs, such as CML. The continued loss of lysine over the same time interval in BSA-G might be due to a faster rate of sugar condensation and/or a slower rate of conversion of FL to CML. In support of this hypothesis, a greater rate of modification of Lysine-524 by glucose compared to fructose was observed by LC-MS while >15 times more $Lys524_{CML}$-Lys533 was observed in BSA-F. Under anaerobic conditions, the N^{ε}-(formylmethyl)lysine would be reduced to N^{ε}-(hydroxyethyl)lysine, which was not detected in our aerobic incubations.

CEL formed at a faster initial rate and yields were higher in BSA-F compared to BSA-G, in line with fructose being more readily degraded to dicarbonyl compounds in solution, compared to glucose (Sakai et al., 2002). McPherson et al. (1988) suggest that the aldehyde group of the open chain form of glucose is more electrophilic than the keto group of fructose and therefore the condensation of the glucose with the epsilon group of lysine residues would be favored over its oxidation to dicarbonyl compounds. Oxidation of fructose and glucose as well as the Schiff base yields 3-deoxyglucosone (3-DG) (Shin et al., 1988; Thornalley et al., 1999) which may further degrade to methylglyoxal (MGO) (Thornalley et al., 1999) a precursor of CEL. Lysozyme incubated with fructose at physiological temperature and pH, forms over twice the amount of 3-DG, compared to equivalent incubations with glucose (Shin et al., 1988). CEL is unlikely to form directly from any of the isomeric forms of the HRP because the hydroxymethyl group formed as a result of cleavage between C_3 and C_4 would have to convert to a methyl group and this is unlikely to occur. The amount of CEL, even in BSA-F at 14 d, is extremely low (0.05 mol CEL/mol BSA) compared to that of CML (5.3 mol CML/mol BSA) suggesting that modification of lysine residues of BSA by 3-DG or MGO during incubation is not substantial.

Knowledge of preferentially modified amino acid residues, how this differs for different sugars, the degree of site specificity of modification and the mechanisms involved have important implications for the design of inhibitory therapeutic strategies *in vivo* (Cohen, 2003). For example, Lysine-199, one of the major glycation sites of HSA (Iberg and Flückinger, 1986), plays a key role in the binding of the anti-inflammatory drug 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid (van Boekel et al., 1992). Such binding has been shown to result in a decrease in glycation of HSA incubated with glucose-6-phosphate *in vitro* (van Boekel et al., 1992), presumably via competitive binding to a favored site of glycation.

In conclusion, this work has shown for the first time that (1) early and advanced glycation of BSA by fructose results in similar site specificity as that of glucose and (2) yields of CML are higher from fructose than from glucose, under the conditions applied. This has implications for the importance of fructose as an AGE precursor *in vivo*. In particular, lens crystallin oligmerization is implicated in the formation of cataracts. The accumulation of fructose in the lens suggests that this sugar may be an important precursor of such crosslinking, as well as non-crosslinking, AGEs in that organ.

Saxena et al. (2000) have proposed that ascorbate is the main precursor of AGEs, including CML, in the lens and have demonstrated that CML-proteins immunoprecipitated from cataractous lens are able to oxidize ascorbate ~4 times faster than proteins isolated from normal lens. Due to the ability of fructose to carboxymethylate protein at a faster rate compared to glucose, we hypothesise that the primary function of fructose in the lens is as a precursor of AGE-protein that subsequently catalyses the ascorbylation of lens proteins.

Acknowledgements

This work was supported by a studentship from the Biotechnology and Biological Sciences Research Council, UK (DJSH). Instrumental analysis was carried out in the laboratory of John Baynes and Suzanne Thorpe (University of South Carolina). We are very grateful to them for their hospitality and numerous helpful suggestions. William Cotham, Andrzej Januszewski and Jonathan Brock (University of South Carolina) are thanked for technical assistance. Harry Nursten (The University of Reading) is thanked for helpful discussions and assistance.

