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Non-enzymic glycation of fibrous collagen: reaction products of glucose and ribose

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Non-enzymic glycation of collagen involves a series of complex reactions ultimately leading to the formation of intermolecular cross-links resulting in changes in its physical properties. During analysis for the fluorescent cross-link pentosidine we identified the presence of an additional component (Cmpd K) in both glucose and ribose incubations. Cmpd K was formed more quickly than pentosidine in glucose incubations and more slowly than pentosidine in ribose incubations. Cmpd K represented 45 % of the total fluorescence compared with 15 % for pentosidine in glucose incubations and 25 % of the total fluorescence compared with 30 % for pentosidine in the ribose incubations. Cmpd K is not an artefact of *in vitro* incubations, as it was shown to be present in dermal tissue from diabetic patients. Subsequent high-resolution h.p.l.c. analysis of glucose-incubated collagen revealed Cmpd K comprise two components (K1 and K2). Further, a similar analysis of Cmpd K from the ribose incubations revealed two different components (K3 and K4). These differences indicate alternative mechanisms for the reactions of glucose and ribose with collagen. The amounts of these fluorescent components and the pentosidine cross-link determined for both glucose and ribose glycation were found to be far too low (about one pentosidine molecules per 200 collagen molecules after 6 months incubation with glucose) to account for the extensive cross-linking responsible for the changes in physical properties, suggesting that a further additional series of cross-links are formed. We have analysed the non-fluorescent high-molecular-mass components and identified a new component that increases with time of *in vitro* incubation and is present in the skin of diabetic patients. This component is present in sufficient quantities (estimated at one cross-link per two collagen molecules) to account for the changes in physical properties occurring *in vitro*.

INTRODUCTION

The non-enzymic glycation of fibrous collagen has generated considerable interest in view of its relevance to long-term diabetic complications [for a review, see Baynes and Monnier (1989)]. The initial reaction with collagen was demonstrated to involve the condensation of glucose with the ϵ-amino groups of lysine and residues along the peptide chain (Robins and Bailey, 1972). Collagen has a long biological half-life, and the level of non-enzymic glycation increases gradually with normal aging (Robins and Bailey, 1973). This correlates with increased resistance to enzyme digestion and swelling in acid (Hamlin et al., 1978; Golub et al., 1978) and greater heat-stability (Yue et al., 1983). In common with other long-lived proteins such as the crystallins (Monnier and Cerami, 1982), non-enzymic glycation of collagen is substantially elevated in diabetes compared with age-matched controls (Le Pape et al., 1982; Cohen et al., 1980; Trueb et al., 1984). Collagenous tissues of long-term diabetics also demonstrate increased stability, a phenomenon which has been referred to as 'accelerated aging' (Hamlin et al., 1975; Schnider and Kohn, 1981. We proposed that the increased stability of collagen in diabetes may be due to cross-linking of collagen molecules via glucose (Andreassen et al., 1981) and later confirmed the formation of high-molecular-mass thermally stable polymers following glycation of collagen *in vitro* (Kent et al., 1985). Bai et al. (1992) recently demonstrated a dramatic increase in fibril packing by electron microscopy, confirming the formation of sugar-derived interfibrillar cross-links following incubation with ribose.

These studies clearly indicated that the hexosyl-lysine and hexosyl-hydroxylysine residues initially formed undergo further reaction to produce covalent intermolecular cross-links. It might be expected that further products of non-enzymic browning or Maillard reaction would lead to fluorescent compounds and, consequently, efforts over the past few years have concentrated on these readily identifiable fluorescent products. Sell and Monnier (1989) reported the identification of a fluorescent imidazopyridinium compound, designated 'pentosidine' on the basis of its apparent derivation from ribose, lysine and arginine. The amount of this compound increased linearly with age in dura mater, increased further in tissue from diabetics and has been positively correlated with the presence of retinopathy and nephropathy in diabetic patients (Sell et al., 1994). In view of the known low levels of ribose in tissues, it was suggested that the pentose arose from oxidative degradation of glucose, fructose or ascorbate (Dyer et al., 1991; Grandee and Monnier 1991). Several workers have demonstrated the importance of oxygen and trace metals (Chace et al., 1991; Fu et al., 1992) and free radicals (Wolff et al., 1991) in the oxidation of the initial hexosyl-lysine residues. The levels of pentosidine in plasma proteins are elevated in uraemia to a much higher degree than in diabetes, despite the absence of an increase in blood sugar levels (Odetti et al., 1992). However, there is an increase in ascorbate levels in ureamia (Chatterjee and Bannerjee 1979), supporting the pro-

