Evidence for glucose-mediated covalent cross-linking of collagen after glycosylation *in vitro*

M. J. Christine KENT,* Nicholas D. LIGHT and Allen J. BAILEY
A.F.R.C. Meat Research Institute, Langford, Bristol BS18 7DY, U.K.

Rabbit forelimb tendons incubated for 15 or 21 days at 35°C in the presence of 8 or 24 mg of glucose/ml were shown to change their chemical, biochemical and mechanical characteristics. The tendons treated with glucose contained up to three times as much hexosyl-lysine and hexosylhydroxylysine as did control tendons as judged by assay of NaB^3H_4-reduced samples. Measurement of the force generated on thermal contraction showed significant increases in glycosylated tendons compared with controls, indicating the formation of new covalent stabilizing bonds. This conclusion was supported by the decreased solubility of intact tendons and re-formed fibres glycosylated *in vitro*, and by the evidence from peptide maps of CNBr-digested glucose-incubated tendons. The latter, when compared with peptide maps of control tendons, revealed the presence of additional high-M_r peptide material. These peptides appear to be cross-linked by a new type of covalent bond stable to mild thermal and chemical treatment. This system *in vitro* provides a readily controlled model for the study of the chemistry of changes brought about in collagen by non-enzymic glycosylation in diabetes.

The first demonstration of non-enzymic glycosylation of a protein was the attachment of glucose to the *N*-terminal valine residue of haemoglobin (Allen *et al.*, 1958). Subsequently, we demonstrated the random glycosylation of the ε-amino group of peptide-bound lysine and hydroxylysine in collagen (Robins & Bailey, 1972). The reaction is most specific for glucose, the detection of mannosyl-lysine in acid hydrolysates of reduced collagen being due to epimerization on reduction of the Amadori-rearranged product (Le Pape *et al.*, 1981). The increased attachment of glucose to the *N*-terminal of haemoglobin in diabetes mellitus raised the interesting possibility of a similar increased attachment to the ε-amino group of peptide-bound lysine in other proteins. Several proteins have now been shown to be glycosylated, notably lens crystallin (Steven *et al.*, 1978), serum albumin (Day *et al.*, 1979; Dolhoffer & Wieland, 1979) and erythrocyte membrane proteins (Bailey *et al.*, 1976; Miller *et al.*, 1980).

During maturation and aging, collagen becomes less soluble and susceptible to proteolytic enzymes and increasingly stronger and more rigid (for review, see Light & Bailey, 1979). These changes have always been attributed to the accumulation of covalent cross-links derived from amino acid side chains and formed between collagen molecules. However, the physico-chemical changes are also concomitant with the accumulation of glycosylated lysine residues in collagen. It appeared from the studies of Robins & Bailey (1972) that these hexosyl-lysine and hexosylhydroxylysine residues, as such, could not act as collagen cross-links and would not be capable of stabilizing the collagen matrix. More recently, this view that glucose cannot mediate collagen cross-link formation has altered, since we reported a correlation between increased hexosyl-lysine content in collagen from diabetic rats and increased tensile strength (Andreassen *et al.*, 1981).

In the present paper we report data obtained with our system *in vitro*, which shows conclusively that non-enzymic glycosylation of collagen causes covalent cross-linking, which leads to increases in hydrothermal isometric tension, increased amounts of polymeric collagen and decreased solubility.

Materials and methods

Materials

NaB³H₄ was bought from Amersham International, Amersham, Bucks., U.K. Penicillin G, streptomycin and CNBr were from Sigma Chemical Co., Poole, Dorset, U.K. Millipore filters were from Millipore Corp., Bedford, MA, U.S.A. Zeokarb 225 and cocktail T 'Scintran' were from BDH Chemicals, Poole, Dorset, U.K. Acetonitrile was from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K. Rabbit forelegs and rat tails were stored at $-20°C$ until used.

Preparation of collagen

Tail tendons from 2-month-old rats were extracted in 1.0M-NaCl/50mM-Tris/HCl (pH 7.4)/10mM-EDTA/2mM-N-ethylmaleimide at $4°C$ for 48h. Solubilized material was precipitated with 2.6M-NaCl, redissolved in 0.5M-acetic acid, precipitated again with 5% (w/v) NaCl and finally taken up in 0.5M-acetic acid (2mg/ml). Non-solubilized material was removed by centrifugation (20000g, 60min), and the supernatant dialysed into 0.1M-acetic acid, freeze-dried and stored at $-180°C$ (Lee, 1983). Whole chains of collagen were examined by SDS/polyacrylamide-disc-gel electrophoresis as described by Laemmli (1970); 10μg of material was run in each track, and was separated in a polyacrylamide gel containing 6% (w/v) acrylamide with a stacking gel containing 4% (w/v) acrylamide.

