by inhibiting cellular enzymes (Guranovski et al., 1987; Suzuki et al., 1987; Yount, 1975).

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REFERENCES

Bass, M. B., & Fromm, H. J. (1985) *Anal. Biochem. 145*, 292–303.

Brevet, A., Coste, H., Fromant, M., Plateau, P., & Blanquet, S. (1987) *Biochemistry 26*, 4763–4768.

Gadian, D. G., Radda, G. K., Dawson, M. J., & Wilkie, D. R. (1982) in *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions* (Nuccitelli, R., & Deamer, D. W., Eds.) pp 61–77, Liss, New York.

Gillies, R. J., Alger, J. R., Den Hollander, J. A., & Shulman, R. G. (1982) in *Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions* (Nuccitelli, R., & Deamer, D. W., Eds.) pp 79–104, Liss, New York.

Granot, J., Mildvan, A. S., Bramson, H. N., & Kaiser, E. T. (1980) *Biochemistry 19*, 3537–3543.

Guranowski, A., Biryukov, A., Tarussova, N. B., Khomutov, R. M., & Jabukowski, H. (1987) *Biochemistry 26*, 3425–3429.

Jentoft, J. E., & Town, C. D. (1985) *J. Cell Biol. 101*, 778–784.

Kay, R. R., Gadian, D. G., & Williams, S. R. (1986) *J. Cell Sci. 83*, 163–179.

Klein, G., & Satre, M. (1986) *Biochem. Biophys. Res. Commun. 138*, 1146–1152.

Martin, J.-B., Foray, M.-F., Klein, G., & Satre, M. (1987) *Biochim. Biophys. Acta 931*, 16–25.

Pople, J. A., Schneider, W. G., & Bernstein, H. J. (1959) *High-Resolution Nuclear Magnetic Resonance*, pp 138–151, McGraw-Hill, New York.

Satre, M., & Martin, J.-B. (1985) *Biochem. Biophys. Res. Commun. 132*, 140–146.

Satre, M., Klein, G., & Martin, J.-B. (1986) *Biochimie 68*, 1253–1261.

Schliselfeld, L. H., Burt, C. T., & Labotka, R. J. (1982) *Biochemistry 21*, 317–320.

Smirnova, I. N., Baykov, A. A., & Avaeva, S. M. (1986) *FEBS Lett. 206*, 121–124.

Suzuki, H., Tanaka, Y., Buonamassa, D. T., Farina, B., & Leone, E. (1987) *Mol. Cell. Biochem. 74*, 17–20.

Tarussova, N. B., Shumiyanzeva, V. V., Krylov, A. C., Karpeisky, M. Ya., & Khomutov, R. M. (1983) *Bioorg. Khim. 9*, 838–843.

Vogel, H. J., & Bridger, W. A. (1982) *Biochemistry 21*, 394–401.

Watts, D. J., & Ashworth, J. M. (1970) *Biochem. J. 119*, 171–174.

Yount, R. G. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 1–56.

Zamecnik, P. (1983) *Anal. Biochem. 134*, 1–10.

Role of Fructose in Glycation and Cross-Linking of Proteins†

John D. McPherson, Brian H. Shilton, and Donald J. Walton*

Department of Biochemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6

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ABSTRACT: Incubation of carbohydrate-free human serum albumin (HSA) with fructose in an aqueous buffer at pH 7.4 resulted in glycation of ε-amino groups of lysyl residues. A recently developed procedure, involving analysis of hexitol amino acids by high-performance liquid chromatography of phenylthiocarbamyl derivatives, was used to show that 85% of the bound hexose was attached to protein via carbon 2 (C-2). The remainder was attached to protein via carbon 1 (C-1). When incubations were conducted with glucose under identical conditions, all the hexose was attached via C-1. Examination of human ocular lens proteins showed that the majority of the covalently bound hexose was connected to ε-amino groups of lysyl residues via C-1; this was attributed mainly to nonenzymatic glucosylation in vivo, which has already been documented. A significant proportion (10–20%) of the bound hexose was connected via C-2. In view of the HSA–hexose incubation results (above), this indicated that the lens proteins had reacted with endogenous fructose; i.e., they had undergone nonenzymatic fructosylation in vivo. The model protein bovine pancreatic ribonuclease A reacted with fructose and glucose at similar rates under physiological conditions. However, covalent, non-disulfide cross-linking, which could be inhibited by D-penicillamine, was induced 10 times more rapidly by fructose than by glucose. It is postulated that some of the protein cross-linking that occurs in vivo is fructose-induced. The possible significance of these processes in diabetic subjects is discussed.

The amino groups of some mammalian proteins react nonenzymatically with glucose, in vivo, to give Schiff bases such as I (Figure 1), which then undergo an Amadori rearrangement to form *N*-(1-deoxyfructos-1-yl) groups (II; Krantz et al., 1986). Subsequent reactions may result in the formation of cross-linked, fluorescent, protein derivatives (Elbe et al., 1983; Pongor et al., 1984), which may account for some of the complications of diabetes, such as cataract formation (Monnier et al., 1979), stiffening of collagen (Vishwanath et al., 1986), and vascular narrowing (Cerami et al., 1986).