Abbreviations used: TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; NFC, non-fluorescent component.

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posal by Monnier and his colleagues that pentosidine is most probably derived from ascorbate (Nagaraj et al., 1991) rather than ribose.

Here we demonstrate: (i) that additional potential cross-links are formed during the reactions of glucose and ribose with collagen; (ii) that the reactions of glucose and ribose are different; (iii) that the changes in physical properties indicate very extensive cross-linking which is inconsistent with the low level of the pentosidine and other fluorescent cross-links; and (iv) that the formation of an as-yet-uncharacterized non-fluorescent high-molecular-mass amino acid appears to be responsible for the extensive cross-linking.

MATERIALS AND METHODS

Materials

Acetonitrile, tetrahydrofuran, trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were from Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland, U.K. Sephadex G-10 was from Pharmacia Fine Chemicals A. B., Uppsala, Sweden; Fractogel TSK HW-40S was from E. Merck, Darmstadt, Germany. Ninhydrin and buffers for amino acid analysis were obtained from Pharmacia Fine Chemicals Ltd. C18 columns were 150 mm × 4.6 mm [S3 (triacylglycerol)] supplied by Phase Separations Ltd., Queensferry, Clwyd, Wales, U.K. Hypercarb S columns were 100 mm × 4.6 mm graphitized carbon supplied by Shandon Scientific Ltd., Runcorn, Cheshire, U.K.

Rat collagen

Rat tail tendon collagen was obtained by stripping the tail tendons from 4-month-old rats, washing them in PBS and keeping them frozen at −20 °C until required.

Human collagen

Skin biopsies of between 1 and 4 mg dry weight were taken from the forearm of diabetic male patients between 16 and 83 years of age and with disease duration of between 10 and 33 years.

Methods

Incubations

Native collagen fibres were incubated in PBS with toluene/chloroform added (20 µl/20 ml of buffer) to prevent bacterial growth. The use of antibiotics was discontinued following the detection of adventitious fluorescent components after hydrolysis. Glycation *in vitro* was performed either with 24 mg/ml glucose or 2 mg/ml ribose, and incubation times ranged from 2 days to 30 weeks as described previously (Kent et al., 1985) Fibres were removed at intervals, and the reaction was terminated by extensive dialysis into PBS at 4 °C. Samples were stored at −20 °C until analysed.

Molecular-exclusion chromatography for high-molecular-mass amino acids

Rat tail tendons rendered insoluble following incubation with glucose or ribose were washed in PBS, hydrolysed in 6 M HCl and the hydrolysate chromatographed on a TSK-40S (Fractogel) column (90 cm × 1.6 cm) using 0.05 M acetic acid as eluent at a flow rate of 1 ml/min. The column was monitored with a Gilson Spectra Glo fluorimeter (excitation at 340 nm; emission at 389 nm), and 5 ml fractions were collected. The column had previously been calibrated using [^{3}H]lysine and adenosine mono-, di- and tri-phosphate as molecular-mass markers (146, 347, 427 and 551 Da respectively).

The fluorescent high-molecular-mass amino acids (resulting from the incubation of the tendons with ribose or glucose) that were eluted from the column were collected and analysed by reverse-phase h.p.l.c. for possible fluorescent cross-links as described below. These same high-molecular-mass components were also analysed by ion-exchange chromatography.

