Characterization of *N*-glycosylated type I collagen in streptozotocin-induced diabetes

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The *N*-glycosylation of lysine and hydroxylysine residues in collagen from streptozotocin-induced-diabetic rats was confirmed and the stability of the complex shown to be due to an Amadori rearrangement. The studies also demonstrate the relative specificities of glucose, galactose and mannose in their reaction with collagen. The glycosylation of lysine *in vitro* occurs with glucose and galactose, but not with mannose, whereas only glucose reacts with hydroxylysine to any significant extent. Glycosylation of collagen occurs slowly during normal aging, but, in contrast with reports suggesting accelerated aging of collagen in diabetic animals, we clearly demonstrated that the apparent increased stability is not due to an acceleration of the normal maturation process involving the reducible cross-links.

Over the past few years the non-enzymic glycosylation of proteins has received considerable attention, with the realization of the possible importance of their uncontrolled glycosylation in diabetes mellitus, and a consequent change in properties of the protein.

Non-enzymic glycosylation under physiological conditions was first demonstrated by the attachment of glucose to the *N*-terminal valine residue of haemoglobin (Allen *et al.*, 1958; Holmquist & Schroeder, 1966). Biosynthetic studies (Bunn *et al.*, 1978a) demonstrated the non-enzymic nature of the glucose binding, and chemical studies (Bookchin & Gallop, 1968; Bunn *et al.*, 1975, 1978b) indicated that the glucose was attached through a stable keto-imine linkage.

The attachment of glucose to the ϵ -amino group of lysine residues within the peptide chain of a protein under physiological conditions was first reported by Robins & Bailey (1972) during studies on age changes in collagen. Isolation of the hexosyl-lysine residues after borohydride reduction demonstrated the presence of glucosyl- and mannosyl-lysine, and it was clear from their structure that the glucose was attached to the ϵ -amino group of lysine and hydroxylysine. The extent of glycosylation was shown to increase steadily with the age of the tissue, and a similar reaction occurs *in vitro*, indicating a non-enzymic reaction. It was later shown that other proteins, such as serum albumin, were glycosylated (Bailey *et al.*, 1976).

More recently, the glucosylation of insulin (Dolhoffer & Wieland, 1979a), serum albumin (Dolhoffer & Wieland, 1979b; Day *et al.*, 1979), and

lens crystallin (Steven *et al.*, 1978; Pande *et al.*, 1979; Cerami *et al.*, 1979) has been demonstrated in subjects with diabetes mellitus. In addition, the collagen of diabetic patients has been reported to be more resistant to collagenase by Hamlin *et al.* (1975, 1978), who suggested an accelerated aging of the collagen. In this context, an increased degree of polymerization of tail-tendon collagen in rats with streptozotocin- and alloxan-induced diabetes has been reported (Golub *et al.*, 1978). This increased cross-linking is consistent with the increased activity of lysyl oxidase demonstrated in living tissue of diabetic rats (Madia *et al.*, 1979). These results all suggested an accelerated aging through increased cross-linking of the collagen.

In the present paper, we examine the specificity of the reaction of hexoses with collagen and demonstrate that the glycosylation does not represent an acceleration of the normal maturation process in collagen involving the reducible cross-links.

Materials and methods

Male Sprague-Dawley rats (Charles River CD strain) fed with commercial pellets and water *ad libitum* were used for this study. Groups of ten animals were killed at 6 and 12 months as controls. Two lots of ten rats (1 month old) were made diabetic by injection of 65 mg of streptozotocin (Calbiochem A grade)/kg (body wt.) in 0.1 M-sodium citrate buffer, pH 4.0, and were killed respectively 5 and 11 months later; the mean blood sugar value as determined by glucose oxidase assay was

385 ± 72 mg/100 ml, and glucosuria was about 8–10 g/litre after the second week of the diabetic state.