In some organs, such as the ocular lens and peripheral nerves, fructose is biosynthesized by oxidation of sorbitol in

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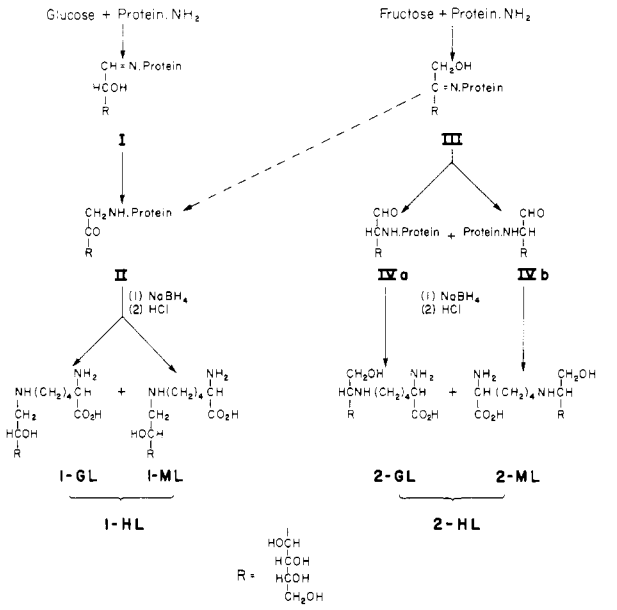


FIGURE 1: Reaction scheme, showing glucosylation and fructosylation of a protein. The degree of glycation of ϵ-amino groups of lysyl residues can be determined by borohydride treatment and hydrolysis of the protein, followed by analysis of 1-HL (from II) and 2-HL (from IVa and IVb).

a reaction catalyzed by polyol dehydrogenase (Gabbay, 1973). In diabetic subjects, concentrations of fructose often approach, and sometimes exceed, those of glucose in ocular lenses (Varma et al., 1979; Jedziniak et al., 1981; Lerner et al., 1984) and in nerves (Dyck et al., 1984; Mayhew et al., 1983).

It has been shown that fructose reacts with proteins in neutral aqueous solutions at 37 °C (Lee et al., 1979; Bunn & Higgins, 1981) and that colored, fluorescent, derivatives are formed (Suarez et al., 1984, 1986). Therefore, by analogy with glucosylation, it is conceivable that nonenzymatic fructosylation of proteins occurs in some mammalian tissues and that this leads to cross-linking which contributes to diabetic complications. However, this hypothesis has received little attention so far.

In nonphysiological solvents, such as dimethyl sulfoxide and acetic acid, ϵ-amino groups of lysyl residues of proteins react with fructose and acquire N^{ϵ}-(2-deoxyglucos-2-yl) groups (IVa) by a Heyns rearrangement of the Schiff base (III) (Heyns & Rolle, 1959). Until now there has been no proof that these derivatives are formed in aqueous solutions under physiological conditions.

We have now reexamined this subject and present a study in which we have established that human serum albumin (HSA) can be nonenzymatically fructosylated in an aqueous solution at pH 7.4 and 37 °C. Analysis of glycated amino acids was used to show that 85% of the attached hexose is connected to ϵ-amino groups of lysyl residues via carbon 2 (C-2) of the hexose chain, the remainder being connected via carbon 1 (C-1). We also present evidence that human ocular proteins possess small quantities of hexoses connected to lysyl residues via C-2, which suggests that these proteins were nonenzymatically fructosylated in vivo. In addition, using pancreatic ribonuclease A (RNase) as a model protein, we have established a connection between nonenzymatic fructosylation, development of fluorescence, and formation of covalent, non-disulfide, cross-links. The latter were formed more rapidly by fructose than by glucose.

Brownlee et al. (1986) reported that the experimental drug aminoguanidine hydrochloride blocks hexose-induced cross-linking of collagen, in vivo, by combining with carbonyl groups of bound hexoses. We report that D-penicillamine, which is used frequently for the treatment of rheumatic diseases (Howard-Lock et al., 1986), inhibits hexose-induced cross-linking of RNase by a similar mechanism.

Materials and Methods

Materials

Bovine pancreatic ribonuclease A, type XII-A (RNase), and D-penicillamine were obtained from the Sigma Chemical Co. Human nonglyco serum albumin (nonglyco-HSA; completely free of bound hexose) was prepared by removal of the glycated component of HSA (Sigma Chemical Co.) with agarose–phenylboronate (Glyco-Gel B, Pierce Chemical Co., Rockford, IL) as described by Flückiger et al. (1984). Human eye lenses were obtained from the local hospital and the Eye Bank of Canada, Toronto. Water-soluble and -insoluble protein fractions were isolated as described by Garlick et al. (1984). Labeled hexoses were purified by TLC[1] on cellulose, in pyridine/ethyl acetate/water/acetic acid (5:5:3:1 v/v). Other chromatographic solvents were as follows (v/v for solvents A–C): (A) 1-propanol/5.9 M ammonia, 3:1; (B) 1-propanol/5.9 M ammonia, 4:1; (C) acetonitrile/water, 3:2; (D) sodium acetate buffer [70 mM in acetate; containing triethylamine (0.5 mL/L); pH as specified under Results].

Synthesis of Reference Compounds

NMR and TLC. ^{1}H NMR spectroscopy, at 400 MHz, of deuterium oxide solutions of *N*-(2-deoxyhexitol-2-yl) amino accids was performed by T. Hvidt, using a Bruker AM 400 spectrometer. It was assumed that, of the eight hexitol protons, H-2 would resonate at the highest field. The other signals were assigned by spin–spin decoupling and by establishment of proton connectivities by 2-D correlation spectroscopy. R_G refers to the rate of migration, on silica gel G TLC (solvent A), relative to that of D-glucose. Compounds were visualized with alkaline $KMnO_4$ (Briggs et al., 1956).

N^{ϵ}-(1-Deoxy-glucitol-1-yl)-L-lysine (1-GL) and N^{ϵ}-(1-deoxy-D-mannitol-1-yl)-L-lysine (1-ML) were prepared as described previously (Walton et al., 1984).