Incubations in vitro

Paired rabbit tendons (i.e. corresponding tendons from the left and right foreleg of one animal; approx. 20mg wet wt.) were incubated separately in 15ml of phosphate-buffered saline, with 100 units of penicillin G/ml and 100μg of streptomycin sulphate/ml added to prevent bacterial growth. One of each pair of tendons acted as a control. The second was incubated with 8–24mg of glucose or mannitol/ml added to the above medium. Incubations were performed in 25ml screw-cap bottles at $35°C$ for 15–21 days. In some instances the incubation medium was changed every 2–3 days throughout the incubation period.

Type I collagen fibrils re-precipitated from neutral-salt-extracted material were incubated with 24mg of glucose/ml in phosphate-buffered saline (plus antibiotics) for 21 days at $35°C$. Controls contained Type I collagen fibrils incubated with phosphate-buffered saline (plus antibiotics) alone. After incubation, collagen fibrils were extensively dialysed against water and freeze-dried.

Hydrothermal isometric-tension experiments

After incubation in vitro, rabbit tendons were dabbed dry and placed between the jaws of a hydrothermal isometric-tension apparatus previously described by Viidik (1968). The length of the tendon between the jaws was measured with Vernier callipers, and the tendon immersed in phosphate-buffered saline at $30°C$. The temperature was raised to $90°C$ at a rate of $10°C$/min, then maintained at $90°C$ for 10min. The tension generated was continuously recorded. After the experiment the tendon was cut close to the jaws with a scalpel, freeze-dried and weighed. Assuming tendons from one rabbit were of uniform density, cross-sectional area of tendons was proportional to dry wt./length. Maximum tension and tension 10min after reaching $90°C$ were measured as N (newtons)/mg dry wt. of tendon per mm, which is proportional to force/unit cross-sectional area.

Solubility experiments

After 21 days incubation at $35°C$, rabbit tendons, control and glycosylated in vitro, were dabbed dry and cut into small pieces with a scalpel. Acetic acid (0.5M) was added to give an approximate concentration of 5mg wet wt. of tendon/ml. After 2h at room temperature with shaking, solubilized material was separated by passage through a 25μm-pore-size Millipore filter, which was rinsed with 0.5M-acetic acid.

The two fractions (filtrate and residue plus Millipore filter) were freeze-dried and hydrolysed in 6M-HCl at $110°C$ for 24h. HCl was removed under vacuum, and hydroxyproline content was measured with a Technicon Autoanalyser as described by Grant (1964).

The solubility of Type I collagen fibrils glycosylated in vitro was compared with that of controls incubated in vitro and collagen stored at $-180°C$. Freeze-dried material (3mg) was taken up in 1.5ml of 0.5M-acetic acid. After 1h at room temperature with shaking, mixtures were stored at $4°C$ overnight. Solubilized material was separated by passage through 25μm-pore-size Millipore filters rinsed with 0.5M-acetic acid. Freeze-dried filtrates and residues (plus Millipore filters) were acid-hydrolysed, and hydroxyproline content was measured as described above.

CNBr peptides

Rabbit tendons or Type I collagen fibrils were taken up in 70% (v/v) formic acid (10mg dry wt./ml). For re-formed type I collagen fibrils, freeze-dried material was rehydrated in distilled water by heating at $60°C$ for 30min; 90% formic acid was then added (final concn. 70%, v/v). An

equal weight of CNBr (2g/ml in acetonitrile) was added, and mixtures were incubated in screw-cap bottles at 30°C for 4h. The reaction was stopped by the addition of 10vol. of water, and digests were filtered through glass wool, evaporated under vacuum at 40°C and freeze-dried. CNBr peptides were examined by SDS/polyacrylamide-disc-gel electrophoresis by the method of Laemmli (1970); 75μg of CNBr peptides per track was run on poly-acrylamide gels containing 10% (w/v) acrylamide, with stacking gels containing 4% (w/v) acrylamide. Gels were stained with Coomassie Brilliant Blue, destained and scanned in a densitometer (Light, 1982).

Hexosyl-lysine and reducible cross-link content

Rabbit tendons and Type I collagen fibrils were examined for reducible cross-link and hexosyl-lysine content by ion-exchange chromatography after acid hydrolysis of NaB^3H_4-reduced samples (Bailey *et al.*, 1970; Robins & Bailey, 1972).