Identification of borohydride-reducible components

The tail tendons were carefully dissected from the tails, washed twice in ice-cold 0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.4, and shredded. A portion (500 mg) of each wet preparation was reduced by addition of 15 mCi of NaB^3H_4 (The Radiochemical Centre, Amersham, Bucks., U.K.) to 20 mg of NaBH_4 in ice-cold 0.15 M-NaCl/0.1 M-sodium phosphate buffer for 1 h as previously described (Robins & Bailey, 1972). The reaction was stopped by addition of acetic acid to lower the pH to 4.0, and the tendon suspension was dialysed against distilled water for 3 days. After acid hydrolysis (6 M-HCl, 100°C for 24 h) of the freeze-dried material, the HCl was removed under vacuum. Each dried sample was dissolved in a 0.1 M-pyridine/formic acid-buffered solution (pH 2.9) and submitted to ion-exchange chromatography on Dowex 50 W $\times$ 8 (0.9 cm $\times$ 60 cm) at 60°C. Elution was performed with a gradient of pyridine formate buffers from pH 2.9 to 5.0 as previously described in detail (Robins & Bailey, 1972). The radioactivity of the fractions (2 ml) diluted in Unisolve (Sochibo, 92100 Boulogne, France) (5 ml) was determined in an Intertechnique Kontron SL 4220 scintillation counter.

Incorporation of ^{14}C -labelled hexoses in vivo

For this, 1 μCi of D-[1- ^{14}C]glucose, -galactose or -mannose (50 Ci/mol; The Radiochemical Centre) per g body wt. in 0.5 mg of non-labelled sugar in 0.15 M-NaCl was administered intravenously to 6-month-old diabetic rats and age-matched controls. Animals were killed 48 h later, and the specific radioactivity of the hexose in the tendon collagen was measured after solubilization of 10 mg of freeze-dried collagen in 5 ml of Soluene 250 (Packard).

Use of a dual-labelling method involving [^3H]-borohydride reduction of the ^{14}C -labelled complex permitted the identification of both the [^{14}C]-glycosylated and the [^3H]-borohydride-reducible components. The components were identified on ion-exchange columns after acid hydrolysis as described above.

Glycosylation of tendon in vitro

To 100 mg wet wt. of shredded rat tail tendon in 5 ml, of 0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.5, 10 μCi of D-[1- ^{14}C]hexose in 50 mg of non-radioactive hexose was added. Incubations were performed with radioactive glucose, galactose and mannose at 37°C for 12 h with continuous stirring.

The individual hexosyl derivatives were identified by the dual-labelling technique after reduction with

NaB^3H_4 and chromatographically separated as described above.

Determination of the extent of glycosylation

A modification of the method described by Fluckiger & Winterhalter (1976) was used to determine the extent of glycosylation. Briefly, 1 mg of freeze-dried tail-tendon collagen from studies *in vivo* or *in vitro* was hydrolysed in 0.5 M-oxalic acid for 5 h at 100°C. The quantity of 5-hydroxy-methyl-2-furaldehyde liberated was then determined colorimetrically at 443 nm by using the thiobarbituric acid reaction.

Assessment of purity of [^{14}C]glucose

Unknown radioactive contaminants present in commercial preparations of [^{14}C]glucose have been reported to bind to proteins, thus producing significant errors in determination of glucose-protein binding. A test of purity was therefore carried out as described by Trüeb *et al.* (1980) with 20 μCi of labelled glucose diluted in 0.2 ml of 0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.4, containing 10 mg of non-radioactive glucose. The glucose was then degraded in the solution by incubating at room temperature with 2 ml of glucose test kit 'Merckotest' (Merck) containing glucose dehydrogenase (EC 1.1.1.47) and mutarotase (EC 5.1.3.3), the reaction being continued until the A_{365} was stable. Controls without dehydrogenase were carried out under the same conditions. The treated and control preparations were then incubated with 10 ml of collagen solution (1 mg/ml) in the presence of non-radioactive glucose (final concn. 0.17 M) at 37°C for 12 h, and the total ^{14}C radioactivity was then determined.

Investigation of the stability of the glucosyl-lysine complex

(a) Acid-soluble collagen was prepared from 1.5-month-old rat tail tendons as described by Chandrakasan *et al.* (1976). A sample (15 ml) of this preparation (1 mg/ml in 0.15 M-NaCl/0.1 M-sodium phosphate buffer, pH 7.4) was incubated with 50 μCi of [U- ^{14}C]glucose (250–300 Ci/mol; C.E.A., Gif-sur-Yvette, France) diluted with non-labelled glucose to a final concentration of 0.17 M. Streptomycin (100 μg /ml) and penicillin (100 units/ml) were added to prevent bacterial contamination. After 8 days at 29°C, unbound glucose was removed by extensive dialysis against the phosphate-buffered saline and the bound radioactivity was determined.

(b) A second dialysis against 0.15 M-D-glucose was subsequently carried out to demonstrate the irreversibility of the glucose-collagen linkage, the radioactivity remaining covalently linked to the collagen being determined up to day 10 of dialysis.