N^{ϵ}-(2-Deoxy-D-glucitol-2-yl)-L-lysine (2-GL). N^{ϵ}-(2-Deoxy-D-glucos-2-yl)-L-lysine [prepared according to Heyns and Noack (1962)] was reduced with $NaBH_4$. The product was purified on a column of silica gel, with solvents B and A successively, and had R_G 0.65 and $[\alpha]^{22}_D$ +18° (*c* 2.0, 6 N HCl). The NMR parameters for 2-GL were compared (Tables I and II) with those of *N*-(2-deoxy-D-glucitol-2-yl)glycine, R_G 0.17 and $[\alpha]^{22}_D$ +11° (*c* 2.1, 6 N HCl), and *N*-(2-deoxy-D-mannitol-2-yl)glycine, R_G 0.19 and $[\alpha]^{22}_D$ 0° (*c* 2.1, 6 N HCl). [The hexitol glycine epimers were prepared by $NaBH_4$ reduction of the *N*-(2-deoxy-D-glucos-2-yl) and *N*-(2-deoxy-D-mannos-2-yl) derivatives of glycine, which have established configurations (Heyns et al., 1957).] Except for the H-2 chemical shift, the NMR parameters of all the deoxyhexitolyl protons of *N*-(2-deoxy-D-glucitolyl)glycine were similar to those of 2-GL, thus confirming that the latter had the *gluco* configuration.

Methods

Incubation of Proteins with Hexose. Solutions of HSA or RNase (20 mg/mL) and hexose (0.5 M; sometimes including 10–20 μCi/mL [U-^{14}C]hexose) in 0.2 M sodium phosphate

[1] Abbreviations: 1-GL, N^{ϵ}-(1-deoxy-D-glucitol-1-yl)-L-lysine; 1-ML, N^{ϵ}-(1-deoxy-D-mannitol-1-yl)-L-lysine; 1-HL, mixture of 1-GL and 1-ML; 2-GL, N^{ϵ}-(2-deoxy-D-glucitol-2-yl)-L-lysine; 2-ML, N^{ϵ}-(2-deoxy-D-mannitol-2-yl)-L-lysine; 2-HL, mixture of 2-GL and 2-ML; PTC, *N*-phenylthiocarbamyl; TLC, thin-layer chromatography.

Table I: ^{1}H NMR Chemical Shifts of Hexitol Amino Acids[a]

proton	*N*-(2-deoxy-D-glucitol-2-yl)glycine	*N*-(2-deoxy-D-mannitol-2-yl)glycine	2-GL
H-1A	3.92	4.03	3.92
H-1B	3.84	3.91	3.83
H-2	3.51	3.59	3.40
H-3	4.18	4.27	4.15
H-4	3.66	3.69	3.65
H-5	3.76	3.76	3.76
H-6A	3.83	3.82	3.84
H-6B	3.65	3.65	3.66
H-α			3.72
H-αA	3.79	3.79	
H-αB	3.69	3.74	
H-β			1.89
H-γ			1.49
H-δ			1.75
H-ϵA			3.17
H-ϵB			3.05

[a] In ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate.

Table II: ^{1}H NMR Coupling Constants (Hz) for Hexitol Amino Acids

	N-(2-deoxy-D-glucitol-2-yl)glycine	*N*-(2-deoxy-D-mannitol-2-yl)glycine	2-GL[a]
$J_{1A,1B}$	12.6	12.3	12.6
$J_{1A,2}$	4.1	4.3	4.2
$J_{1B,2}$	4.9	6.7	5.0
$J_{2,3}$	6.8	3.0	7.8
$J_{3,4}$	<1.0	<1.0	<1.0
$J_{4,5}$	9.3	8.1	9.1
$J_{5,6A}$	2.4	2.2	2.3
$J_{5,6B}$	*b*	5.8	6.0
$J_{6A,6B}$	11.7	11.0	11.5
$J_{\alpha A,\alpha B}$	16.5	15.9	
$J_{\alpha,\beta}$			6.2

[a] Coupling constants for H-β to H-ϵ were not obtained. [b] Value not obtained.

buffer, pH 7.4, containing 0.01% gentamycin sulfate, were incubated at 37 °C in screw-capped tubes. In most cases air was admitted daily. Otherwise, solutions were kept under nitrogen and contained diethylenetriaminepentaacetic acid (1 mM).

Incorporation of Labeled Hexose. Aliquots (0.1 mL) of incubation solutions were diluted with water (0.9 mL) and dialyzed against water. Protein concentrations (Lowry method; Hartree, 1972) and radioactivities were then measured.

Electron-Impact Mass Spectrometry. Hexitol amino acids were converted into methyl esters of per-*N*,*O*-acetyl derivatives as described by Adams (1974) and Ahmed et al. (1986). Acetonitrile solutions were applied to a Hewlett-Packard Model 5985B spectrometer which was operated by Dr. K. Nakatsu, Department of Pharmacology. The probe temperature was increased at 20 deg/min; derivatives were volatilized at 140–150 °C.

Analysis of the Hexitol Lysines, 1-HL and 2-HL, in Hydrolysates of Borohydride-Treated Proteins. Each protein sample (1–5 mg) was treated with an excess of $NaBH_4$ (Walton & McPherson, 1986) and then hydrolyzed in 4 N HCl at 110 °C for 24 h; 45% of 1-HL and 34% of 2-HL were degraded under these conditions. An internal standard, *N*-methyl-D-glucamine, was added. Hexitol amino acids and the internal standard were isolated by affinity chromatography on a phenylboronate column (Affi-Gel 601, Bio-Rad Laboratories; Walton & McPherson, 1986) and converted into *N*-phenylthiocarbamyl (PTC) derivatives (Walton & McPherson, 1987). The latter were examined by HPLC on a C18 column (Whatman Partisil ODS-3; 4.6 × 250 mm), with a gradient composed of solvents C and D, as described previously (Walton & McPherson, 1987). HPLC was also conducted on a cyanoalkyl column (I.B.M., 4.6 × 250 mm), using the same gradient.

