Diabetologia (1985) 28: 687-691

Diabetologia

Thermal stability of collagen in relation to non-enzymatic glycosylation and browning in vitro

T. T. Andreassen and H. Oxlund

Department of Connective Tissue Biology, Institute of Anatomy, University of Aarhus, Aarhus, Denmark

Summary. Thermal stability measured by isometric contraction-relaxation force was examined in rat tail tendons after incubation in vitro in glucose or hydroxymethylfurfurale solutions at pH 7.4 using buffer systems of either phosphate or tris (hydroxymethyl)aminomethan. In the phosphate system, incubation with glucose (170 mmol/l) for 12 days was found to increase the thermal stability of the tendons by a factor 3. At the same time, glucose was found to be attached to the lysine and hydroxylysine residues of collagen, and reactive carbonyl compounds were formed in the solution. In the tris(hydroxymethyl)aminomethan system containing reactive amino groups (pK 8.1), glucose was also attached to the lysine and hydroxylysine residues, but only very small amounts of reactive carbonyl compounds were formed in the solutions and no changes in thermal stability were recorded. Incubation with hydroxymethylfurfurale itself was found to increase the thermal stability rapidly and markedly in the phosphate buffer systems. This effect was inhibited when the tris(hydroxymethyl)aminomethan buffer system was used. Buffer solutions with tris(hydroxymethyl)aminomethan, containing large amounts of free amino groups compared to the free amino groups of collagen, might interfere with the formation of cross-links formed by carbonyl groups derived from metabolic glucose and amino groups of collagen. The non-enzymatic glycosylation of lysine and hydroxylysine itself does not influence the thermal stability. Additional reactions appeared to be transformation into reactive carbonyl compounds, such as hydroxymethylfurfurale, with subsequent formation of thermally stable cross-links between the collagen molecules.

Key words: Collagen, thermal stability, non-enzymatic glycosylation, browning reaction.

In recent years it has been well documented that several proteins are glycosylated non-enzymatically, and that such glycosylation of proteins is increased in patients with diabetes mellitus and in animals with experimental diabetes [1]. Changes in the functional properties of proteins caused by this glycosylation might be of importance for the etiology of the complications in diabetes mellitus [1, 2].

Collagenous proteins play important roles in the function of several tissues. They are especially exposed to non-enzymatic glycosylation, because they contain several lysine and hydroxylysine residues with free ε-amino groups, have a very slow turnover rate [3], and are exposed to ambient levels of extracellular glucose.

The solvability of collagen is reduced in skin from diabetic patients [5] and in rat tail tendons [6]. The mechanical stiffness is increased in the skin and tail tendons of rats [7-9]. The thermal stability of rat tail tendons is increased [8, 10]. These changes in the biophysical properties have been attributed to the non-enzymatic glycosylation of the tissue collagenous proteins, supported by in vitro studies of the thermal stability of tendons incubated with glucose [10, 11]. The mechanism of this increase in the stability and alteration in the biophysical properties of the tissues, however, is not clarified. An increase in thermally stable cross-links between the collagen molecules would be expected.

Storage of non-enzymatically glycosylated proteins results in formation of compounds with highly reactive carbonyl groups [12, 13]. One of these compounds is 5-hydroxymethylfurfurale (HMF), which is known to condense with amino groups forming carbonyl protein polymers called melanoidins (Maillard or non-enzymatic browning reaction). These browning reaction products have recently been detected in increased amounts in the dura mater collagen of patients with diabetes mellitus [14], and after in vitro non-enzymatical glycosylation of rat tail tendon where the thermal stability was enhanced [15], supporting the hypothesis that these compounds may be involved in formation of cross-links between collagenous proteins, resulting in alterations in the tissue functions. The purpose of the present study was to elucidate the changes in the biophysical properties of collagen induced by in vitro incu-