Fluorescent components: h.p.l.c. analysis

The incubated and control tendons were freeze-dried, weighed and hydrolysed with 6 M HCl, evaporated to dryness, redissolved in water and either chromatographed directly on the h.p.l.c. system, or first chromatographed on the TSK-40S column or eluted from the CF1 (cellulose fibre) column (Whatman International, Maidstone, Kent, U.K.). Samples for the CF1 column were dissolved in butanol/acetic acid/water (4:1:1, by vol.) and the standard amino acids eluted from the column. Experience showed that, in order to retain the pentosidine on the CF1 column, the weight of material put on the column had to be 0.5–1.0% of the CF1 dry weight and the volume of organic phase kept to 3 column vol., otherwise the pentosidine was eluted in the organic phase. The fluorescent 'cross-links' were then eluted with water, freeze-dried, and separated on a reversed-phase h.p.l.c. system (LKB, Loughborough, U.K.).

(a) C_{18} column. Initial separations were carried out using a linear gradient from 5 to 35% acetonitrile in water, each containing 0.05 M HFBA, at a flow rate of 1 ml/min. Compounds were detected by fluorescence using an LS5 luminescence spectrometer (Perkin–Elmer) (excitation at 335 nm; emission at 385 nm).

(b) Hypercarb column. High-resolution separations were carried out using a succession of shallow gradients from 0 to 11% tetrahydrofuran in water, both containing 0.5% TFA, at a flow rate of 1 ml/min and detected by fluorescence as described above.

Fluorescence yield of components

After preliminary purification using a semi-preparative ODS 1 column (Spherisorb; 10 µm particle size; 250 mm × 20 mm), the fluorescent compounds were further purified on the Hypercarb S graphitic column. The fractions were filtered (0.2 µm-pore-size filter) and freeze-dried in acid-washed microvials and accurately weighed (approx. 200 µg each sample) using a Mettler (AE240) five-place balance. Each component was rehydrated with 200 µl of filtered 1% TFA and an aliquot (5 µl) re-analysed using the Hypercarb S column. The fluorescent peak area was correlated with the known weight of the sample to give the fluorescence yield.

Non-fluorescent components: ion-exchange analysis

The high-molecular-mass material from the Fractogel column was submitted to the technique developed for identification of the lysinaldehyde-derived cross-links (Sims and Bailey, 1992). Briefly the potential cross-linking amino acids were concentrated on a CF1 column using organic solvents and then separated on an amino acid analyser (Pharmacia Alpha Plus) using the modified gradient and ninhydrin detection previously described, except that the gradient was extended by an additional 38 min step of 0.5 M citrate/borate buffer, pH 8.60, and the column temperature was maintained at 90 °C throughout the analysis. The elution point of the known cross-linking amino acids occurs between phenylalanine and hydroxylysine, but the gradient was continued until arginine was eluted.

Molecular mass of the non-fluorescent component

The major non-fluorescent component isolated from glycated rat tail tendon after hydrolysis and CF1 cellulose separation was chromatographed on a calibrated TSK HW 40 s column as described above to determine its molecular mass.

Chromatography of [^{14}C]carbohydrate products

A 350 mg (wet wt.) portion of rat tail tendon was incubated at 37 °C in 5 ml of 0.15 M PBS, pH 7.4, containing either 250 mM glucose, pH 7.4, spiked with 10 μCi of [^{14}C]glucose for 30 days, or 250 mM ribose spiked with 2 μCi of [^{14}C]ribose for 1–3 days. The tendons were then washed in physiological saline to remove excess sugar, hydrolysed in 6 M HCl and the hydrolysate separated on CF1 cellulose as previously described. The aqueous eluant from the CF1 column was applied to the amino acid analyser using the extended analysis program referred to above. Fractions were collected every minute after passage through the ninhydrin reaction coil, and the radioactivity in these fractions measured in an LKB–Wallac 1219 Rackbeta liquid-scintillation counter (Pharmacia Biotech Ltd., St. Albans, Herts., U.K.) using Scintran Cocktail-T scintillation fluid (Merck, Poole, Dorset, U.K.).