Table III: Hexitol Lysine Content of Borohydride-Treated, Glycated HSA[a]

hexose used for incubation	mol/mol of HSA: attached hexose (counting)[b]	1-HL (HPLC)	2-HL (HPLC)	1-HL + 2-HL (analyzer)[c]
glucose	12	14	0	*d*
fructose	1.5	0.2	1.3	1.7

[a] Nonglyco-HSA that had been incubated with hexose for 1 week in the presence of air (for conditions, see Methods). [b] Total hexose incorporated during an incubation with [U-^{14}C]hexose. [c] Amino acid analyzer used to determine hexitol lysine content. Retention times of 2-GL, 1-ML, and 1-GL were 29.1, 29.2, and 29.4 min, respectively. 1-ML and 1-GL gave a shoulder on the 2-GL peak. The retention time of 2-ML is unknown. [d] Not determined.

Fluorescence Spectroscopy. Dialyzates of samples of incubation mixtures (see above) were diluted 10-fold with water. Fluorescence emission, at 405 nm, was measured with a Perkin-Elmer Model MPF-66 instrument: excitation, 330 nm; 350-nm cutoff filter in emission beam.

Electrophoresis. The conditions for sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and staining of gels with Coomassie blue were those described by Elbe et al. (1983). Samples of incubation mixtures were not dialyzed prior to electrophoresis.

Removal of Hexose from Incubation Mixtures. The method is based upon that described by Penefsky (1979). An aliquot (0.1 mL) of an incubation mixture was applied to a 1-mL column of Sephadex G-25 Superfine (Pharmacia, Sweden; in a plastic syringe) that had already been centrifuged at 4300*g* for 2 min. The column was spun at 120*g* for 1 min, and incubation buffer (0.1 mL) was applied. On recentrifugation (120*g* for 1 min), all the protein and <0.02% of the unreacted hexose were collected.

RESULTS

Products of Glucosylation and Fructosylation of HSA. In the following studies, samples of hexose–protein incubation mixtures were always dialyzed exhaustively against water prior to analysis, and it was assumed (Bisse et al., 1982) that this would result in complete hydrolysis of Schiff bases, such as I and III (Figure 1), but would not affect rearrangement products, such as II and IV.

At first we set out to gauge the extent to which C-1- and C-2-attached hexoses (Figure 1; II and IV, respectively) were present in a protein that had been incubated with glucose or fructose at pH 7.4. HSA was chosen for this purpose, as it can be glycated only at lysyl ϵ-amino groups (Garlick & Mazer, 1983). The approach was as follows (see Figure 1). The glycated protein was treated with $NaBH_4$ and then hydrolyzed with acid. 1-HL and 2-HL that were formed from structure II, or IVa and IVb, respectively, were then analyzed by reverse-phase HPLC of their PTC derivatives (Walton & McPherson, 1987), with synthetic 1-GL, 1-ML, and 2-GL as reference compounds.[2]

Incubation of nonglyco-HSA with 0.5 M glucose for 1 week in an aqueous buffer at 37 °C gave 1-HL (representing II in

[2] The ratio of 2-GL to 2-ML in the 2-HL from hydrolysates of borohydride-treated proteins was unknown. Judging from previous studies (Heyns & Rolle, 1959; Heyns & Noack, 1962), 2-GL would preponderate.

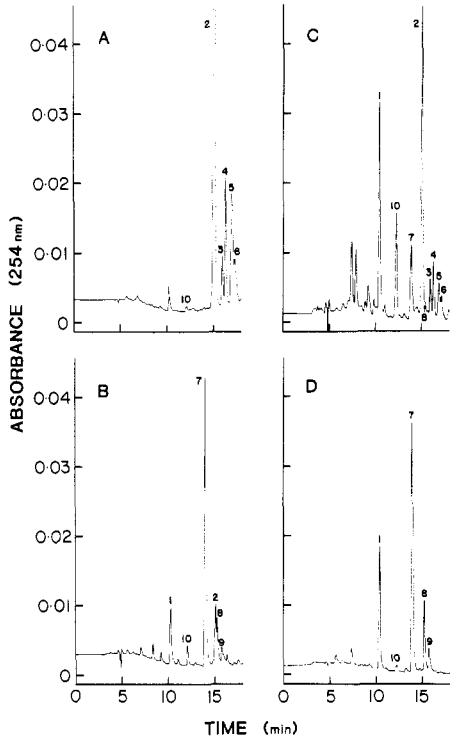


FIGURE 2: Separation of PTC hexitol amino acids by HPLC, using a C18 column with the solvent at pH 4.5 (see Methods). Peaks are numbered as follows: 1, *N*-methylglucamine (internal standard); 2, 1-HL; 3–6, acid degradation products of 1-HL; 7, 2-HL; 8 and 9, acid degradation products of 2-HL; 10, ammonia. Panels A–C represent hydrolysates of borohydride-treated proteins: (A) nonglyco-HSA, incubated with glucose for 1 week; (B) nonglyco-HSA, incubated with fructose for 1 week (for incubation conditions, see Methods); (C) water-soluble fraction of lens protein from 60–69 year old diabetic subjects. (D) Synthetic 2-GL, after being heated in 4 N HCl at 110 °C for 24 h.

the protein) but no 2-HL (representing IV; see results in Table III and Figure 2A). Incubation with fructose under similar conditions gave both 1-HL and 2-HL, in a ratio of 15:85 (Table III and Figure 2B). The identities of 1-HL and 2-HL were confirmed by electron-impact mass spectrometry of methyl esters of per-*N*,*O*-acetyl derivatives of hexitol amino acids formed from HSA that had been incubated with fructose (Figure 3). Diagnostically useful ions had values of *m*/*z* 215 (from 1-HL acetate) and *m*/*z* 287 and 329 (from 2-HL acetate). When either glucose or fructose was used, the hexitol lysines accounted for all of the attached sugar, which was measured by counting after incubation with labeled hexose (Table III). No differences in rates of incorporation of radioactivity were observed when incubations were conducted in air, or nitrogen, indicating that oxidative cleavage of the attached hexose (Ahmed et al., 1986) was not significant here.