(c) The acid-soluble collagen prepared as

described above, but incubated with non-radioactive glucose, was further tested for stability of the hexosyl-lysines by the following treatment: (i) thermal denaturation for 30 min at 45°C, (ii) cleavage into peptides by digestion with an equal weight of CNBr in 70% (v/v) formic acid, (iii) enzymic digestion to small peptides with pepsin at pH 3.7.

The products of these three treatments were reduced with NaB^3H_4 , and the presence of hexosyl-lysines was detected, after acid hydrolysis on ion-exchange columns eluted with pyridine/formate buffers (Robins & Bailey, 1972).

Extent of Amadori rearrangement

In order to determine the extent of the Amadori rearrangement hexoses labelled at different sites, one of which is known to be involved in the rearrangement, were studied. Acid-soluble collagen was incubated as described above, but with specifically labelled D-[2- ^3H]glucose or D-[5- ^3H]glucose (13 Ci/mmol; C.E.A., Gif-sur-Yvette, France). The radioactivity of the glucosylated preparation was determined after 8 days incubation.

Results

Identification of reducible components in normal and diabetic rats

Fig. 1 presents the elution pattern of the radioactive components separated from the acid hydrolysate of rat tail tendon (100 mg wet wt.) from 12-month-old diabetic rats as compared with age-matched controls. The specific radioactivity of the reduced aldimine cross-link hydroxylysionorleucine (peak B) and the artefact of borohydride reduction histidino-hydroxymerodesmosine (peak C) appears unchanged in diabetic rats compared with controls. The amount of peak B decreased by about 20% from 6 to 12 months (Table 1). These results agree with findings previously shown to be related to a stabilization *in vivo* of dehydrohydroxylysionorleucine during aging (Robins *et al.*, 1973).

In contrast, a dramatic augmentation of the hexosyl-hydroxylysine (pA), hexosyl-lysine (A_1) and their acid-dehydration products (A_2) was noticeable in the diabetic animals when compared with the controls. The variations of the specific activities are reported in Table 1 and show increases of the

Fig. 1. Elution pattern from ion-exchange column of ^3H -labelled reduced components separated from an acid hydrolysate of NaB^3H_4 -reduced collagen from normal (●—●) and diabetic (—) rats

Rat tail-tendon collagen (100 mg wet wt.) was reduced as described in the text, hydrolysed and eluted from the column with pyridine/formate buffer. Key: pA, hexosyl-hydroxylysine; A_1 , hexosyl-lysine; A_2 , acid-dehydration product of hexosyl-lysine; B, hydroxylysionorleucine; C, histidino-hydroxymerodesmosine

Table 1. Identification and determination of the amount of borohydride-reducible components in rat tendon collagen during development of streptozotocin-induced diabetes

Results are expressed as d.p.m./mg wet wt. of material, quenching correction being performed after external standardization. Mean values $\pm$ S.E.M. for ten determinations (from ten different animals) after reduction of 10 mg of tendon are given. Key (see Fig. 1): pA, hexosyl-hydroxylysine; A₁, hexosyl-lysine; A₂, acid-dehydration product of hexosyl-lysine; B, hydroxylysine norleucine; C, histidinohydroxymerodesmosine.

Reducible component	Normal (6 months)	Diabetic (6 months)	Normal (12 months)	Diabetic (12 months)
pA	52 $\pm$ 6	129 $\pm$ 33	138 $\pm$ 19	298 $\pm$ 60
A ₁	141 $\pm$ 30	419 $\pm$ 127	334 $\pm$ 43	801 $\pm$ 152
A ₂	100 $\pm$ 28	290 $\pm$ 101	219 $\pm$ 25	537 $\pm$ 108
B	494 $\pm$ 111	423 $\pm$ 134	385 $\pm$ 51	387 $\pm$ 69
C	852 $\pm$ 149	1041 $\pm$ 252	1051 $\pm$ 215	1047 $\pm$ 302
Total	1639	2302	2127	3070

Table 2. Glycosylation *in vivo* by [¹⁴C]hexoses of tendon collagen from normal and streptozotocin-induced-diabetic rats

(i) ¹⁴C results are expressed as d.p.m./mg wet wt of tendons. Mean values $\pm$ S.E.M. of determinations on five separate animals are given. (ii) ¹⁴C radioactivity is shown of fractions identified after reduction with NaB³H₄, expressed as d.p.m./mg wet wt. of collagen. Key: pA, hexosyl-hydroxylysine; A₁, hexosyl-lysine; A₂, acid-dehydration product of hexosyl-lysine.

