Non-enzymic glycation of collagen inhibits binding of oxidized low-density lipoprotein

Norman KALANT,*†‡ Suzanne McCORMICK* and Michael A. PARNIAK*†

*Lady Davis Institute for Medical Research of the Jewish General Hospital, and †Department of Medicine, McGill University, Montreal, Quebec H3T 1E2, Canada

We have examined the effect of non-enzymic glycation of native soluble collagen, in solution or in gels, on binding of oxidized low-density lipoprotein (LDL). We found the following. (1) Glycation markedly inhibited binding of LDL. This is contrary to results previously reported; the difference may be attributable to the use of detergent- and heat-denatured collagen, covalently bound to agarose beads, in the earlier study. (2) With increased duration of glycation, collagen solution would not gel, and preformed gels dissolved. (3) [¹⁴C]Glucose bound to collagen gels dissociated slowly, even at pH 5, suggesting that it was not present as a Schiff's base; in addition, ketoamines, pentosidine

and fluorescent advanced glycation products were not detectable in glycated collagen gels, although they accumulated in tendon collagen glycated under the same conditions. It is hypothesized that the difference in glycation effects between gel and tendon may be due to the strength of cross-linking before glycation: the increase in intermolecular distance in collagen fibrils which results from glycation disrupts the fibrils in gels, preventing binding of LDL and formation of glycation-dependent cross-links, whereas the extensive cross-linking in tendon maintains the intermolecular distances within a range which permits formation of glycation cross-links.

INTRODUCTION

Atherosclerosis is characterized by the deposition of lipid in the arterial wall; although some of the lipid is within foam cells, most is deposited extracellularly [1], where it is associated with the intercellular matrix. Binding of low-density lipoprotein (LDL) to certain proteoglycans [2] and to collagen [3] may be a mechanism by which such deposition occurs. Oxidation of LDL by cellular or non-cellular mechanisms greatly increases its binding to collagen [4]. Because of its large binding capacity and its abundance (approx. 30% of the dry defatted weight of the aorta [5]), collagen may play a very important role in such a process. Diabetics are particularly prone to development of atherosclerosis, even though their plasma total- and LDL-cholesterol levels are, on average, normal. It has been reported that advanced glycosylation end-products resulting from non-enzymic glycation of collagen increase the capacity of collagen to bind LDL [6]; this observation might explain the increased susceptibility to atherogenesis. In that work denatured collagen was covalently linked to agarose beads, then incubated with native LDL; under the experimental conditions used the LDL would have undergone non-cellular oxidation. Since glycated collagen catalyses lipid peroxidation [7], the increased binding may have resulted from increased LDL oxidation, increased binding of oxidized LDL, or both. We have examined the effect of glycation of native collagen on the oxidation and binding processes.

MATERIALS AND METHODS

Materials

LDL was prepared from pooled normolipemic serum, by precipitation with heparin and MgCl₂ [3]; the very-low-density lipoprotein which co-precipitated was removed by flotation at

$d = 1.006$. The purified LDL was dialysed against 0.9% NaCl containing 0.01% EDTA, iodinated by an ICl procedure [8] and sterilized by filtration through a 0.22 μ m-pore membrane. Specific activities were > 150 c.p.m./ng of LDL protein.

Assays for binding of LDL to collagen were performed with gels of collagen type I. Collagen was extracted from tail tendons of young rats with dilute acetic acid [9] and stored at 4 °C. Samples were mixed with appropriate volumes of 2 × concentrated Dulbecco's modified minimal essential medium (DMEM) or of 0.3 M NaCl containing 7.4 g/l NaHCO₃ to provide a final concentration of 1 mg/ml collagen in 1 × concentrated DMEM (DMEM gel) or in 0.15 M NaCl (saline gel), both at pH 7.4. Portions (1 ml) of this were pipetted into 35 mm plastic culture dishes and incubated at 37 °C for 1 h to permit gel formation. The gels were then covered with 1 ml of DMEM or of 0.15 M NaCl containing 50 μ g (as protein) of ¹²⁵I-LDL, either oxidized or native, as indicated, and incubated for 48 h. After thorough washing [3], the gels were dissolved in 3 ml of 1 M NaOH; samples were taken for measurement of ¹²⁵I and of collagen protein. Under these conditions native LDL may undergo non-cellular oxidation and subsequent binding to collagen; oxidation occurs readily in saline gels, but is minimal in DMEM gels [4]. The term 'binding of native LDL' refers to the binding after such oxidation.