Results

Hydrothermal isometric tension

Tension recorded across the rabbit forelimb tendons as the temperature of the surrounding medium was raised from 30 to 60°C was extrapolated to give a baseline. An increasing tension was generated in the tendons as they were heated above denaturation temperature. Two different types of isometric tension curve were consistently shown by tendons that had been preincubated in phosphate-buffered saline, the type of response depending on the anatomical site of origin of the tendon. Either the isometric tension reached a maximum around 90°C and then the tendon relaxed (as shown in Fig. 1a, continuous line), or the tension was maintained for a further 10min at 90°C (Fig. 1b, continuous line).

A study of reducible cross-link content and CNBr peptide maps showed that it was rabbit tendons with a high proportion of the heat-stable cross-link hydroxylysino-5-oxo-norleucine and high-M_r cross-linked peptides (poly-α_1CB6; see Light & Bailey, 1980) that consistently gave isometric tension curves similar to that shown in Fig. 1b (continuous line). Tendons with low proportions of the heat-stable cross-link and poly-α_1CB6 gave a curve similar to that shown in Fig. 1a (continuous line). In these cases the heat-labile reducible cross-link, dehydrohydroxylysino-norleucine, was always more predominant.

Both types of tendons became non-enzymically glycosylated to a similar extent on incubation with glucose *in vitro*, as shown by their content of hexosyl-lysine derivatives, both of which were up
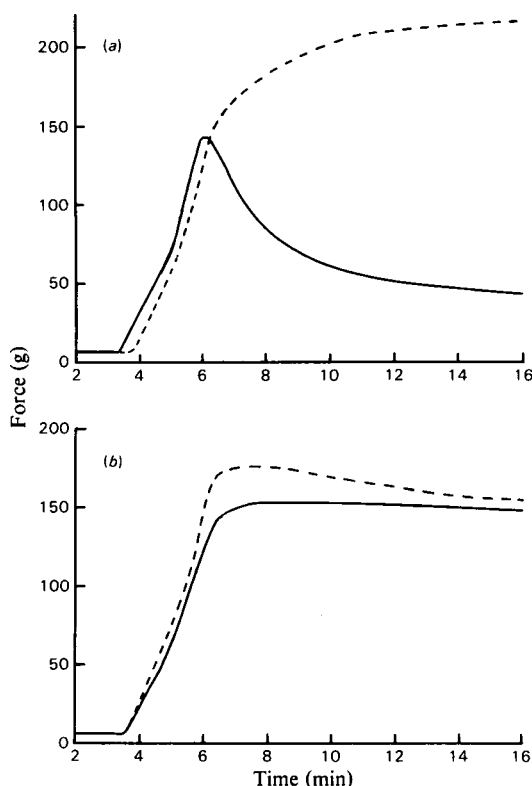

Fig. 1. *Hydrothermal isometric-tension curves of paired rabbit tendons*
One tendon of each pair was preincubated at 35°C for 21 days in phosphate-buffered saline (———). The second tendon was preincubated with 24mg of glucose/ml in phosphate-buffered saline (– – – –). (a) 'Heat-relaxing' tendon; (b) 'heat-resistant' tendon. The temperature of the hydrothermal isometric-tension bath was raised at 10°C/min to 90°C (at 6min), then maintained at 90°C for 10min.

to 3-fold greater than that of control incubated tendons.

To describe the two types of isometric-tension response, the following ratio was calculated for tendons preincubated in phosphate-buffered saline alone (i.e. without glucose or mannitol):

$$\frac{\text{Maximum isometric tension}}{\text{Isometric tension 10 min after reaching } 90°C}$$

Where this ratio was high (as in Fig. 1a, continuous line) tendons were described as 'heat-relaxing', and where it was low (as in Fig. 1b, continuous line) tendons were 'heat-resistant'.

After incubation with glucose (24mg/ml in phosphate-buffered saline) at 35°C for 21 days, 'heat-relaxing' tendons showed an increase in isometric tension of 2–7N/mg dry wt. per mm

10 min after reaching 90°C (Fig. 1a, broken line), whereas 'heat-resistant' tendons showed only slightly increased isometric tension, usually less than 0.5 N/mg dry wt. per mm (Fig. 1b, broken line).