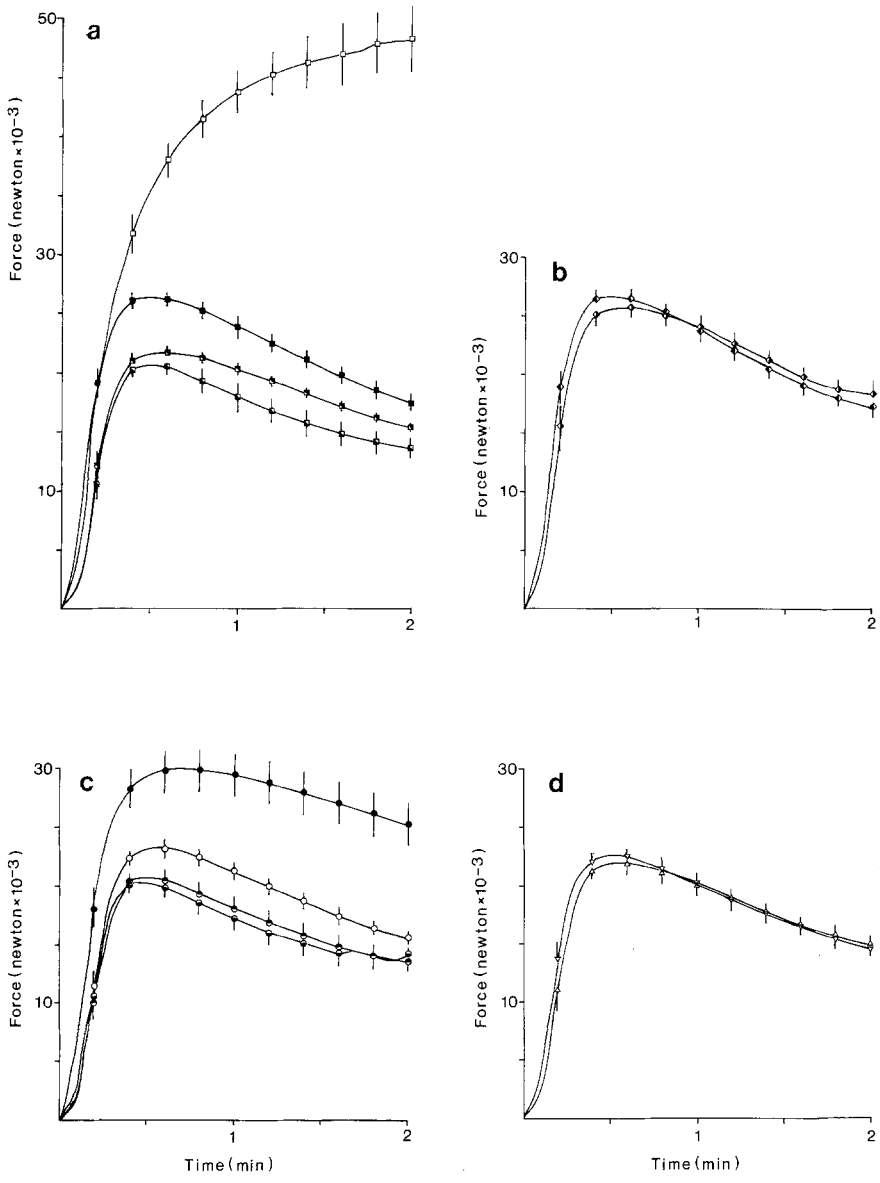

Fig. 1a–d. Thermal isometric contraction-relaxation curves from rat tail tendons after in vitro incubation. Tension values are normalized to mg wet wt./cm tendon length and plotted against time. Mean values ± SEM are given. **a** Incubation in phosphate buffer systems with and without glucose for 2 and 12 days respectively: (□) glucose in phosphate for 12 days; (■) phosphate for 12 days; (◹) glucose in phosphate for 2 days; (◺) phosphate for 2 days. **b** Incubation in Tris buffer systems with and without glucose for 12 days: (◇) glucose in Tris; (◆) Tris. **c** Incubation in phosphate buffer systems with and without hydroxymethylfurfurale (0.2 mM, 2 mM and 20 mM) for 2 days: (●) HMF 20 mmol/l in phosphate; (○) HMF 2 mmol/l in phosphate; (◓) HMF 0.2 mmol/l; (◒) phosphate. **d** Incubation in Tris buffer systems with and without hydroxymethylfurfurale (2 mM) for 2 days: (△) HMF in Tris; (▽) Tris