The products were similar to those resulting from reactions of the two hexoses with free amino acids (Heyns et al., 1957; Heyns & Paulsen, 1959; Heyns & Noack, 1962); i.e., glucosylation gave *N*-(1-deoxyfructos-1-yl) amino acids (equivalent to II), while fructosylation gave a mixture of *N*-(2-deoxyhexos-2-yl) (equivalent to IVa and IVb) and *N*-(1-deoxyfructos-1-yl) amino acids (equivalent to II), the 2-deoxyglucos-2-yl derivative predominating. The formation of 2-deoxyhexos-2-yl groups involves a Heyns rearrangement of the Schiff base, but the mechanism of formation of 1-deoxyfructos-1-yl groups as a result of fructosylation is not fully understood (Reynolds, 1965).

Evidence for in Vivo Fructosylation of Human Lens Proteins. Since human ocular lens proteins are long lived and are continuously exposed to fructose as well as glucose, we examined them for the presence of N^{ϵ}-(2-deoxyhexos-2-yl)lysyl residues (IV) that might have been formed in vivo. Hexitol amino acids derived from borohydride-treated lens proteins

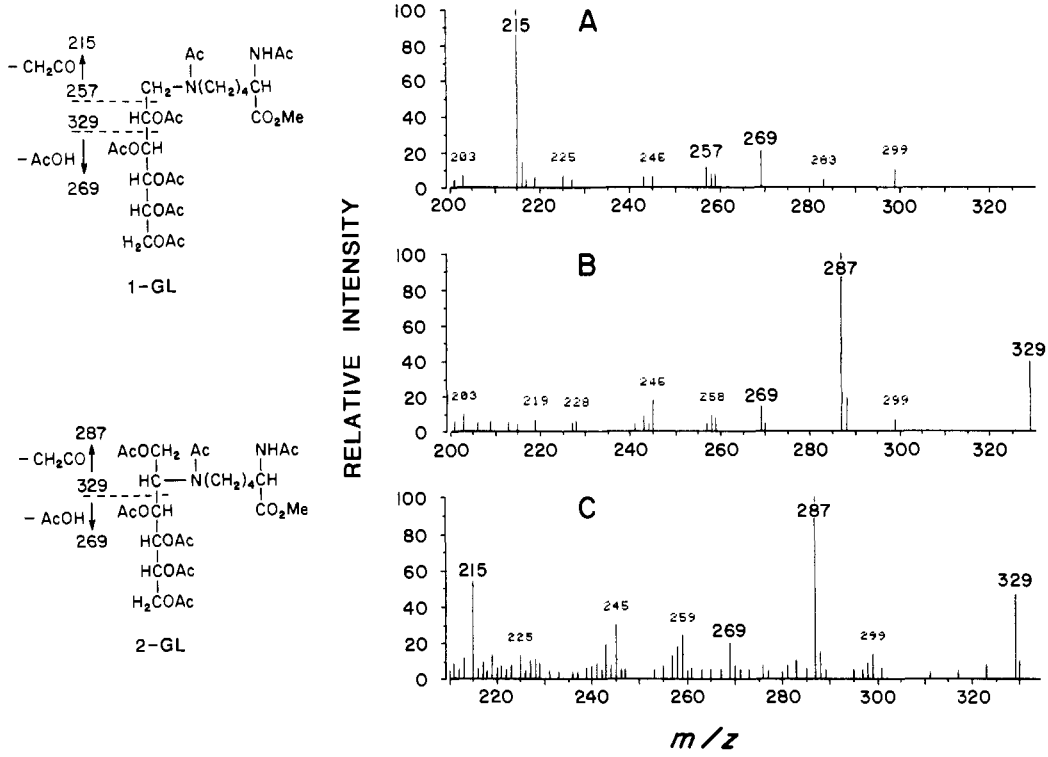


FIGURE 3: Electron-impact mass spectra of methyl esters of per-*N*,*O*-acetyl derivatives of (A) synthetic 1-GL, (B) synthetic 2-GL, and (C) hexitol amino acids obtained from HSA (not fractionated by agarose–phenylboronate) that had been incubated with fructose for 1 week, treated with $NaBH_4$, and hydrolyzed. Analysis of the HSA, before incubation with fructose, gave 0.32 mol of 1-HL/mol of protein and no 2-HL; the mass spectrum of derived methyl esters of acetates showed an ion of *m*/*z* 215 (from 1-HL). Ions of *m*/*z* 287 and 329 (from 2-HL) were absent.

Table IV: Hexitol Lysine Content of Borohydride-Treated Proteins from Human Lenses[a]

age (years)	with (+) or without (−) cataract	diabetic (D) or nondiabetic (N)[b]	hexitol lysine content (mmol/mol of protein)[c] soluble 1-HL	soluble 2-HL	insoluble 1-HL	insoluble 2-HL
41–55	−	N	16	2	16	6
41–55	−	N	14	1	14	1
41–55	−	N	5	1	6	1
41–55	+	N	16	3	11	2
41–55	+	N	9	1	10	2
41–55	+	N	10	2	*d*	*d*
56–70	−	N	15	4	14	4
56–70	−	N	5	1	5	1
56–70	−	N	6	5	*d*	*d*
60–69	+	D	32[e]	5[e]	61	6
60–69	+	D	34	8	61	6
60–69	+	D	*d*	*d*	56	7

[a] The data on each line were obtained from a pool of four lenses. [b] The severity of diabetes of the donors was unknown. [c] Corrected for degradation during hydrolysis (see Methods). Values are based upon crystallin monomer, M_r 20000. [d] Not determined. [e] Use of the amino acid analyzer gave a value of 38 mmol/mol for 1-HL + 2-HL; for retention times, see footnote *c* of Table III. (2-GL + 1-ML) and 1-GL appeared as two unresolved peaks.