To test whether the effect of incubating tendons with glucose was due to variations in the osmolality of the medium, rabbit tendons were incubated with mannitol (24 mg/ml in phosphate-buffered saline) at 35°C for 21 days. 'Heat-relaxing' tendons showed only small increases in isometric tension compared with paired tendons incubated in phosphate-buffered saline alone. 'Heat-resistant' tendons also demonstrated little change (results not shown).

The temperature at which isometric tension began to increase rapidly (63–64°C for phosphate-buffered-saline-incubated tendons) was often raised by 3 or 4°C for glucose-incubated tendons (see, e.g., Fig. 1a). A similar effect was seen after very mild glutaraldehyde treatment [0.4% (w/v) glutaraldehyde in phosphate-buffered saline at room temperature for 10 min] as shown in Fig. 2. Isometric tension began to increase at 75–77°C after this treatment, and tension developed 10 min after reaching 90°C was greater than for the phosphate-buffered-saline-treated control.

Hydrothermal isometric tension developed by rabbit tendons varied from a maximum of 10.4 to 1.4 N/mg dry wt. per mm at 90°C. In order to compare the response of different tendons, the following ratio was calculated:

$$\frac{\text{Isometric tension for glucose (or mannitol)-incubated tendon}}{\text{Isometric tension for the phosphate-buffered-saline-incubated paired tendon}}$$

in each case taking isometric tension 10 min after reaching 90°C. This ratio was plotted against the ratio:

$$\frac{\text{Maximum isometric tension}}{\text{Isometric tension 10 min after reaching 90°C}}$$

for tendons incubated in phosphate-buffered saline as shown in Fig. 3.

Tendons preincubated in 24 mg of glucose/ml for 21 days (●, Fig. 3a) demonstrate a linear relationship between response to glucose and heat-susceptibility of the phosphate-buffered-saline-incubated paired tendon. When incubated under the same conditions for 15–18 days (▲, Fig. 3a), tendons show a similar relationship, but to a lesser extent.

Tendons incubated in 8 mg of glucose/ml for 21 days (●, Fig. 3b) or 15–18 days (▲, Fig. 3b) gave a similar response to those incubated with 24 mg of
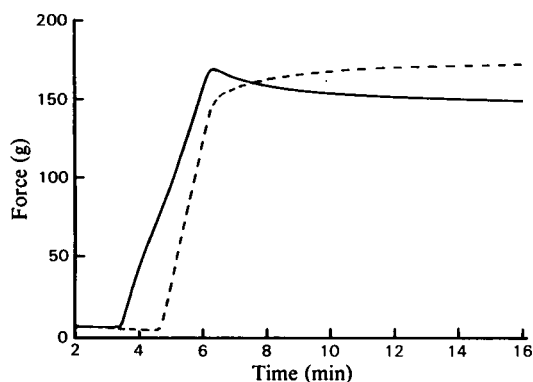

Fig. 2. *Effect of glutaraldehyde treatment on hydrothermal isometric tension of rabbit tendons*
One of a pair of rabbit tendons was preincubated with 0.4% (w/v) glutaraldehyde in phosphate-buffered saline at room temperature for 10 min (– – – –) and the other preincubated in phosphate-buffered saline alone (———). The temperature of the hydrothermal isometric-tension bath was regulated as in Fig. 1.

glucose/ml. It appears that the length of incubation *in vitro* at 35°C had more effect on the increased stability of tendons at 90°C than the concentration of glucose in the incubation medium. When the incubation medium *in vitro* was changed every 2 to 3 days over a 21-day incubation period, 'heat-relaxing' tendons showed a similar increased ability to maintain higher isometric tension at 90°C for 10 min (not shown).

Incubation in the presence of 8 or 24 mg of mannitol/ml provides an environment of similar osmolality to that of 8 or 24 mg of glucose/ml. Non-enzymic glycosylation of collagen does not occur in the presence of mannitol, owing to the absence of a suitable aldehyde group to form a Schiff base with the ε-amino group of lysine or hydroxylysine. Tendons incubated with 8 or 24 mg of mannitol/ml were not markedly different from phosphate-buffered-saline-incubated controls in their ability to maintain isometric tension at 90°C (○, △, Figs. 3a and 3b). This suggests that increased stability to heat was a function of non-enzymic glycosylation rather than of elevated osmolality of incubation media *in vitro* and must be due to an increase in heat-stable covalent bonds.