bation with glucose or hydroxymethylfurfurale. The extent of reactive carbonyl compounds formed in the glucose solutions used was determined. Two buffer systems were used, a phosphate buffer and a tris(hydroxymethyl)aminomethan (Tris) buffer system. The phosphate buffer system was not expected to interfere with the reactions. In contrast the Tris buffer system might inhibit the formation of cross-links between the ε-amino groups of collagen and the carbonyl groups of the transformed glucose molecules, because the Tris buffer contains large amounts of free amino groups compared to the free amino groups of collagen.

Materials and methods

Animals

Tail tendons from 28 male Wistar rats aged 140–145 days were used. The rats were killed by an overdose of nembutal (125 mg/kg body wt.) and the tails were removed and stored at −20 °C in airtight containers until testing.

Thermal isometric contraction-relaxation

Single tendon fibres were pulled from the proximal end of the tail, wrapped in plastic film and kept in airtight containers. The wet weight and length of each tendon were determined. Each tail contained approximately 25 tendons suitable for experimentation. In each of the following groups five or six tendons from each of four or six animals were tested as follows:

Tendons from six rats were divided into four groups and incubated for 12 days in glucose and buffer (pH 7.4): 1) glucose (170 mmol/l) in phosphate (50 mmol/l), 2) glucose (170 mmol/l) in Tris (70 mmol/l), 3) phosphate (50 mmol/l) and 4) Tris (70 mmol/l).

Tail tendons from four rats were divided into four groups and incubated for 2 days in HMF and buffer (pH 7.4): 1) HMF (0.2 mmol/l) in phosphate (50 mmol/l), 2) HMF (2.0 mmol/l) in phosphate (50 mmol/l), 3) HMF (20 mmol/l) in phosphate (50 mmol/l) and 4) phosphate (50 mmol/l).

Tail tendons from six rats were divided into four groups and incubated for 2 days in glucose or HMF and buffer (pH 7.4): 1) glucose (170 mmol/l) in phosphate (50 mmol/l), 2) phosphate (50 mmol/l), 3) HMF (2.0 mmol/l) in Tris (70 mmol/l) and 4) Tris (70 mmol/l).

One group of tail tendons from six rats was tested immediately after thawing.