	[¹⁴ C]Glucose		[¹⁴ C]Galactose		[¹⁴ C]Mannose	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
(i) Whole tendon collagen	460 $\pm$ 73	1066 $\pm$ 92	545 $\pm$ 56	1213 $\pm$ 108	137 $\pm$ 19	151 $\pm$ 14
(ii) Borohydride-reduced components						
pA	32 $\pm$ 3.1	81 $\pm$ 10.5	17 $\pm$ 2.6	22 $\pm$ 4.1	19 $\pm$ 2.0	15 $\pm$ 4.7
A ₁	97 $\pm$ 5.0	331 $\pm$ 17.6	116 $\pm$ 10.0	205 $\pm$ 15.3	44 $\pm$ 6.8	56 $\pm$ 8.2
A ₂	68 $\pm$ 5.7	230 $\pm$ 11.3	80 $\pm$ 8.4	137 $\pm$ 9.7	30 $\pm$ 5.9	39 $\pm$ 7.5
Total	197	642	213	364	93	110

hexosyl-lysine (A₁) component for 6- and 12-month-old diabetic rats of 197% and 140% of normal respectively. There was a slightly smaller increase in hexosyl-hydroxylysine (148% at 6 months and 116% at 12 months).

Specificity of hexose binding during glycosylation *in vivo*

The specific radioactivities of tendons as shown in Table 2(i) demonstrate an increased incorporation of [¹⁴C]glucose and [¹⁴C]galactose in diabetic animals of about 130%, whereas in contrast [¹⁴C]mannose was not incorporated to any significant extent. After borohydride reduction, hydrolysis and examination of the specific radioactivity of the fractions isolated by ion-exchange chromatography demonstrate selective attachment of glucose and galactose to lysine rather than hydroxylysine residues [Table 2(ii)]. Hydroxylysine appears to be selectively glycosylated by galactose, but only to a relatively small extent. Mannose is only minimally incorporated on hydroxylysine (pA) or lysine (A₁ and A₂).

Specificity of glycosylation *in vitro*

The results shown in Table 3 demonstrate that the reactivity of tendon collagen isolated from diabetic rats when incubated *in vitro* with [¹⁴C]glucose and [¹⁴C]galactose is markedly lower than that of collagen from control rats. This reduction must clearly be due to the prior blocking of reactive sites by glucose in the diabetic rats.

As in the studies *in vivo*, the data also indicate that glucose is the most readily bound carbohydrate, in contrast with the incorporation of mannose, which is not significant.

By using the dual-labelling technique with ¹⁴C and ³H to identify the individual components, a difference in the specificity of the hexose to N^ε-glycosylation of lysine and hydroxylysine is shown in Table 3(ii). The reaction of galactose with hydroxylysine appears to be negligible, and mannose is not incorporated [Table 3(ii)].

Determination of the extent of N-glycosylation

(i) *In vivo*. The extent of N-glycosylation *in vivo*

was determined as nmol of 5-hydroxymethyl-2-furaldehyde per mg of collagen. The value for collagen from normal tendon was 1.1 ± 0.2 nmol/mg,

and 2.6 ± 0.3 nmol/mg for tendon collagen from diabetic rats.

(ii) *In vitro*. Higher extents of N-glycosylation

Table 3. Glycosylation *in vitro* by [^{14}C]hexoses of tendon collagen extracted from normal and streptozotocin-induced-diabetic rats

(i) ^{14}C radioactivity after solubilization of 10g of intact tendon, expressed as d.p.m./mg. (ii) ^{14}C radioactivity of fractions identified after reduction with NaB^3H_4 , and expressed as d.p.m./mg wet wt. of material. Results are mean values $\pm$ S.E.M. for five separate incubations. Key: pA, hexosyl-hydroxylysine; A₁ hexosyl-lysine; A₂, acid-dehydration product of hexosyl-lysine.