Oxidized LDL was prepared by exposure of ¹²⁵I-LDL to endothelial cells (EC) (for cell-oxidized LDL) or to a saline gel (for non-cell-oxidized LDL). EC from pig aorta were maintained in DMEM supplemented with 1 mM non-essential amino acids, 3.7 g/l NaHCO₃ and 10% (v/v) defined supplemented calf serum (Hyclone Laboratories), in plastic dishes coated with human fibronectin [10]. To obtain cell-oxidized LDL, cells were seeded in 60 mm tissue-culture dishes and grown to confluence in DMEM; the medium was then removed and 400 μ g of ¹²⁵I-LDL protein in 2 ml of Ham's F-12 medium was added. After 24 h the conditioned medium was centrifuged to remove cell debris, then

Abbreviations used: BHT, butylated hydroxytoluene; DMEM, Dulbecco modification of minimal essential medium; EC, endothelial cells; HMF, 5-hydroxymethylfurfural; LDL, low-density lipoprotein; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

‡ To whom correspondence should be addressed, at: Jewish General Hospital, 3755 Cote Ste. Catherine Road, Montreal, Quebec H3T 1E2, Canada.

assayed for cold-trichloroacetic-acid-precipitable ^{125}I to determine the concentration of oxidized LDL (usually $\sim 90\%$ of the LDL added). Non-cellularly oxidized LDL was prepared in a similar manner by incubation of ^{125}I -LDL in 0.15 M NaCl with a saline gel, for 24 h at 37°C . For experiments in which DMEM gels were glycated by exposure to glucose or ribose, DMEM was prepared without glucose, and the glycating agent was added to provide the concentration indicated.

Protein was measured by the method of Lowry et al. [11]. Electrophoresis was performed on agarose-gel films in barbital buffer, pH 8.6, at 200 V for 45 min; gels were stained with Sudan Black B. Relative mobility was expressed as the ratio of the migration distance of the sample to that of native LDL in the same electrophoretic run. Thiobarbituric acid (TBA)-reactive substances (TBARS) were measured by the method of Schuh et al. [12] against standards of malondialdehyde bis(dimethyl acetal) containing ribose at the same concentration as in the samples; standards and samples were extracted with butan-1-ol/pyridine (15:1, v/v) to remove interfering sugars [13]. The presence of advanced glycosylation end-products was determined fluorimetrically; samples were digested with collagenase (Sigma type IV) in Tris buffer as described by Monnier et al. [14], and emission spectra were obtained at an excitation wavelength of 370 nm. Glucose incorporation into collagen gels was measured by incorporation of $[\text{U-}^{14}\text{C}]$ glucose (ICN), previously incubated with collagen for 48 h at 37°C to remove radiochemical impurities [15]; after incubation with purified glucose for 48 h the gels were washed as for binding of LDL, then dissolved in 3 M NaOH, and a measured sample was counted for radioactivity in a liquid-scintillation counter. Ketoamines in collagen gels exposed to sugars were assayed as 5-hydroxymethylfurfural (HMF) released by hydrolysis in 1 ml of 0.5 M oxalic acid for 3 h at 100°C ; HMF was measured colorimetrically by reaction with thiobarbituric acid (TBA) [16], and fluorimetrically (excitation at 360 nm, emission at 430 nm) [17] after removal of oxalic acid on a 4 cm $\times$ 0.4 cm column of Dowex-1 (acetate form), followed by drying under vacuum.

Pentosidine was measured fluorimetrically after hydrolysis in 6 M HCl for 24 h at 105°C under N_2 [18]. The acid hydrolysate was dried under vacuum, taken up in a small volume of water and fractionated by reverse-phase chromatography on a 4.6 mm $\times$ 250 mm column of Econosil C_{18} , 10 μ (Alltech, Deerfield, IL, U.S.A.) by using a linear gradient of 2–18% acetonitrile in water (0.1% trifluoroacetic acid counter-ion), with a flow rate of 1 ml/min. Fractions (1 ml) were collected, and their fluorescence was determined (excitation at 335 nm, emission at 380 nm); emission spectra were obtained on fractions showing the greatest fluorescence.