Determination of cross-links in tissues

Skin obtained from diabetic and control subjects was homogenized, defatted with chloroform/methanol, reduced with $NaBH_4$ in PBS, dried and hydrolysed with 6 M HCl and then treated as for the glycated rat tail tendon. The hydrolysate was prefractionated on CF1-cellulose columns and then analysed for cross-links using both the Hypercarb h.p.l.c. system and the LKB automatic amino acid analyser as described above.

RESULTS

Rates of formation and yields of pentosidine

The yields of pentosidine found during incubation with glucose, ascorbic acid and ribose were determined from the fluorescence after separation of the hydrolysate on the h.p.l.c. system. The rate of formation of pentosidine from ribose was 50 times greater, and from ascorbic acid 10 times greater, than than that from glucose. Despite the virtual insolubility of ribose-incubated collagen, after 3 days the yield of pentosidine was surprisingly low, namely about one molecule per 30 collagen molecules. The yield of pentosidine from glucose was not detectable below 30 days, and the yield after 60 days only about one pentosidine molecule per 300 collagen molecules

Molecular-mass exclusion chromatography

Molecular-sieve chromatography of the insoluble collagen after acid hydrolysis revealed two major high-molecular-mass peaks from both glucose and ribose incubations. The two peaks from ribose-treated collagen were present in a ratio of 1:2 and had molecular masses of about 550 and 520 respectively. The glucose-treated collagen after a longer incubation (6 months) revealed peaks with molecular masses of 550 and 520 in the ratio of 2:1. The peaks were then analysed on the h.p.l.c. system and the ion-exchange system for potential cross-links. The first peak from both the glucose and the ribose incubations had a molecular mass of about 550 kDa and contained a component designated 'Cmpd K' (see below), whereas the second peak had a molecular mass about 520 kDa and contained both Cmpd K and pentosidine. Although the values are high (the molecular mass of pentosidine being 379 kDa), the results indicate Cmpd K has a slightly higher molecular mass than pentosidine.

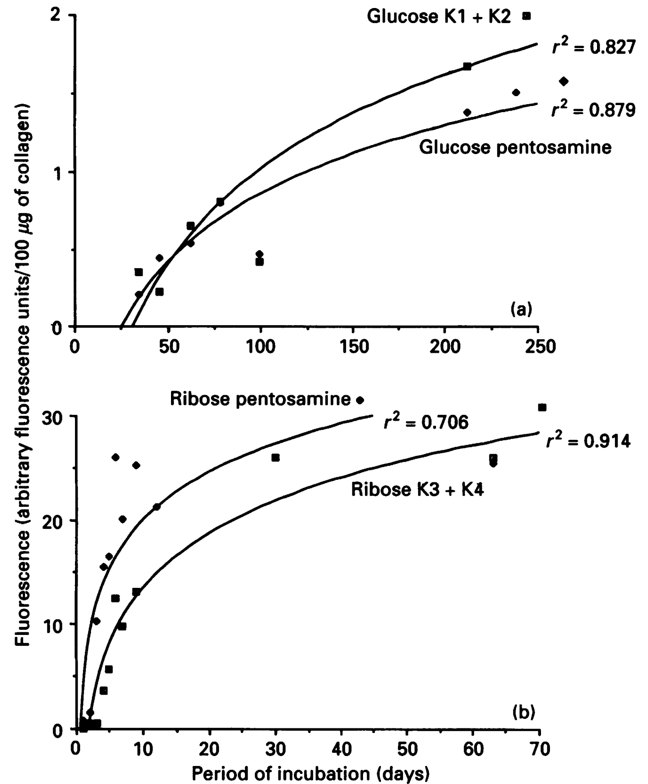

Figure 1 Development of fluorescence of pentosidine and Cmpd K species during incubation of rat tail tendon collagen fibres

Acid hydrolysates were separated using a Hypercarb column h.p.l.c. system and the components detected with a fluorimeter. (a) With glucose at 37 °C (note: values for K1 and K2 are summed); (b) with ribose at 37 °C (note: values for K3 and K4 are summed).