were therefore analyzed by HPLC of PTC derivatives, with solvent at pH 4.5. Every protein fraction examined gave a peak at the PTC-1-HL position, together with an additional, smaller peak with a retention time identical with that of authentic PTC-2-GL (see Figure 2C). When the pH of the HPLC solvent was varied, the relative retention time of the small peak changed from 1.46 at pH 4 to 1.29 at pH 5 and was always exactly the same as that of PTC-2-HL. [The relative retention time of PTC-1-GL is also pH sensitive (Walton & McPherson, 1987).] The new substance behaved identically with PTC-2-HL when HPLC was performed with a cyanoalkyl column.[3] Relative retention times were 1.47, 1.38, and 1.32 at pH 4.0, 4.5, and 5.0, respectively. The PTC-2-HL peak from lens proteins (Figure 2C), like that derived from fructosylated HSA (Figure 2B), was asymmetrical and probably represented unequal amounts of the epimers, 2-GL and 2-ML, but this was not substantiated.[2]

The hexitol lysine contents of borohydride-treated lens proteins from several human age groups were analyzed. The results are shown in Table IV. The main component, 1-HL, was present in quantities that were 2–4 times larger in proteins of diabetic subjects than in those of nondiabetics, as has already been reported (Garlick et al., 1984; Liang et al., 1986). The content of 2-HL was always smaller than that of 1-HL, and there were no significant differences between values for samples from cataractous and noncataractous lenses of nondiabetic groups. The average value of 2-HL content was only slightly larger for the proteins from diabetics than for those from nondiabetics.

Hexose-Induced Fluorescence and Cross-Linking in RNase. RNase was chosen for this part of the work, since it is a useful model protein for studying hexose-induced cross-linking (Elbe et al., 1983). It possesses several reactive lysyl residues (Watkins et al., 1985), and covalently cross-linked oligomers of RNase can easily be determined by SDS–PAGE.

Glucose and fructose reacted at similar rates on incubation with RNase for 1 week at 37 °C (Figure 4), in contrast to

[3] Although PTC-1-HL and PTC-2-HL were well resolved on the cyanoalkyl column, peaks were sometimes obscured by reagent-derived peaks that were eluted early. Therefore, this column is not recommended for assays of hexitol amino acids.

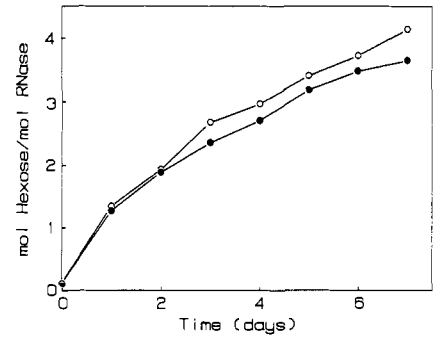


FIGURE 4: Incorporation of [U-^{14}C]glucose (closed circles) and [U-^{14}C]fructose (open circles) into RNase. For conditions, see Methods.

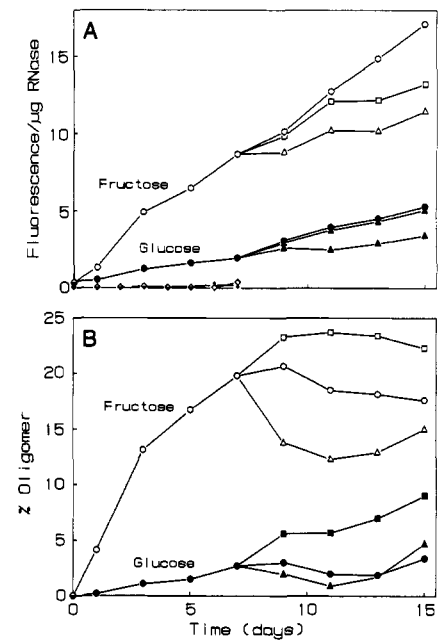


FIGURE 5: (A) Development of fluorescence (excitation, 330 nm; emission, 405 nm) of RNase after incubation with hexoses. (B) Cross-linking of RNase, measured by densitometric scanning of SDS–PAGE gels. The ordinate scale represents the fraction of the protein in the form of covalently linked oligomers (for incubations with glucose, only the dimer was formed; for incubations with fructose, the dimer was formed with small amounts of trimer and tetramer). Note that values of <0.5% oligomer were obtained in solutions that were incubated with 100 mM D-penicillamine for 15 days and that oligomerization was not inhibited by dithiothreitol (10 mM). Symbols for panels A and B: (closed or open symbols) glucose or fructose, respectively, included in incubation mixture at the start; (circles) solution contained RNase and hexose (0.5 M); (diamonds) solution contained hexose (0.5 M) and D-penicillamine (0.1 M). At 7 days, each solution was divided into three portions: (i) no change (circles), (ii) hexose removed by gel filtration (squares), and (iii) hexose removed and D-penicillamine (0.1 M) added (triangles).

incubations with HSA (Table III), when glucose reacted at 8 times the rate of fructose. Glucose reacts more slowly than fructose with hemoglobin (Bunn & Higgins, 1981) and liver alcohol dehydrogenase (Tsai & White, 1983). This disparate behavior may be due to differences in local environments at sites of glycation, where charged protein groups influence rates of glycation by (a) affecting nucleophilicities of amino groups and (b) catalyzing Amadori or Heyns rearrangements of Schiff bases (Watkins et al., 1985; Iberg & Flückiger, 1986).