Solubility of tendons and Type I collagen fibrils glycosylated in vitro

Some 0.38 ± 0.12% of rabbit tendons glycosylated *in vitro* were solubilized in 0.5 M-acetic acid after 2 h at room temperature (mean ± s.d. of two observations), compared with 1.61 ± 0.18% of non-
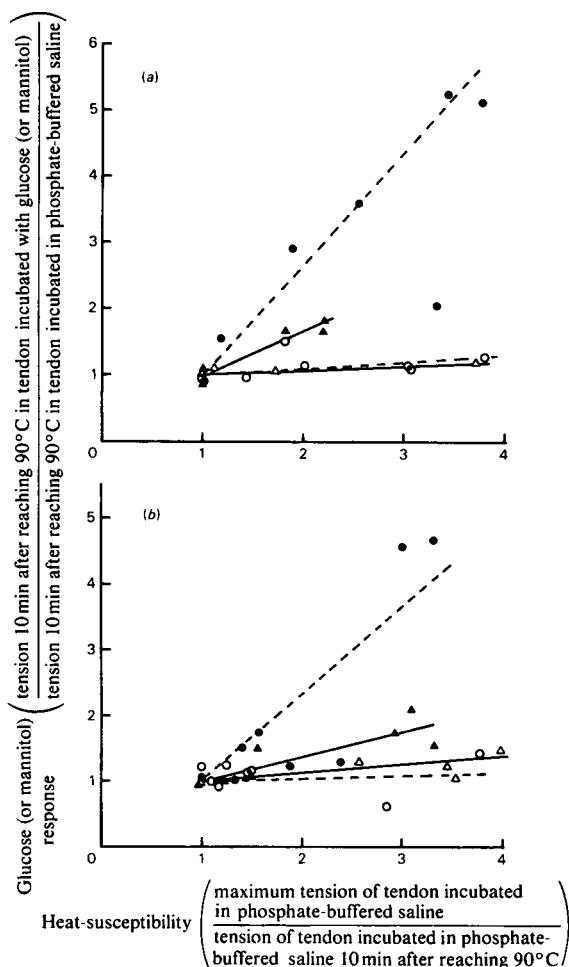
Fig. 3. *Comparison of the hydrothermal isometric tension developed by different rabbit tendons preincubated with glucose or mannitol in phosphate-buffered saline with paired tendons preincubated in phosphate-buffered saline alone*

(*a*) One tendon of each pair incubated in phosphate-buffered saline alone and one incubated with 24 mg of glucose/ml (●, ▲) or with 24 mg of mannitol/ml (○, △). (*b*) One tendon of each pair incubated in phosphate-buffered saline alone and one incubated with 8 mg of glucose/ml (●, ▲) or with 8 mg of mannitol/ml (○, △). Tendons were preincubated at 35°C for 21 days (●, ○) or for 15–18 days (▲, △).
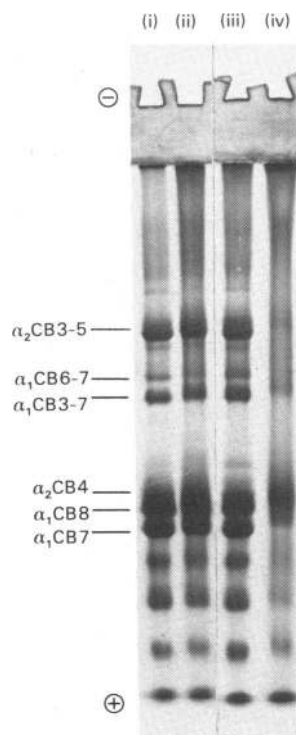

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of rabbit tendon CNBr peptides after incubation in vitro*

One of each pair of tendons was preincubated in phosphate-buffered saline [tracks (i) and (iii)] and the other preincubated with 24 mg of glucose/ml in phosphate-buffered saline [tracks (ii) and (iv)]. Tracks (i) and (ii) refer to a 'heat-relaxing' tendon incubated for 15 days at 35°C, and tracks (iii) and (iv) refer to a 'heat-resistant' tendon incubated for 21 days at 35°C.

glycosylated tendons incubated *in vitro*, as shown by hydroxyproline content of filtrate and residue.

Type I collagen fibrils glycosylated *in vitro* showed $6.2 \pm 2.8\%$ solubilization in 0.5 M-acetic acid after 1 h at room temperature and 18 h at 4°C, whereas $42.8 \pm 7.3\%$ of phosphate-buffered-saline-incubated controls was solubilized, and control fibrils that had been stored at −180°C showed $94.5 \pm 2.8\%$ solubilization.