All fluorescence measurements were made with an SLM-Aminco SPF500 spectrofluorimeter.

RESULTS

Binding of LDL to glycated collagen

Collagen solution (3.7 mg/ml) and sterile ribose solution (800 mM) were mixed in a ratio of 3:1 (v/v) to give final concentrations of 2.8 mg/ml collagen and 200 mM ribose. Another portion of collagen solution was similarly diluted with sterile water. The two preparations were kept at room temperature for up to 21 weeks; at intervals DMEM collagen gels were prepared from the two solutions and used to assay binding of native LDL and of freshly prepared EC-oxidized LDL; binding of EC-oxidized LDL to gels made from collagen stored at 4°C was determined at each interval to obtain a 'control'

value. Binding of native LDL was not different for collagen stored at 4°C , non-ribosylated collagen and ribosylated collagen, and did not change with time: the values for the three collagen preparations were (in μg of LDL/mg of collagen) 0.26 ± 0.02 , 0.25 ± 0.02 , and 0.33 ± 0.05 . Binding of EC-oxidized LDL to collagen which had been glycated by exposure to ribose decreased progressively with time, and by 17 weeks was virtually abolished (Figure 1); by contrast, binding to collagen maintained under the same conditions in the absence of ribose was unchanged. It was noted that, at 17 weeks, the gel formed by the ribosylated collagen was softer and more fragile than control gels; by 21 weeks the ribosylated collagen would not gel. In a similar experiment the ribosylated and control collagen solutions were used to prepare saline gels to which native LDL was exposed; measurements were made of the release of TBARS into the medium, of LDL electrophoretic mobility, and of binding of LDL to the saline gel. As shown in Table 1, TBARS production and collagen binding were diminished, but the increase in mobility was not.

When collagen was incubated with 20 mM glucose at 37°C , its ability to bind EC-oxidized LDL decreased from $10.8 \pm 0.03 \mu\text{g}/\text{mg}$ of collagen on day 0 to 8.7 ± 0.4 on day 4 and 7.9 ± 0.5 on day 8. This decrease did not occur when the collagen/glucose solution was incubated at 4°C .

Effect of glycation of collagen gels

Collagen gels were prepared in saline, then overlaid with saline or with 200 mM ribose in saline. After 48 h at 37°C the overlying fluid was removed and native LDL was added to the gels for a further incubation period of 48 h at 37°C ; control gels bound $9.29 \pm 0.04 \mu\text{g}/\text{mg}$ of collagen, whereas ribose-exposed gels bound $0.24 \pm 0.01 \mu\text{g}/\text{mg}$. By 72 h the gels incubated with ribose had dissolved.

Saline collagen gels were overlaid with an equal volume of ribose or glucose in 0.15 M NaCl; after incubation at 37°C for intervals up to 5 days the overlying fluid was replaced with native ^{125}I -LDL in 0.15 M NaCl for measurement of binding. At a final concentration of 10 mM ribose (in the combined volume of gel and overlying fluid), binding fell rapidly to a low of 8% after 3 days of incubation (Figure 2). The rate of fall increased with increasing ribose concentration; with glucose the rate of fall was about 50% of that with ribose (Figure 3).

Figure 1 Binding of EC-oxidized LDL to ribosylated (●) and non-ribosylated (○) collagen

Collagen was incubated at room temperature, in the absence and presence of 200 mM ribose. At the times shown, portions were used to prepare gels in DMEM, for assay of binding of LDL which had previously been oxidized by exposure to EC. At each time oxidized LDL was also bound to collagen solution stored at 4°C , to provide a control value. Symbols represent means $\pm$ S.D. of four replications.

Table 1 Effect of glycation of collagen on non-cellular oxidation of LDL and binding to collagen

Collagen solution was stored at room temperature with or without 200 mM ribose, then used to prepare gels in saline. ^{125}I -LDL (50 μg) was added to each gel and incubation was carried out for 48 h at 37 °C; measurements were made of LDL binding to the gel, TBARS in the overlying fluid, and relative electrophoretic mobility of LDL in the overlying fluid. Values for binding are means $\pm$ S.E.M. for quadruplicate samples: *for difference from 2-week values with ribose, and 6-week value without ribose, $P < 0.05$; **for difference from 2-week values with ribose, and 10-week value without ribose, $P < 0.01$. Values for TBARS are means of triplicate samples F value for comparison of $-/+$ ribose is significant at $P < 0.01$.