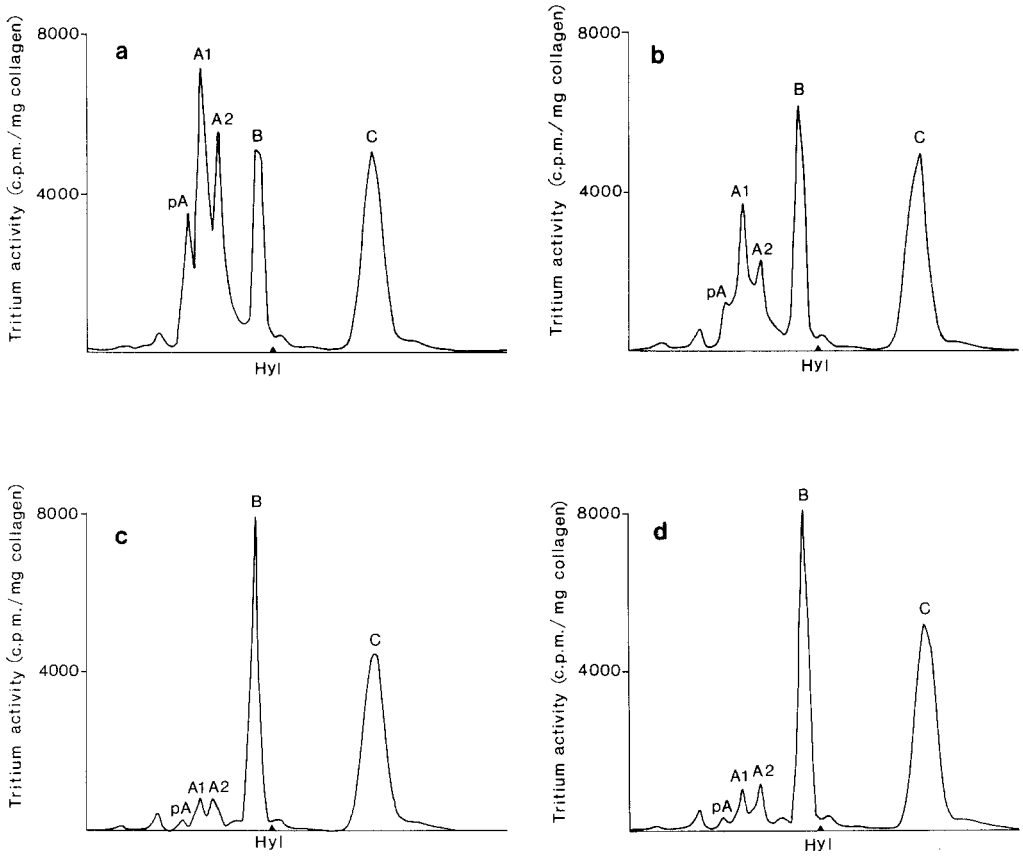

Fig. 2a–d. Elution chromatograms of radioactive components separated by ion-exchange chromatography after reduction with tritiated potassium borohydride and acid hydrolysis. The peaks are identified as glucosyl-hydroxylysine (pA), glucosyllysine (Al) and its acid degradation product (A2), hydroxylysinonorleucine (B) and histidinohydroxymerodesmosine (C). **a** and **c** Rat tail tendon collagen after incubation for 12 days in phosphate buffer systems with and without glucose. **b** and **d** Rat tail tendon collagen after incubation for 12 days in Tris buffer systems with and without glucose

Osmolarity at 300 mmol/l was achieved by adding the necessary NaCl to the solutions. Penicillin (300,000 IU/l), Neomycinsulphate (100 mg/l) and Nystatin (50,000 units/l) were added to prevent bacterial and fungal contamination. pH was adjusted at 7.4. Incubation was performed at 29 °C and each tendon was kept in its own chamber with about 100 ml solution. Each chamber was intensively shaken the first day of incubation and shaken 2 or 3 times the following days. At the end of the incubation the pH of the solutions was measured and each tendon was extensively washed in a buffered Ringer's solution.

The thermal stability of the rat tail tendons was measured as isometric contraction-relaxation force when the tendons were immersed in a buffered Ringer's solution at 62 °C. A measuring apparatus according to Viidik [17] was used and the force was continuously recorded against time on a strip chart recorder (Hewlett Packard, 7100 BM). The force obtained was read into a calculator by a digitizer (Hewlett Packard 9830 A) and was normalized to mg wet weight/cm tendon length. Based on these data mean curves with SEM were calculated.

The formation of reactive carbonyl compounds in the solution during incubation was assessed by measuring their reaction with thiobarbituric acid (TBA). These reactions depend on pH and temperature and the measurement was performed in accordance with Keeney and Bassette [18]. Four ml of the incubated solution was added 1 ml TBA (50 mmol/l), oxalic acid and trichloroacetic acid to final concentrations of 0.3 mol/l and 0.5 mol/l, respectively. After 30 min at 40 °C, the absorbance at 443 nm was measured. HMF (one of the reactive carbonyl compounds) was applied as a standard. Also, the optical density of the incubated solutions was determined at 284 nm. HMF has a maximal absorbance at this wavelength and was used as standard.

Reducible hexosyl-lysines and cross-links

The amount of glucose attached to the collagen through the ε-amino groups of lysine and hydroxylysine was determined by reduction of the collagen fibers with tritiated potassium-borohydride as described in [20–22]. Two probes from each group were analyzed and each probe contained the tendons from three rats. The tendons were washed extensively, immersed in physiological saline and reduced by adding a solution of KB^3H_4. After 1 h the solution was acidified to pH 4.0 and dialyzed against water for 48 h.