CNBr peptides of rabbit tendons glycosylated in vitro

Rabbit tendons that had been glycosylated *in vitro* for 15–21 days were poorly digested by CNBr at 30°C for 4 h. SDS/polyacrylamide gels of CNBr peptides are shown in Fig. 4. Glycosylated tendons showed more high-M_r material, and a smaller amount of low-M_r CNBr peptides. This was particularly evident when tendons had been glycosylated *in vitro* for 21 days, and is demonstrated more clearly by densitometric scans of the gels (Fig. 5). Peaks labelled 1 and 2 refer to high-M_r material, and were increased in size on glycosylation (broken lines). Fig. 5(*a*) refers to a 'heat-relaxing' tendon after 15 days incubation *in vitro*, and Fig. 5(*b*) refers to a 'heat-resistant' tendon after 21 days incubation *in vitro*. Peaks 3, 5, 6 and 7 were initially conserved, whereas low-M_r material was decreased (Fig. 5*a*). After 21 days, glycosylation *in vitro*, all but the high-M_r CNBr peptides
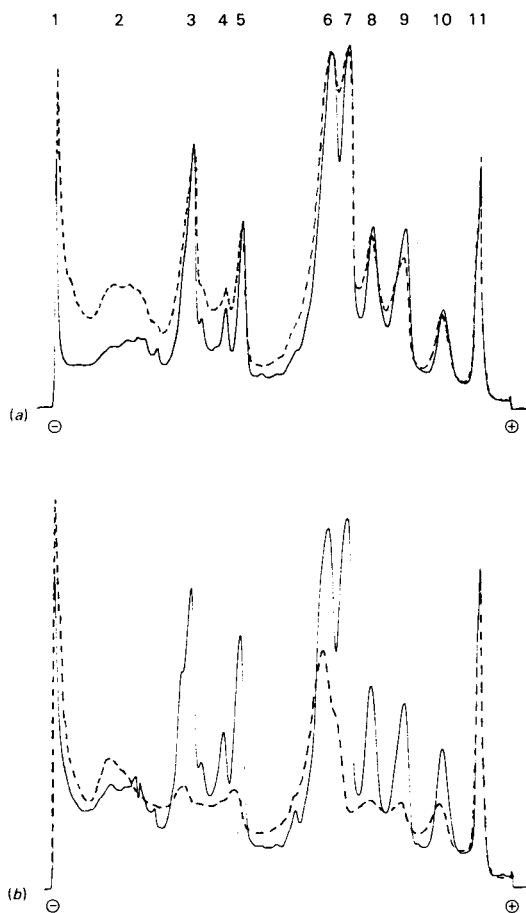
Fig. 5. *Densitometry scans of rabbit tendon CNBr peptides separated by SDS/polyacrylamide-gel electrophoresis, as shown in Fig. 4*

Tendons were pre-incubated in phosphate-buffered saline (——) or with 24 mg of glucose/ml in phosphate-buffered saline (————). Preincubation was for (*a*) 15 days or (*b*) 21 days at 35°C. Peaks are labelled 1–10 in order of decreasing M_r. Peak 11 refers to the dye front.
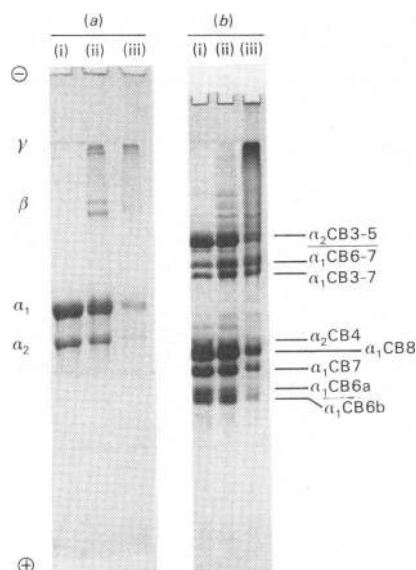

Fig. 6. *SDS/polyacrylamide-gel electrophoresis of Type I collagen*

(*a*) Whole chains separated on polyacrylamide gels containing 6% (w/v) acrylamide. (*b*) CNBr peptides separated on polyacrylamide gels containing 10% (w/v) acrylamide. Track (i), Type I collagen stored at -180°C. Track (ii), reprecipitated fibrils incubated in phosphate-buffered saline at 35°C for 21 days. Track (iii), reprecipitated fibrils incubated with 24 mg of glucose/ml in phosphate-buffered saline at 35°C for 21 days.

were decreased in quantity, and a considerable amount of high-M_r material was often retained in the stacking gel.