When solutions of RNase were incubated with glucose or fructose, a non-tryptophan type of fluorescence (excitation and emission maxima, 330 and 405 nm, respectively) developed at constant rates that was 3 times greater for fructose than for glucose (Figure 5A; circles). This was accompanied by the formation of cross-linked oligomers (mainly the dimer)

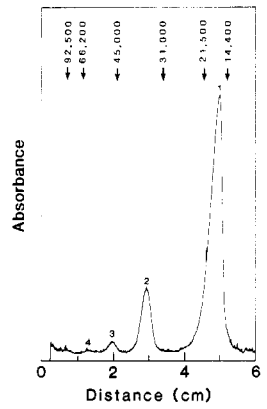


FIGURE 6: Laser densitometer scan of SDS–PAGE gel, stained with Coomassie blue. The sample was RNase that had been incubated with fructose for 7 days. Peaks are numbered as follows: 1, monomer; 2, dimer; 3, trimer; 4, tetramer.

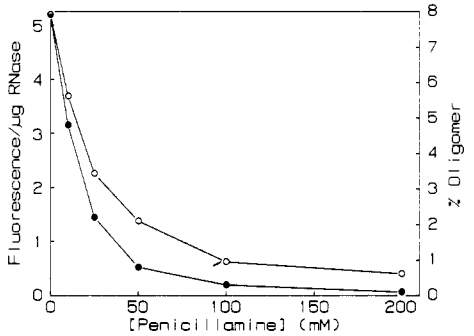


FIGURE 7: Effect of concentration of D-penicillamine upon development of fluorescence (open circles) and cross-linking (closed circles) in RNase that had been incubated with glucose (0.5 M) for 9 days.

that were quantitated by scanning of SDS–PAGE gels (e.g., Figure 6). For the first 5 days of incubation, fructose was 10 times as effective as glucose at inducing cross-linking (Figure 5B; circles). After 10 days, cross-linking ceased while fluorescence continued to develop.

When free hexoses were removed from portions of the incubation mixtures at 7 days, fluorescence development and cross-linking continued to take place (Figure 5; squares); cross-linking occurred faster in the absence of hexose than in its presence.

Effects of D-*Penicillamine.* Glucose-induced cross-linking and fluorophore formation were decreased significantly by D-penicillamine. In 9-day incubations of solutions of RNase and glucose, 88–96% inhibition was obtained at a D-penicillamine concentration of 0.1 M, and the effects leveled off at higher concentrations of the drug (Figure 7). A D-penicillamine concentration of 0.1 M was therefore used in further studies. Similar results were obtained when incubations were conducted in the presence of aminoguanidine hydrochloride (data not shown).

Glycation of RNase by the two hexoses was affected by the drug to different extents. In 7-day incubations with labeled hexoses in the presence of 0.1 M D-penicillamine, the total incorporation of glucose or fructose was 91 or 52%, respectively, of that obtained in absence of the drug.

In another experiment, designed to examine the effect of D-penicillamine upon postglycation reactions, hexoses were removed after 7 days of incubation without D-penicillamine. The drug was then added, and incubation was continued for 4 days. Fluorescence developed more slowly in the presence of D-penicillamine than in its absence (Figure 5A; triangles); cross-linking was reversed at first, but resumed later (Figure 5B; triangles).

Hexose-induced cross-linking of RNase was not affected by 10 mM dithiothreitol (see legend for Figure 5).

DISCUSSION

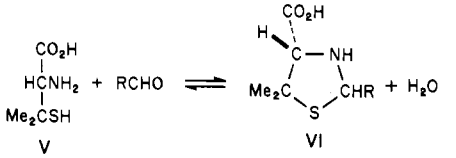
Judging from the results that are summarized in Table III, the identification of 2-HL in the hydrolysate of a borohydride-treated protein can be used as evidence that ε-amino groups of lysyl residues were nonenzymatically fructosylated. Identification of 1-HL alone indicates that they were glucosylated. If both 1-HL and 2-HL are obtained, some of the former originates from lysyl residues that were fructosylated. If these principles are used to interpret the results of analysis of hexitol amino acids in lens proteins, it must be concluded that most of the observed 1-HL represents lysyl residues that were glucosylated and a small fraction is attributable to those that were fructosylated. the 2-HL derived from lens proteins must represent lysyl residues that had undergone nonenzymatic fructosylation in vivo. As far as we are aware, this is the first study in which this type of posttranslational modification has been confirmed by detailed chemical methods.

In the RNase study the main finding was that covalent cross-linking was induced more rapidly when the protein had been fructosylated than when it had been glucosylated to the same extent. Cross-linking appeared to precede fluorophore formation, since fluorescence continued to increase when cross-linking had stopped. This is consistent with a mechanism proposed by Pongor et al. (1984) for the formation of a fluorophore on incubation of polylysine with glucose.

The continuation of cross-linking after removal of glucose has already been noted by Elbe et al. (1983), who attributed it to stable, protein-attached hexoses. Similar structures [presumably, *N*-(2-deoxyhexos-2-yl) groups] are probably involved in fructose-induced cross-linking, since this continued to occur after removal of fructose by gel filtration. The higher rate of cross-linking in the absence of hexose than in its presence, also observed by Elbe et al. (1983), may be ascribed to competition between free and bound hexoses for reactive sites on the protein.

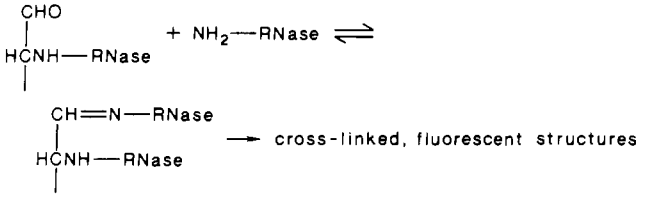
Processes similar to those observed in the RNase experiments, in which fructosylated proteins undergo cross-linking faster than glucosylated ones, may therefore occur in the lens and in other tissues in which the polyol pathway is operative.