Time (weeks)	Ribose ...	Binding ($\mu\text{g}/\text{mg}$ of collagen)		TBARS ($\mu\text{mol}/\text{mg}$ of collagen)		Relative electrophoretic mobility (native = 1)	
		—	+	—	+	—	+
2		8.15 $\pm$ 0.20	8.88 $\pm$ 0.31	7.5	2.1	3.6	4.5
6		8.76 $\pm$ 0.23	6.31 $\pm$ 0.58*	5.4	0.8	4.2	4.6
10		10.18 $\pm$ 0.33	3.53 $\pm$ 0.15**	6.2	1.6	4.6	4.6

Figure 2 Effect of glycation of collagen gels on ability to bind LDL

Collagen gels were prepared in 0.15 M NaCl and overlaid with 10 mM ribose in 0.15 M NaCl. At the times shown, the ribose solution was removed and LDL in 0.15 M NaCl was added to the gels; binding of LDL was measured 48 h later.

Figure 3 Effect of sugar concentration during glycation on subsequent binding of LDL

Collagen gels in 0.15 M NaCl were exposed for 24 h to ribose (●) or glucose (○) at the concentrations shown. The sugar solution was then removed and LDL was added; binding of LDL was measured after 48 h.

To determine the effect of glycation directly on binding, oxidized LDL was incubated with glycated saline gels; 20 mM butylated hydroxytoluene (BHT), which has no effect on the binding process, was added to prevent further oxidation. As seen in Table 2, binding of both EC-oxidized and non-cell-oxidized LDL was inhibited by glycation of saline gels.

There were several important differences in the glycation procedures used in the experiments shown in Figures 1 and 2,

Table 2 Effect of glycation of collagen gel on binding of oxidized LDL

LDL which had been oxidized by previous incubation with EC in F-12 medium, or with F-12 in the absence of cells, was incubated with collagen gels prepared in 0.15 M NaCl for 48 h and bound LDL was then measured. Results are means $\pm$ S.E.M. ($n = 4$): *for difference from control value, $P < 0.01$.

LDL	LDL bound ($\mu\text{g}/\text{mg}$ of collagen)		
	Control	Ribosylated (48 h)	Glycosylated (14 days)
EC-oxidized	6.23 $\pm$ 0.20	0.46 $\pm$ 0.08*	1.98 $\pm$ 0.24
Non-cell-oxidized	10.06 $\pm$ 0.50	0.48 $\pm$ 0.02*	3.82 $\pm$ 0.35*

Table 3 Effect of preincubation of collagen gels with ribose on binding of oxidized LDL

Values for LDL binding are means $\pm$ S.E.M. ($n = 4$). Collagen gels were prepared in 0.15 M NaCl, and preincubated (except where marked NA) for 48 h at 37 °C; EC-oxidized LDL was then added with or without ribose (to bring the concentration to 10 mM). Binding of LDL was measured after 48 h incubation.

Preincubation (10 mM ribose present)	Incubation (10 mM ribose present)	LDL binding ($\mu\text{g}/\text{mg}$ of collagen)
—	—	11.62 $\pm$ 0.25
—	+	11.05 $\pm$ 0.48
+	+	0.64 $\pm$ 0.03
NA	+	9.8 $\pm$ 0.36

and these may have been responsible for the fact that there was no effect on LDL binding after 2 weeks of glycation in the first (Figure 1) and a major effect after 1 day of glycation in the second (Figure 2), even though in both cases the collagen gel was exposed to sugar for 48 h at 37 °C during the binding of LDL. These results imply that the inhibitory effect of glycation may depend on interaction of sugar with collagen before addition of LDL. To test this directly, saline gels were preincubated for 48 h at 37 °C in the absence or in the presence of 10 mM ribose; then EC-oxidized LDL was added and the extent of binding analysed after a further 48 h incubation. As seen in Table 3, the inhibitory effect of ribose required that the collagen be exposed to the sugar