Phosphate-buffered-saline-incubated controls show more high-M_r material (Peak 2, continuous line) in 'heat-resistant' tendons compared with 'heat-relaxing' tendons, owing to their higher poly-α_1CB6 content. Peak 11 refers to the dye front.

Glycosylation in vitro of Type I re-formed collagen fibrils

Non-incubated collagen (stored at -180°C), control (phosphate-buffered saline) incubated and glycosylated *in vitro* Type I collagen fibrils were taken up (at 2 mg/ml) in SDS/polyacrylamide-gel-electrophoresis sample buffer [2% (w/v) SDS, 125 mM-Tris/HCl, pH 6.8, 1% (w/v) glycerol and Bromophenol Blue]. After 90 min at 60°C, followed by 30 min at 100°C, glycosylated material remained poorly solubilized. Samples were centrifuged at 9000 *g* for 5 min, and 5 μl of supernatant was run on SDS/polyacrylamide gels (Fig. 6*a*). Collagen, which was largely monomeric (mainly α-chains) before incubation (track i), contained α-, β- and γ-chains after 21 days in phosphate-buffered saline at 35°C (track ii). After glycosylation *in vitro* for 21 days, collagen showed very little α and β material but large amounts of very-high-M_r material, which hardly penetrated the gel (track iii). It should be noted, however, that the three tracks in Fig. 6(*a*) do not contain the same amount of material, owing to different solubilities in sample buffer.

CNBr peptides were made from neutral-salt-extracted Type I collagen stored at -180°C and from Type I collagen fibrils glycosylated *in vitro*. CNBr peptides were separated by SDS/polyacrylamide-gel electrophoresis, shown in Fig. 6(*b*). Unlike in Fig. 6(*a*), all three tracks contain the

same amount of material. It is clear that glycosylation *in vitro* under these conditions causes an accumulation of high-M_r CNBr peptides, with loss of lower-M_r material (Fig. 6*b*, track iii).

Discussion

During the last 15 years, many studies on diabetic tissues have indicated that certain proteins become non-enzymically glycosylated by endogenous free glucose. It is also known that collagen can become similarly glycosylated during normal aging. Several workers have suggested that, by some unknown mechanism, some or all of these proteins may become cross-linked through this glycosylation process. However, glycosylation may affect several other factors increasing the stability of collagen *in vivo*, including increased normal cross-linking, stabilization of collagen by non-covalent bonds, decreased synthesis resulting in a higher proportion of stable mature collagens, and altered disulphide-bond formation. Therefore there is little direct evidence from studies *in vivo* for glucose-mediated covalent cross-linking.

Day *et al.* (1980) reported that incubation of glycosylated albumin *in vitro* with albumin led to the time-dependent appearance of dimerized albumin, which was subsequently shown to contain lysylglucitol-lysine, although Rucklidge *et al.* (1983) reported that this could not be repeated. Eble *et al.* (1983) found that glycosylated ribonuclease A would continue to polymerize even in the absence of added glucose, a process that could be inhibited by 100mM-lysine. The model system used relied on the detection of very small amounts of polymeric material and did not allow any mechanical tests to be performed to assess the stability of the apparent cross-linking. Andreassen *et al.* (1981) suggested that increased hydrogen-bonding and steric hindrance in glycosylated collagen could account for increased native tensile strength and decreased acid solubility, but that increased thermal contraction and decreased relaxation of diabetic-rat tail tendons must be due to an increase in thermally stable cross-links. A similar conclusion was also deduced by Yosha *et al.* (1983) on studying isometric tension of diabetic-rat tail skin. Schneir *et al.* (1979) warned against the interpretation of results from diabetic-animal material, suggesting that an apparent increase in cross-linked collagen may be due to preferential loss of less stable material.

Clearly, a system was needed in which tissues or purified proteins could be glycosylated and then unambiguously tested both chemically and mechanically, so that the hypothesis that glucose could cross-link proteins by modifying amino acid side chains could be checked. We devised our incubation system *in vitro* for this purpose. By using paired tendons from rabbits we have been able to demonstrate unequivocally that these glycosylated tendons contain new, heat-stable, cross-links.