D-Penicillamine (V) interferes with collagen cross-linking by combining with enzymatically formed aldehyde groups, to form 2-substituted 5,5-dimethylthiazolidine-4-carboxylic acid



V VI

(VI; Deshmukh & Nimni, 1969). Our experiments show that D-penicillamine inhibits both glycation and postglycation reactions of RNase. The effects observed after removal of glucose (reversal of cross-linking and slowing of fluorescence development) may be explained by assuming that D-penicillamine influences the first reaction of a sequence such as



by reacting with the bound hexose of the glycated protein (at left of scheme). Further Schiff base formation is therefore inhibited, and existing Schiff bases are cleaved by a displacement of the equilibrium to the left. D-Penicillamine might have inhibited cross-linking of RNase by reducing disulfide bonds, thus altering its conformation and decreasing the reactivities of lysyl amino groups. This seems implausible in view of the fact that the rate of hexose-induced cross-linking of RNase was not affected by dithiothreitol.

D-Penicillamine should be a useful tool for investigating these reactions in vivo and for designing drugs to limit hexose-induced cross-linking of proteins in diabetic subjects.

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REFERENCES

Adams, R. F. (1974) *J. Chromatogr. 95*, 197–212.
Ahmed, M. U., Thorpe, S. R., & Baynes, J. W. (1986) *J. Biol. Chem. 261*, 4889–4894.
Bisse, E., Berger, W., & Flückiger, R. (1982) *Diabetes 31*, 630–633.
Briggs, D. R., Garner, E. F., & Smith, F. (1956) *Nature (London) 178*, 154–155.
Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P., & Cerami, A. (1986) *Science (Washington, D.C.) 232*, 1629–1632.
Bunn, H. F., & Higgins, P. J. (1981) *Science (Washington, D.C.) 213*, 222–224.
Cerami, A., Vlassara, H., & Brownlee, M. (1986) *J. Cell. Biochem. 30*, 111–120.
Deshmukh, K., & Nimni, M. E. (1969) *J. Biol. Chem. 244*, 1787–1795.
Dyck, P. J., Sherman, W. R., Hallcher, L. M., Service, F. J., O'Brien, P. C., Grina, L. A., Palumbo, P. J., & Swanson, C. J. (1980) *Ann. Neurol. 8*, 590–596.
Elbe, A. S., Thorpe, S. R., & Baynes, J. W. (1983) *J. Biol. Chem. 258*, 9406–9412.
Flückiger, R., Woodtli, T., & Berger, W. (1984) *Diabetes 33*, 73–76.
Gabbay, K. H. (1973) *N. Engl. J. Med. 288*, 831–836.
Garlick, R. L., & Mazer, J. S. (1983) *J. Biol. Chem. 258*, 6142–6146.
Garlick, R. L., Mazer, J. S., Chylack, L. T., Tung, W. H., & Bunn, H. F. (1984) *J. Clin. Invest. 74*, 1742–1749.
Hartree, E. F. (1972) *Anal. Biochem. 48*, 422–427.
Heyns, K., & Paulsen, H. (1959) *Justus Liebigs Ann. Chem. 622*, 160–174.
Heyns, K., & Rolle, M. (1959) *Chem. Ber. 92*, 2451–2460.
Heyns, K., & Noack, H. (1962) *Chem. Ber. 95*, 720–727.
Heyns, K., Breuer, H., & Paulsen, H. (1957) *Chem. Ber. 90*, 1374–1386.
Howard-Lock, H. E., Lock, J. L., Mewa, A., & Kean, W. F. (1986) *Semin. Arthritis Rheum. 15*, 261–281.
Iberg, N., & Flückiger, R. (1986) *J. Biol. Chem. 261*, 13542–13545.
Jedziniak, J. A., Chylack, L. T., Cheng, H. M., Gillis, K. M., Kalustian, A. A., & Tung, W. H. (1981) *Invest. Ophthalmol. Visual Sci. 20*, 314–326.
Krantz, S., Lober, M., & Henschel, L. (1986) *Exp. Clin. Endocrinol. 88*, 257–269.
Lee, H. S., Sen, L. C., Clifford, A. J., Whitaker, J. R., & Feeney, R. E. (1979) *J. Agric. Food Sci. 27*, 1094–1098.
Lerner, B. C., Varma, S. D., & Richards, R. D. (1984) *Arch. Ophthalmol. (Chicago) 102*, 917–920.
Liang, J. N., Hershorin, L. L., & Chylack, L. T. (1986) *Diabetologia 29*, 225–228.
Mayhew, J. A., Gillon, K. R. W., & Hawthorne, J. N. (1983) *Diabetologia 24*, 13–15.
Monnier, V. M., Stevens, V. J., & Cerami, A. (1979) *J. Exp. Med. 150*, 1098–1107.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.
Pongor, S., Ulrich, P. C., Bencsath, F. A., & Cerami, A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2684–2688.
Reynolds, T. M. (1965) *Adv. Food Res. 14*, 167–283.
Suarez, G., Novick, J., & Oronsky, A. L. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 2022.
Suarez, G., Rajaram, R., & Oronsky, A. L. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1539.
Tsai, C. S., & White, J. H. (1983) *Biochem. J. 209*, 309–314.
Varma, S. D., Schocket, S. S., & Richards, R. D. (1979) *Invest. Ophthalmol. Visual Sci. 18*, 237–241.
Vishwanath, V., Frank, K. E., Elmets, C. A., Dauchot, P. J., & Monnier, V. M. (1986) *Diabetes 35*, 916–921.
Walton, D. J., & McPherson, J. D. (1986) *Carbohydr. Res. 153*, 285–293.
Walton, D. J., & McPherson, J. D. (1987) *Anal. Biochem. 164*, 547–553.
Walton, D. J., Ison, E. R., & Szarek, W. A. (1984) *Carbohydr. Res. 128*, 37–49.
Watkins, N. G., Thorpe, S. R., & Baynes, J. W. (1985) *J. Biol. Chem. 260*, 10629–10636.