Table 4 Effect of LDL on binding of glucose to collagen gels

Values for [^{14}C]glucose binding are means $\pm$ S.E.M. ($n = 8$). Collagen gels were prepared in 0.15 M NaCl. In A, gels were preincubated for 48 h at 37 °C with 10 mM [^{14}C]glucose; EC-oxidized LDL in 0.15 M NaCl or an equal volume of saline was added, and gels were incubated for a further 48 h. Binding of [^{14}C]glucose was then measured. In B, 10 mM [^{14}C]glucose, with or without EC-oxidized LDL, was added, gels were incubated for 48 h and binding of [^{14}C]glucose was measured. NA = no preincubation.

	Preincubation	Incubation (addition)	[^{14}C]Glucose bound (d.p.m./mg of collagen)
A	Glucose	—	2388 $\pm$ 125
	Glucose	LDL	2550 $\pm$ 85
B	NA	Glucose	1962 $\pm$ 43
	NA	Glucose, LDL	2019 $\pm$ 109

Figure 4 Binding of glucose to collagen gels

(a) Collagen gels in 0.15 M NaCl (pH 7.4) were incubated for 48 h at 37 °C with [^{14}C]glucose in 0.15 M NaCl, at the concentrations shown. The gels were then washed and the amount of glucose bound to the collagen was measured. (b) Collagen gels were incubated with 50 mM [^{14}C]glucose and binding was measured at the times shown. Each point is the mean of four gels.

Figure 5 Dissociation of glucose from collagen gels

Collagen gels in 0.15 M NaCl were incubated with [^{14}C]glucose (50 mM) for 48 h. Gels were removed from their plastic wells, blotted rapidly and gently on filter paper, placed in tubes containing 2 ml of 50 mM unlabelled glucose, and shaken gently at 37 °C. At the times shown, gels ($n = 4$) were washed thoroughly and assayed for radioactivity, expressed as a percentage of that at zero time.

in the absence of LDL; this was not due to LDL inhibition of glycation as measured by [^{14}C]glucose binding (Table 4).

Nature of glycation of collagen gels

Incorporation of glucose into collagen increased with glucose concentration and with time of incubation (Figure 4). After a 48 h exposure the incorporated glucose was not discharged by lowering the pH to 5 (results not shown), and dissociation at neutral pH was only about 20 % in 24 h (Figure 5). The amount of glucose incorporated was not affected by sodium cyanoborohydride (20 mM) under standard incubation conditions (37 %, pH 7.4). To determine the nature of the glycation product(s), collagen gels and samples of minced rat tail tendon were incubated in the absence or presence of ribose, then analysed for several known glycation products. The results (Table 5; Figures

Table 5 Development of glycation products in collagen gels and tendon

Rat tail tendon and collagen gels were incubated at 37 °C in the absence or presence of 100 mM ribose. Portions of dried tendon (10 mg) and pooled gels containing 10 mg of collagen were analysed for ketoamines colorimetrically and fluorimetrically, for pentosidine, and for advanced glycation end-products (AGE). ND, not detected.

	Gels			Tendon			
	Incubation (days) ...			2		6	
	Ribose ...			0	100 mM	0	100 mM
Colour	—	—	—	—	pale yellow	—	amber
Ketoamines							
TBA reaction (nmol/mg)	ND	ND	ND	ND	0.87	ND	3.03
Relative fluorescence*	0.14	0.26	0	0.1	1.82	0	3.96
Pentosidine							
Relative fluorescence†	0.3	0.3	0.3	0.4	1.3	0.4	3.6
AGE							
A_{350}	0.155		0.171	0.157	0.251	0.159	0.483
Relative fluorescence‡	2		2.2	2.3	3.8	2.3	7.2

* Values corrected for fluorescence of reagent blank run on effluent from the Dowex-1 column used for removal of oxalic acid.

† Values from Figure 6.

‡ Values from Figure 7.