	[^{14}C]Glucose		[^{14}C]Galactose		[^{14}C]Mannose	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
(i) Whole tendon collagen	1317 $\pm$ 241	531 $\pm$ 93	885 $\pm$ 112	306 $\pm$ 56	84 $\pm$ 39	139 $\pm$ 43
(ii) Borohydride-reduced components						
pA	218 $\pm$ 32	167 $\pm$ 30	81 $\pm$ 21	28 $\pm$ 9	17 $\pm$ 12	19 $\pm$ 10
A ₁	372 $\pm$ 51	101 $\pm$ 18	306 $\pm$ 47	134 $\pm$ 25	21 $\pm$ 9	36 $\pm$ 17
A ₂	332 $\pm$ 58	125 $\pm$ 26	342 $\pm$ 62	103 $\pm$ 16	25 $\pm$ 7	31 $\pm$ 14
Total	922	393	628	265	63	86

Scheme 1. Formation of N^ε-glucosyl-lysine and subsequent reduction of the Amadori-rearranged Schiff-base complex to produce N^ε-glucosyl- and N^ε-mannosyl-lysine

could be obtained by extending the incubation time *in vitro*, i.e. 2.75 nmol/mg after 2 days and 9.45 nmol/mg after 8 days.

Purity of the [^{14}C]glucose

The purity control performed by glucose dehydrogenase treatment on labelled [^{14}C]glucose batches before incubation *in vitro* led to decreases in formation of hexosyl-lysines of 95% for D-[1- ^{14}C]glucose and 97% for D-[U- ^{14}C]glucose, clearly demonstrating the purity of the material. It can therefore be concluded that the radioactivities determined relate to hexose binding and not to unknown impurities as described by Trüeb *et al.* (1980). (Note: labelled material was used immediately after receipt from the manufacturer.)

Studies on the stability of the glycosylated components

(i) No significant release of [1- ^{14}C]glucose could be detected up to day 10 of dialysis against glucose solutions, indicating the irreversibility of the interaction. The initial radioactivity of 1213 d.p.m./mg was decreased to 1155 d.p.m./mg in 7 days and 1083 d.p.m./mg in 10 days.

(ii) Treatment of the *N*-glycosylated collagen with CNBr, enzymes or heat as described in the Materials and methods section failed to decrease the proportion of hexosyl-lysines (as in Fig. 1), as detected in the acid hydrolysate after reduction with NaB $^3\text{H}_4$.

(iii) The Amadori rearrangement involves the loss of the hydrogen on C-2 of the hexose (Scheme 1). Thus comparison of the activities of hexoses labelled at C-2 and C-5 should confirm that the Amadori rearrangement is taking place and provide an estimate of its extent. From similar initial radioactivities in each sample (D-[2- ^3H]glucose + collagen, 14.3×10^6 d.p.m./ml; D-[5- ^3H]glucose + collagen, 15.3×10^6 d.p.m./ml), the specific radioactivities obtained after an 8-day incubation were as follows: D-[2- ^3H]glucosyl-collagen, 3375 d.p.m./ml; D-[5- ^3H]glucosyl-collagen, 8445 d.p.m./ml. These data, when balanced with regard to initial radioactivities, gave a 61% loss in specific radioactivity for the D-[2- ^3H]glucosyl-collagen sample. Assuming a high specificity of the labelled C-2, this result confirms that the initial product of condensation of the ϵ -amino group of lysine and the aldehyde group of the hexose to form a Schiff base rapidly undergoes the Amadori rearrangement, but may not be complete.

Discussion

The results presented clearly demonstrate that, as with some other proteins such as haemoglobin (Rahbar *et al.*, 1969), serum albumin (Day *et al.*, 1979), erythrocyte membrane proteins (Miller *et al.*,

1980), lens crystallin (Steven *et al.*, 1978; Cerami *et al.*, 1979) and aortic collagen (Rosenberg *et al.*, 1979), *N* $^{\epsilon}$ -glycosylation of tendon collagen occurs in diabetes mellitus. Direct determination of the [^{14}C]-hexose-labelled collagen clearly demonstrated that glucose bound to a greater extent than galactose, whereas reaction with mannose was barely detectable.

In a more detailed analysis, by the use of a double-labelling technique of incorporating ^{14}C -labelled hexoses and subsequent reduction of the glycosylamine with NaB $^3\text{H}_4$, we have been able to distinguish the relative reactivities of the specific residues involved. Glucose and galactose react with the ϵ -amino group of lysine to a similar extent, whereas mannose barely reacts. On the other hand, the reaction with hydroxylysine is even more specific and is virtually confined to glucose (Table 2). Similar differences in reactivity were obtained from the incubations *in vitro* (Table 3). Furthermore, the decreased interaction of diabetic collagen with these hexoses indicated that similar sites were involved, some of which must already be blocked by glucose complexes.