References

Ahmed MU, Thorpe SR, Baynes JW (1986) Identification of N-epsilon-(carboxymethyl)lysine as a degradation product of fructoselysine in glycated protein. J Biol Chem 261: 4889–4894

Ahmed MU, Brinkmann-Frye E, Degenhardt TP, Thorpe SR, Baynes JW (1997) N^{ε}-(Carboxyethyl)lysine, a product of the chemical modifica-

tion of proteins by methylglyoxal, increases with age in humen lens proteins. Biochem J 324: 565–570

van Boekel MAM, Hoenders HJ (1992) Glycation of crystallins in lenes from aging and diabetic individuals. FEBS Lett 314: 1–4

Brock JWC, Hinton DJS, Cotham WE, Metz TO, Thorpe SR, Baynes JW, Ames JM (2003) Proteomic analysis of the site specificity of glycation and carboxymethylation of ribonuclease. J Proteome Res 2: 506–513

Casey EB, Zhao HR, Abraham EC (1995) Role of glycine 1 and Lysine 2 in the glycation of bovine γB-crystallin. J Biol Chem 270: 20781–20786

Cohen MP (2003) Intervention strategies to prevent pathogenic effects of glycated albumin. Arch Biochem Biophys 419: 25–30

Cotham WE, Metz TO, Ferguson PL, Brock JWC, Hinton DJS, Thorpe SR, Baynes JW, Ames JM (2004) Proteomic analysis of arginine adducts on glyoxal-modified ribonuclease. Mol Cell Proteomics 3: 1145–1153

Dunn JA, Patrick JS, Thorpe SR, Baynes JW (1989) Oxidation of glycated proteins: age-dependent accumulation of N^{ε}-(carboxymethyl)lysine in lens proteins. Biochemistry 28: 9464–9468

Dunn JA, McCance DR, Thorpe SR, Lyons TJ, Baynes JW (1991) Age-dependent accumulation of N^{ε}-(carboxymethyl)lysine and N^{ε}-(carboxymethyl)hydroxylysine in human skin collagen. Biochemistry 30: 1205–1210

Gabbay KH, Kinoshita JH (1972) Mechanism of development and possible prevention of sugar cataracts. Isr J Med Sci 8: 1557–1561

Garlick RL, Mazer JS (1983) The principal site of nonenzymatic glycosylation of human serum albumin in vivo. J Biol Chem 258: 6142–6146

Glomb MA, Monnier VM (1995) Mechanism of protein modification by glyoxal and glycoaldehyde, reactive intermediates of the Maillard reaction. J Biol Chem 270: 10017–10026

Heaf DJ, Galton DJ (1975) Sorbitol and other polyols in lens, adipose tissue and urine in diabetes mellitus. Clin Chim Acta 63: 41–47

Hers HG (1956) Le mécanisme de la transformation de glucose en fructose par les vésicules séminales. Biochim Biophys Acta 22: 202–203

Heyns K, Müller G, Paulsen H (1967) Quantitative investigation of the reaction of hexoses with amino acids. Justus Liebigs Ann Chem 703: 202–214

Iberg N, Flückinger R (1986) Nonenzymic glycosylation of albumin in vivo: Identification of multiple glycosylation sites. J Biol Chem 261: 13542–13545

Jedziniak JA, Chylack LT Jr, Cheng HM, Gillis MK, Kalustian AA, Tung WH (1981) The sorbitol pathway in the human lens: aldose reductase and polyol dehydrogenase. Invest Ophthalmol Vis Sci 20: 314–326

Lapolla A, Fedele D, Reitano R, Aricò NC, Seraglia R, Traldi P, Marotta E, Tonani R (2004) Enzymatic digestion and mass spectrometry in the study of advanced glycation end products/peptides. Am J Mass Spectrom 15: 496–509

Leonil J, Molle D, Fauquant J, Maubois JL, Pearce RJ, Bouhallab S (1997) Characterization by ionisation mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. J Dairy Sci 80: 2270–2281

Macdonald I, Keyser A, Pacy D (1978) Some effects in man, of varying the load of glucose, sucrose, fructose or sorbitol on metabolites in blood. Am J Clin Nutr 31: 1305–1311

Makino H, Shikata K, Kushiro M, Hironaka K, Yamasaki Y, Sugimoto H, Ota Z, Araki N, Horiuchi S (1996) Roles of advanced glycation end-products in the progression of diabetic nephropathy. Nephrol Dial Transpl 11: 76–80