Figure 6 Fluorescence emission spectra (excitation at 335 nm) of pentosidine obtained from acid hydrolysates (6 M HCl, 24 h, 105 °C) of collagen gels and tail tendon incubated with ribose

Hydrolysates were fractionated by h.p.l.c.; emission spectra were obtained on fractions with greatest fluorescence at 385 nm. (a) Synthetic pentosidine; (b) tendon incubated for 6 days with 100 mM ribose; (c) tendon incubated for 2 days with 100 mM ribose; (d,e) tendon incubated for 6 and 2 days respectively without ribose; (f-h) collagen gels incubated for 2 days with ribose at 0, 10 and 100 mM respectively.

Figure 7 Fluorescence emission spectra (excitation at 360 nm) of collagenase digests of collagen gels and tail tendon after incubation with ribose

Spectra, from top to bottom, are: tendon + 100 mM ribose for 6 days, tendon + 100 mM ribose for 2 days, tendon without ribose for 6 days, tendon without ribose for 2 days, gels with 100 mM ribose for 2 days, gels without ribose for 2 days.

6 and 7) demonstrate that glycated tendon collagen accumulated increasing amounts of ketoamines, pentosidine and fluorescent advanced glycation products; none of these substances was detectable in collagen gels. To determine if the inhibitory effect of collagen glycation on LDL binding involved autooxidation of the sugar and oxygen-radical attack on the collagen, the effects

of a metal chelator and of an oxygen-radical scavenger during glycation with ribose were assessed. Ribose alone decreased binding from 6.17 ± 0.32 to 0.27 ± 0.04 μg of LDL/mg of collagen; this effect was not altered by 10 mM EDTA (0.51 ± 0.09 $\mu\text{g}/\text{mg}$) or 20 μM BHT (0.33 ± 0.02 $\mu\text{g}/\text{mg}$).

DISCUSSION

Native LDL binds to a collagen gel to a limited extent [3]; LDL which has been oxidized by cellular action (in the present work by endothelial cells) or by a non-cellular process catalysed by trace amounts of Cu^{2+} binds to a much greater degree [3,4]. Both routes of oxidation lead to peroxidation of the LDL fatty acids [19]; adducts formed between lipid peroxidation products such as malondialdehyde [20] and 4-hydroxynonenal [21] and the ϵ -amino groups of lysine in the apo-LDL increase the protein net negative charge and thus the relative electrophoretic mobility. In addition, both forms of oxidation lead to changes in the apo-LDL which increase binding to collagen fibres in a hydrated lattice. Thus in the present context three separate processes can be identified: lipid peroxidation, apoprotein oxidation and binding of oxidized apoprotein to collagen.

In the present work we report that glycation of collagen had three effects on LDL oxidation and binding. (a) It inhibited LDL lipid peroxidation during non-cellular oxidation; however, the level of peroxidation and subsequent formation of adducts with apo-LDL were sufficient to change the LDL electrophoretic mobility. (b) It inhibited binding of LDL previously oxidized by either cellular or non-cellular mechanisms. (c) It interfered with gel formation and stability.

It had previously been reported that binding of LDL was increased by non-enzymic glycosylation of collagen, in proportion to the amount of advanced glycation end-products [6]. In these experiments LDL was incubated with collagen in PBS at 37 °C without added antioxidants, conditions which would permit non-cellular oxidation of the LDL; it may therefore be assumed that the binding values were for oxidized LDL. However, the amount of binding even to glycosylated collagen was low (~ 0.6 μg of LDL/mg of collagen, at an LDL concentration of 50 $\mu\text{g}/\text{ml}$) compared with that in collagen gels (~ 10 $\mu\text{g}/\text{mg}$ of collagen). These differences in glycation and in binding may be due to differences in the experimental procedures used.

(a) In the earlier study collagen was immobilized by covalent attachment to agarose beads; in the present work collagen was present in a hydrated gel of collagen fibrils as it exists *in vivo*. Intermolecular cross-linking occurs in such a gel, but it is not clear whether or how it might exist between collagen molecules bound to the agarose.

(b) The previous results were obtained with collagen which had been denatured with SDS. In addition, glycation was carried out at 44 °C for 2 weeks. Since thermal denaturation of collagen type I, with transition of molecular structure from helical to random coil, occurs over the range 35–40 °C, with a midpoint of 38.5 °C [22], it is probable that all the collagen had been so denatured when LDL was added. This might have altered the sites to which LDL can bind. The temperature during LDL binding was 37 °C, but it is not known if any renaturation occurred during this process. We used native collagen, the glycation temperature was ~ 20 °C, well below that of the melting transition, or 37 °C, and binding of LDL was at 37 °C; at this temperature about 90% of the collagen would have been in the helical state, as it presumably is *in vivo*.