The probes were then freeze-dried and hydrolyzed with HCl (6 N). The hydrolysates were submitted to ion-exchange chromatography and the reducible hexosyl-lysines were identified by the elution positions of the radioactive peaks, representing their reduced forms. Relevant fractions were rechromatographied on an extended basic column of an amino acid analyzer (Beckman 120C) [21].

Statistical analysis

When achieving normal distribution and homogeneity of variances, analysis of variances and Student's t-test were performed, else statistical analysis employing Kruskal-Wallis nonparametric test followed by Wilcoxons two sample tests were used [19]. $p < 0.05$ was considered as statistically significant.

Results

Data from the thermal isometric contraction-relaxation experiment of tendons incubated in glucose and phosphate buffer or glucose and Tris buffer are presented in Figure 1a and b. Incubation in glucose buffered with phosphate increased the contraction force after 2 days, and very pronouncedly after 12 days of incubation. Incubation with glucose in Tris buffer did not influence the thermal stability after 12 days of incubation. Incubation with HMF in phosphate buffer also increased the contraction force of tendons, and the resulting relax-

Table 1. Amount of reactive carbonyl compounds formed in the different incubation solutions after 12 days of incubation. The data are converted into μmol/l by using HMF as standard system

Solutions	Absorption at 443 nm after reaction with TBA (μmol/l)	Absorption at 284 nm (μmol/l)
Phosphate (50 mmol/l)	1	1
Tris (70 mmol/l)	2	1
Phosphate (50 mmol/l) + glucose (170 mmol/l)	7	11
Tris (70 mmol/l) + Glucose (170 mmol/l)	2	3

ation was retarded (Fig. 1 c). This increased stability was correlated to the concentration of HMF. The influence of HMF in concentrations of 2 mmol/l could be prevented by Tris buffer (Fig. 1 d). Incubation itself resulted in an increase in the stability. This in vitro effect was similar in the two buffer systems used. At the end of the incubations, the pH of the solutions ranged between 7.2 and 7.4. The pattern of reducible hexosyl-lysines and collagen cross-links eluted by the ion-exchange chromatography is given in Figure 2. The radioactive peaks of glucosyl-hydroxylysine (pA), glucosyl-lysine (Al) and its acid degradation product (A2) are the products of non-enzymatic glycosylation of collagen. The hydroxylysinonorleucine (B) is a reducible aldimine cross-link of collagen. Also, the histidinohydroxymerodesmosine (C), an artefact of the reduction procedure [23], is shown.

The amounts of hexosyl-hydroxylysine and hexosyllysine of the tendon collagen were increased after incubation with phosphate buffer and glucose, as well as after incubation with Tris buffer and glucose, but the glycosylation was more pronounced when the phosphate buffer system was used (Fig. 2). No difference in reducible cross-link formation was found in relation to glucose incubation. The variation of double determinations of the tritium activity of these peaks was about 4%.

Under incubation in glucose phosphate buffer system, reactive carbonyl compounds were formed. The amounts measured by their reaction to TBA at the end of incubation is shown in Table 1. About three quarters of the material recorded by ultraviolet spectrophotometry was found by this TBA-reactive method.

Discussion

Collagen shrinks when exposed to heat as a result of the breakdown of the ordered crystalline structure of the collagen to a preferred random coil state [24]. When collagenous tissue specimens are heated under isometric conditions, there is a quick increase in tension up to a point of maximum tension, after which a gradual relaxation follows. In isometric thermal contraction-relaxation experiments on rat tail tendons, it has been shown that the maximum tension becomes increased and the following relaxation less pronounced with age [8, 25]. The decrease in relaxation of tissue with age has been attributed to an increase in thermally stable cross-links of the collagen [26].