Marotta E, Lapolla A, Fedele D, Senesi A, Reitano R, Witt M, Seraglia R, Traldi P (2003) Accurate mass measurements by Fourier transform mass spectrometry in the study of advanced glycation end products/peptides. J Mass Spectrom 38: 196–205

McPherson JD, Shilton BH, Walton DJ (1988) Role of fructose in glycation and cross-linking of proteins. Biochemistry 27: 1901–1907

Nakamura Y, Horii Y, Nishino T, Shiiki H, Sakaguchi Y, Kagoshima T, Dohi K, Makita Z, Vlassara H, Bucula R (1993) Immunohistochemical localization of advanced glycosylation endproducts in coronary atheroma and cardiac tissue in diabetes-mellitus. Am J Pathol 143: 1649–1656

Ortwerth BJ, Slight SH, Prabhakaram M, Sun Y, Smith JB (1992) Site-specific glycation of lens crystallins by ascorbic acid. Biochim Biophys Acta 1117: 207–215

Pam P, McLuckey SA (2003) Electrospray ionization of protein mixtures at low pH. Anal Chem 75: 1491–1499

Pennington J, Harding JJ (1994) Identification of the site of glycation of γ-II-crystallin by (^{14}C)-fructose. Biochim Biophys Acta 1226: 163–167

Reddy S, Bichler J, Wells-Knecht KJ, Thorpe SR, Baynes J (1995) N-Epsilon-(carboxymethyl)lysine is a dominant advanced glycation endproduct (AGE) antigen in tissue proteins. Biochemistry 34: 10872–10878

Ruttkat A, Ebersdobler HF (1995) N^{ε}-Carboxymethyllysine is formed during heating of lysine with ketoses. J Sci Food Agric 68: 261–263

Sakai M, Oimomi M, Kasuga M (2002) Experimental studies on the role of fructose in the development of diabetic complications. Kobe J Med Sci 48: 125–136

Saxena P, Saxena AK, Cui XL, Obenovich M, Gudipaty K, Monnier VM (2000) Transition metal-catalyzed oxidation of ascorbate in human cataract extracts: possible role of advanced glycation end products. Invest Opthalmol Vis Sci 41: 1473–1481

Shin DB, Hayase F, Kato H (1988) Polymerization of proteins caused by reaction with sugars and the formation of 3-deoxyglucosone under physiological conditions. Agric Biol Chem 52: 1451–1458

Smith MA, Taneda S, Richey PL, Miyata S, Yan S-D, Stern D, Sayre LM, Monnier VM, Perry G (1994) Advanced Maillard reaction end products are associated with Alzheimer's disease pathology. Proc Natl Acad Sci USA 91: 5710–5714

Suarez G, Rajaram R, Oronsky AL, Gawinowicz MA (1989) Nonenzymic glycation of bovine serum albumin by fructose (fructation). Comparison with the Maillard reaction initiated by glucose. J Biol Chem 264: 3674–3679

Thornalley PJ, Langborg A, Minhas HS (1999) Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J 344: 109–116

Valencia JV, Weldon SC, Quinn D, Kiers GH, DeGroot J, TeKoppele JM, Hughes TE (2004) Advanced glycation end product ligands for the receptor for advanced glycation end products: biochemical characterization and formation kinetics. Anal Biochem 324: 68–78

Wells-Knecht MC, Thorpe SR, Baynes JW (1995) Pathways of formation of glycoxidation products during glycation of collagen. Biochemistry 34: 15134–15141

Yeboah FK, Alli I, Yaylayan VA (1999) Reactivities of D-glucose and D-fructose during glycation of bovine serum albumin. J Agric Food Chem 47: 3164–3172

Zhao HR, Smith JB, Jiang XY, Abraham EC (1996) Sites of glycation of beta B-2-crystallin by glucose and fructose. Biochem Biophys Res Commun 229: 128–133

Authors' address: Dr. D. J. S. Hinton, Hugh Sinclair Unit of Human Nutrition, School of Food Biosciences, The University of Reading, Whiteknights, Reading, RG6 6AP, U.K.,
Fax: +44-118-931-0080, E-mail: d.j.s.hinton@reading.ac.uk