In the present work glucose or ribose was present during exposure of LDL to the collagen. The concentration of sugar at that time was half that present during glycation: for example, if

the collagen glycation was effected at 200 mM, this was the concentration within the gel fluid as well as in the overlying medium; when the 1 ml of medium was replaced with 1 ml of LDL, the sugar within the gel would have equilibrated rapidly within the total volume of 2 ml [3] to give a concentration of 100 mM. As shown by the results after short exposure times (e.g. Figure 1 and Table 3), the presence of free sugar at this concentration had no effect on oxidation or binding.

Hicks et al. [7] observed that glucose alone and glycosylated collagen decreased the lag time to onset of peroxidation of fatty acid vesicles; this effect was attributed to reaction of the sugar with lipid hydroperoxide to produce glycosyl and alkoxy radicals, which propagate the peroxidation chain reaction [23]. We did not observe these effects with LDL. However, in the present work glycosylated collagen actually inhibited lipid peroxidation; the mechanism involved remains to be determined.

Two pathways have been proposed to explain glycation and resulting 'damage' to proteins.

(a) The 'classical' pathway involving formation of Schiff bases as adducts of sugar molecules to free amino groups, followed by Amadori rearrangement to form ketoamines; the latter may react with arginine residues to form the fluorescent cross-link, pentosidine, or undergo oxidation. Oxygen radicals formed during oxidation of the ketoamine may attack the protein and produce changes in structure and function [24].

(b) Hunt et al. [25] distinguish between protein damage, leading to formation of fluorophores, and binding of sugar to the protein. They propose that the sugar first undergoes autooxidation, catalysed by a heavy metal, leading to formation of hydroxyl radicals, which degrade the protein and cause formation of fluorophores; the oxidized sugar products then bind to the altered protein. The lack of effect of EDTA, a metal chelator, or of BHT, a free-radical scavenger, on ribose inhibition of LDL binding to collagen argues against a role for sugar autooxidation in glycation.

During the short glycation periods which led to decreased binding of oxidized LDL and to disruption of collagen gels, sugar was bound to the collagen, but the nature of the reaction product is not apparent: (a) the failure to dissociate rapidly at acid pH and the slow rate of dissociation at neutral pH are not compatible with a Schiff's base; (b) we were unable to detect ketoamine, pentosidine or fluorescent advanced glycation end-products. The absence of the usual glycation products from collagen gels and their presence in tendon collagen suggests major differences in the pathway and effects of glycation between soluble collagen, from which gels were prepared, and tightly cross-linked insoluble collagen as in tendon. We observed that glycation interfered with the formation and stability of collagen gels. This is in keeping with the results of Hayashi and Nagai [26], who reported that several saccharides, including glucose and ribose, inhibit formation of collagen fibrils *in vitro*, without denaturing the collagen molecules; this is accompanied by a decrease in lysyl oxidase-catalysed formation of cross-links [27]. Paradoxically, it has also been shown that glycation of tendon leads to formation of intermolecular cross-links [24,28], resulting in increased rigidity of collagen [28]. It has been demonstrated by X-ray diffraction that glycation causes an increase in the intermolecular spacing in the collagen fibril [29]; expansion of the

molecular packing increases with time of exposure to reducing sugar, and the lattice structure is ultimately lost.

We hypothesize that the increase in intermolecular distance in minimally cross-linked gel collagen is sufficient to prevent the formation of glycation-related cross-links. In collagen which is already tightly cross-linked, the increased intermolecular distance leads to increased rigidity of the collagen but the proximity and order of the collagen chains are sufficiently maintained to permit the formation of advanced glycation products such as pentosidine and fluorescent advanced glycation end-products. We also suggest that the increased intermolecular distance disrupts the binding sites for oxidized LDL.

Collagen in the arterial intima and media is largely present as fine reticular fibres rather than as large bundles, and therefore resembles collagen gels rather than tendon; our results may then raise questions concerning the proposal [30] that glycation of arterial-wall collagen is responsible for accelerated atherogenesis in diabetes.

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