Tendons glycosylated *in vitro* showed elevated hydrothermal contraction forces and high retained tensions even at 90°C. As all non-covalent bonds and heat-labile intermolecular cross-links (aldimines) would be broken at temperatures in excess of 75°C, only the presence of covalent, heat-stable, intermolecular bonds can explain this phenomenon. This interpretation is supported by our results on solubility of the tendon glycosylated *in vitro*. Such samples were poorly solubilized by CNBr and released 4 times less material on incubation with 0.5M-acetic acid. Re-formed collagen fibres glycosylated *in vitro* also showed vastly decreased solubility in acetic acid and were poorly dissolved by SDS even at 100°C. Finally, CNBr-peptide maps of both glycosylated tendons and re-formed collagen fibres showed the significant accumulation of high-M_r material, which was not disaggregated by SDS or by heating to 100°C. This material was similar to that seen in normal aged collagenous tissue, which has been isolated and shown to be a covalently cross-linked oligomeric peptide mixture (Light & Bailey, 1980). An investigation of the SDS-solubilized material present in neutral-salt-soluble collagen re-formed fibres incubated in phosphate-buffered saline for 21 days and the same material incubated in phosphate-buffered saline containing glucose showed extensive polymerization of the glycosylated material compared with the controls.

All of this data conclusively proved the existence, in glycosylated collagen, of intermolecular bonds which are either formed from or mediated by the amino acid side chains modified by glucose. It should be noted that the effects we report are not due to the existence in our incubation of long-term oxidation products of the glucose, as we obtained exactly the same result when the incubation medium was renewed every other day.

Gekko & Koga (1983) found that a 0.01% (w/v) aqueous solution of polyols and polyhydric compounds increased the transition temperature of acid-soluble collagen, probably by stabilizing the triple helix through ionic interactions. The increased heat-stability of tendons that we have demonstrated after non-enzymic glycosylation was not found after incubation with mannitol, but was similar to that shown after mild glutaraldehyde cross-linking. This shows that only glucose-mediated covalent cross-linking, rather than changes in hydration state or ionic interactions, could be responsible for the increased stability of collagen that we observed at 90°C.

The interesting observation of variability of isometric-tension curves displayed by different rabbit tendons was explained by their different contents of heat-stable cross-link. It is probable that tendons showing a 'heat-relaxing' response, which contained lower amounts of the heat-stable cross-link hydroxylysino-5-oxonorleucine, may be more rapidly turned over, and therefore contain less of the mature cross-linked complex, poly-α_1CB6. 'Heat-resistant' control tendons showed more high-M_r peptide material on CNBr fingerprints than did 'heat-relaxing' tendons. This material is almost certainly associated with polymeric peptides (poly-α_1CB6) cross-linked by the mature collagen cross-links (see Light & Bailey, 1980) and contributes to the higher heat-resistance of these tendons. For comparison of the response of different rabbit tendons incubated in vitro under a number of conditions, we related the response in each case to that of a paired tendon (the corresponding tendon from the opposite foreleg of the same animal) incubated in phosphate-buffered saline alone. When the control tendon demonstrated a 'heat-resistant' isometric-tension response, very little change was detected after glycosylation in vitro, presumably because any increased stability at 90°C conferred by glycosylation cross-links would not be detectable in an already stable tissue.

The mechanism by which glucose-mediated cross-linking takes place is not clear. It is theoretically possible that the Amadori-rearranged hexosyl-lysine in glycosylated collagen could undergo further reaction through the glucose oxo group with an additional lysine ε-amino group of an adjacent molecule. This reaction could occur at any site along the collagen molecule, a fact supported by consideration of the CNBr-peptide maps of glycosylated material, which indicate that all the peptides along the collagen molecule participate in glucose-mediated cross-linking. In contrast, formation of the reducible cross-links hydroxylysino-5-oxonorleucine and dehydrohydroxylysinorleucine occurs only at the N- and C-terminal ends of the molecule, as this requires the production of hydroxylysine or lysine aldehydes by the site-specific enzyme lysyl oxidase. Normal 'mature' cross-linking also involves only one or two specific peptides.

No evidence for the formation of the novel compound lysylglucitol-lysine was found on incubating ε-N-fructosyl-lysine with lysine at neutral pH in the presence of NaBH$_3$CN (Rucklidge et al., 1983). Cerami et al. (1979) have found, however, that prolonged incubation of bovine lens crystallin with glucose was necessary before pigment formation occurred, and dimers and trimers formed that were stable to reduction.

During the course of this work Pongor et al. (1984) proposed an imidazole compound incorporating two glucose molecules as a possible cross-linking group in poly-L-lysine or bovine serum albumin incubated in vitro with 1 g of glucose/ml. A similar compound may be responsible for covalent cross-linking observed in the present studies. Certainly our system of glycosylation in vitro, in which collagen undergoes modifications characteristic of cross-linking, is ideally suited to the investigation of the chemical nature of the glucose-mediated covalent intermolecular cross-links.

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