The reaction occurs to an even greater extent *in vitro* with pure components, and is concentration- and pH-dependent, similar to the reaction of glucose with insulin (Dolhoffer & Wieland, 1979a) and albumin (Dolhoffer & Wieland, 1979b). The reaction therefore appears to be non-enzymic. This type of reaction is strongly pH-dependent, and proceeds through the nucleophilic attack by the unprotonated ϵ -amino group on C-1 of the carbohydrate. Hence the lower reactivity of the hydroxylysine is due to the lower pK of its ϵ -amino group. Because the glycosylation of albumin occurs more rapidly at high pH (Dolhoffer & Wieland, 1979b), it is possible that, under the conditions of the borohydride treatment, free hexoses could be attached to the ϵ -amino groups of the lysine and hydroxylysine and subsequently stabilized by reduction. However, the stability of the hexosyl-lysines was surprisingly high. Incubation *in vitro* of the [^{14}C]glucose-labelled collagen with excess non-labelled glucose failed to decrease the radioactivity of the collagen significantly, indicating a stable irreversible glucose-lysine linkage. Similarly, dilute acetic acid treatment of the collagen, peptide-bond cleavage by enzyme digestion under acid conditions, and CNBr digestion in formic acid, produced peptides retaining the hexose attachment, as demonstrated by total radioactivity and identification when subsequently reduced with NaBH $_4$.

The use of [^{14}C]glucose to demonstrate *N*-glycosylation has been criticized on the basis of non-specific binding of impurities (Trüeb *et al.*, 1980). In these studies we have confirmed that ^{14}C radioactivity is associated with *N*-glycosylation by

identification of the borohydride-reduced hexosyl-lysines, and by the formation of 5-hydroxymethyl-2-furaldehyde with thiobarbituric acid. We have also demonstrated that at least 95% of the radioactivity was associated with the hexose, and not with an impurity in the commercial preparations used.

The structure of the hexosyl-lysine involves the formation of a Schiff base, but in view of the evidence for the stability of the interaction the product clearly undergoes an Amadori rearrangement (Gottschalk, 1972) (Scheme 1). This type of reaction has also been suggested by Rosenberg *et al.* (1979), based on the liberation of 5-hydroxymethyl-2-furaldehyde in a reaction accepted to be specific for hexoses bound by the keto-imine linkage to aortic collagen. The formation of the keto form through an Amadori rearrangement was confirmed by incubation of the collagen with D-[2-³H]glucose and D-[5-³H]glucose. The ³H from C-2 is lost during the rearrangement, hence incubation with D-[5-³H]glucose should produce the higher specific radioactivity of the collagen, and indeed such results were obtained, confirming that an Amadori rearrangement had occurred. Analysis of the reduced hexosyl-lysines in the present and previous studies (Robins & Bailey, 1972) reveals the presence of glucosyl-lysine and mannosyl-lysine, yet the incorporation of radioactive mannose was barely significant. However, taken with the present results confirming an Amadori rearrangement to the keto derivative, this apparent anomaly can be explained. After reduction of the keto group at C-2, a mixture of N^ε-glucosyl-lysine and its epimer N^ε-mannosyl-lysine is produced (Scheme 1). The identification of the mannosyl derivative therefore supports the proposal that the glucosyl-lysine must have rearranged to the keto form before reduction.

A similar increase in N-glycosylation, but to a much lesser extent, also occurs during normal aging of collagen and is concomitant with a decrease in the proportion of reducible cross-links (Robins & Bailey, 1972; Robins *et al.*, 1973). In contrast, we could detect no analogous decrease in the reducible intermolecular cross-links in the collagen of diabetic animals, as would be expected by 'accelerated aging' (Hamlin *et al.*, 1975, 1978; Golub *et al.*, 1978; Schnider & Kohn, 1980). It would therefore appear that the changes in diabetes do not involve precisely the same mechanism as the normal aging of collagen. However, the formation of this stable non-enzymically glucosylated collagen in diabetes must lower the basicity of the collagen and thereby affect some of its functional properties. The studies of Steven *et al.* (1978) and Cerami *et al.* (1979) have demonstrated that N^ε-glucosylation of lens crystallin affects its properties quite markedly. Similarly collagen from diabetic subjects (Hamlin *et al.*, 1975, 1978; Schnider & Kohn, 1980; Andreassen *et al.*,

1981) shows properties consistent with increased mechanical and chemical stability. Since this stabilizing reaction has been shown to be unrelated to normal cross-linking, further studies are needed to elucidate the mechanism. Whether the extent of N^ε-glucosylation *in vivo* is sufficient to result in serious deleterious changes remains to be demonstrated, but the effect cannot be ignored and its role must be carefully evaluated.

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