The present study shows that the thermal stability of collagen fibres incubated in the phosphate buffered glucose solution is increased, at the same time as an increased non-enzymatic glycosylation of their lysine and hydroxylysine residues is observed. Incubation in a Tris buffered glucose solution also results in an increased glycosylation of the lysine and hydroxylysine residues of collagen fibres, but with no change in the thermal stability. No differences in the thermal stability of collagen fibres were observed after incubation in the two buffer systems without glucose. These results indicate that the alterations in the thermal stability of collagen fibres are not simply caused by increased non-enzymatic glycosylation. Additional reactions must be involved. Tris buffer seems to interfere with these reactions, inhibiting the formation of thermally stable cross-links between the collagen molecules.

It is known that glycosylation and subsequent cross-linking occur in food proteins that are stored in the presence of sugars, and are due to the Maillard or non-enzymatic browning reaction [13, 18]. Glucose, for example, is bound non-enzymatically to free amino groups of proteins. An Amadori rearrangement follows with the formation of a more stable l-amino-l-deoxyfructose, which may then undergo a series of dehydrations, forming among other products the Schiff base of HMF [1, 13]. Occurrence of similar reactions in proteins with slow turnover rates, such as collagen, lens crystallines and myelin, influencing the function of tissues, have been hypothesized [1]. Compounds similar to those of the non-enzymatic browning reactions have been detected in collagen from the dura mater of aged and diabetic individuals [14] and in rat tail tendons after non-enzymatical glycosylation in vitro [15]. In the present study, incubation in vitro of collagen fibres with HMF resulted in a rapid and marked increase in thermal stability, supporting the hypothesis that this compound may be involved in the formation of thermally stable collagen cross-links. The major collagen type of tendons is Type I collagen, which contains about 35 lysine and hydroxylysine residues per 1000 aminoacid residues [27], with ε-amino groups, which may participate in the cross-linking reaction, for example by formation of bonds to the carbonyl groups of HMF.

In conclusion, the data show that glycosylation per se does not alter the thermal stability of collagen fibres, and support the hypothesis that non-enzymatic glycosylation of collagen, followed by transformations into reactive carbonyl compounds and formation of thermally stable cross-links between the collagen molecules, results in changes in the functional properties of connective tissues in patients with diabetes and in animals

with experimental diabetes [1, 2, 28, 29]. The importance of studies of these cross-linking mechanisms of collagen is not limited to long-term complications of diabetes. An increase in the amount of hexosyl-lysines of collagen is also seen with age [21]. At the same time, a decrease in reducible collagen cross-links is observed with age and an increase in thermal stability of collagenous tissues, the latter indicating a subsequent formation of thermally stable cross-links. These cross-linking reactions of proteins might be important factors in the process of aging of tissue proteins.

Acknowledgements. For skilled technical assistance the authors thank E. K. Mikkelsen, N. Kappel and L. P. Kristensen. This work was supported by grants from the Danish Medical Research Council (Nos. 12-3872 and 12-4966).

References

1. Monnier VM, Cerami A (1982) Non-enzymatic glycosylation and browning of proteins in diabetes. Clin Endocrinol Metab 10: 432–452
2. Kennedy L, Baynes JW (1984) Non-enzymatical glycosylation and the chronic complications of diabetes: an overview. Diabetologia 26: 93–98
3. Robins SP (1980) Turnover of collagen and its precursors. In: Viidik A, Vuust J (eds) Biology of Collagen, Academic Press, New York, London, pp 135–152
4. Trüeb B, Holenstein CG, Fischer RW, Winterhalter KH (1980) Nonenzymatic glycosylation of proteins. J Biol Chem 255: 6717–6720
5. Francis MJO, Ellis JP, Hockaday TDR (1974) Skin collagen in diabetes in relation to treatment. Proc R Soc Med 67: 35–36
6. Golub LM, Greenwald RA, Zebrowski EJ, Ramamurthy NS (1978) The effect of experimental diabetes on the molecular characteristics of soluble rat-tail tendon collagen. Biochim Biophys Acta 534: 73–81
7. Galeski A, Kastelic J, Baer E, Kohn RR (1977) Mechanical and structural changes in rat tail tendon induced by alloxan diabetes and aging. J Biomech 10: 775–792
8. Andreassen TT, Seyer-Hansen K, Bailey AJ (1981) Thermal stability, mechanical properties and reducible cross-links of rat tail tendon in experimental diabetes. Biochim Biophys Acta 677: 313–317
9. Andreassen TT, Seyer-Hansen K, Oxlund H (1981) Biomechanical changes in connective tissues induced by experimental diabetes. Acta Endocrinol 98: 432–436
10. Yue DK, McLennan S, Delbridge L, Handelsman DJ, Reeve T, Turtle JR (1983) The thermal stability of collagen in diabetic rats: correlation with severity of diabetes and non-enzymatic glycosylation. Diabetologia 24: 282–285
11. Andreassen TT, Seyer-Hansen K (1981) In vitro effects of glucose on the biophysical properties of collagen. Diabetologia 21: 242–243
12. Reynolds TM (1963) Chemistry of nonenzymic browning I. The reaction between aldoses and amines. Adv Food Res 12: 1–52
13. Reynolds TM (1965) Chemistry of nonenzymic browning II. Adv Food Res 14: 167–283
14. Monnier VM, Kohn RR, Cerami A (1984) Accelerated age-related browning of human collagen in diabetes mellitus. Proc Natl Acad Sci 81: 583–587
15. Kohn RR, Cerami A, Monnier VM (1984) Collagen aging in vitro by nonenzymatic glycosylation and browning. Diabetes 33: 57–59
16. Yue DK, McLennan S, Handelsman, Delbridge L, Reeve T, Turtle JR (1984) The effect of salicylates on nonenzymatic glycosylation and thermal stability of collagen in diabetic rats. Diabetes 33: 745–751
17. Viidik A (1968) An apparatus for the measurement of small tensions. Experientia 24: 861–862
18. Keeney M, Bassette R (1958) Detection of intermediate compounds in the early stages of browning reaction in milk products. J Dairy Sci 42: 945–960
19. Sokal RR, Rohlf FJ (1981) Biometry. WH Freeman, San Francisco
20. Bailey AJ, Peach CM, Fowler LJ (1970) Chemistry of the collagen cross-links. Isolation and characterization of two intermediate intermolecular cross-links in collagen. Biochem J 117: 819–831
21. Robins SP, Shimokomaki M, Bailey AJ (1973) The chemistry of the collagen cross-links. Age-related changes in the reducible components of intact bovine collagen fibres. Biochem J 131: 771–780
22. Oxlund H, Sims T, Light ND (1982) Changes in mechanical properties, thermal stability, reducible cross-links and glycosyl-lysines in rat skin induced by corticosteroid treatment. Acta Endocrinol 101: 312–320
23. Robins SP, Bailey AJ (1973) The chemistry of the collagen cross-links. The characterization of fraction C, a possible artifact produced during the reduction of collagen fibres with borohydride. Biochem J 135: 657–665
24. Flory PJ (1956) Theory of elastic mechanisms in fibrous proteins. J Am Chem Soc 80: 4836–4848
25. Viidik A (1977) Thermal contraction-relaxation and dissolution of rat tail tendon collagen in different ages. Akt Gerontol 7: 493–498
26. Allain JC, Le Lous M, Bazin S, Bailey AJ, Delaunay A (1978) Isometric tension developed during heating of collagenous tissues. Relationships with collagen cross-linking. Biochim Biophys Acta 533: 147–155
27. Stoltz M, Furthmayr H, Timpl R (1973) Increased lysine hydroxylation in rat bone and tendon collagen and localization of the additional residues. Biochim Biophys Acta 310: 461–468
28. Rosenbloom AL, Silverstein JH, Lezotte DC, Richardson K, McCallum M (1981) Limited joint mobility in childhood diabetes mellitus indicates increased risk for microvascular disease. N Engl J Med 305: 191–194
29. Knowles HB Jr (1981) Joint contractures, waxy skin, and control of diabetes. N Engl J Med 305: 217–218

Received: 5 February 1985
and in revised form: 5 July 1985

Dr. T. T. Andreassen
Institute of Anatomy C
University of Aarhus
DK-8000 Aarhus C
Denmark