H.p.l.c. analysis of fluorescent cross-links

(i) C_{18} column analysis

(a) Glucose. After about 30 days the presence of an unknown component (designated Cmpd K) could be detected using a fluorescence detection system (excitation at 335 nm; emission at 385 nm), and this component increased rapidly with increasing incubation time. Pentosidine was not detected until 10–15 days and then increased at a rate similar to that shown by Cmpd K (Figure 1a).

(b) Ribose. The pentosidine content reached a maximum after only 15 days, whereas Cmpd K increased at a slightly lower rate to a maximum at 25 days. The levels of both pentosidine and Cmpd K were of an order of magnitude greater than the levels following glucose incubation over the same time periods (Figure 1b).

(ii) High-resolution Hypercarb h.p.l.c. column

The Cmpd K isolated from both glucose and ribose incubations was further analysed using the Hypercarb column, which was found to have a greater resolving power. The Cmpd K from the glucose incubation produced two components, designated K1

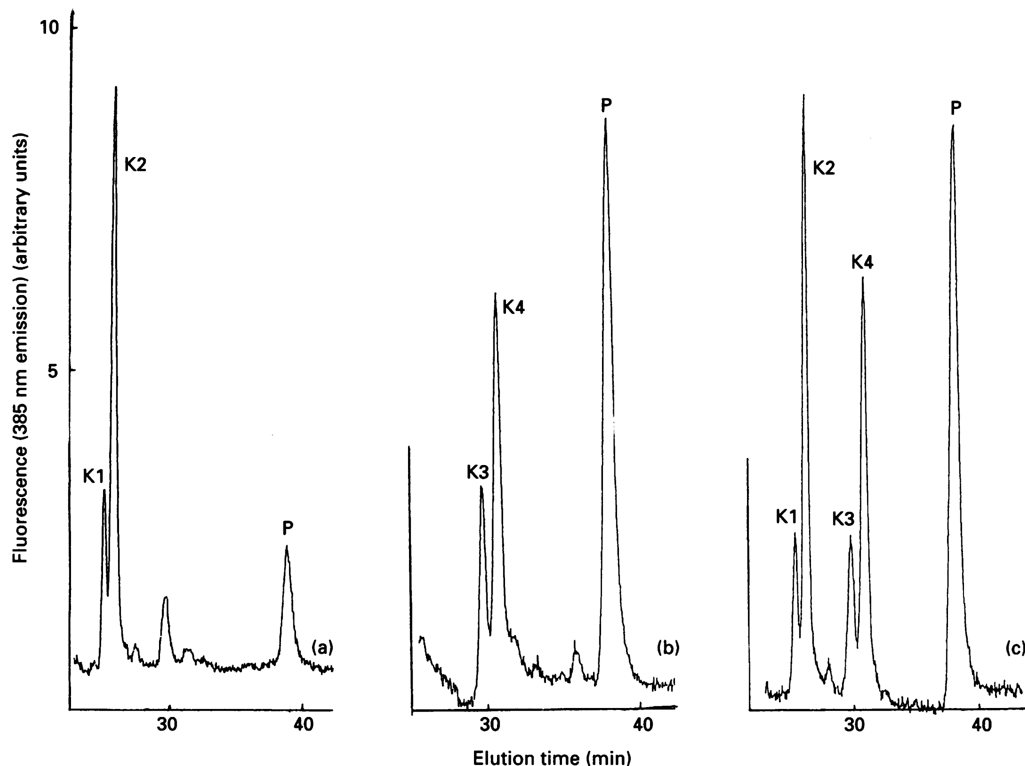

Figure 2 Elution profile of pentosidine (P) and Cmpd K using the high-resolution Hypercarb column on the h.p.l.c. system

Acid hydrolysates of the glycated collagen were eluted through a CF1 cellulose column to concentrate potential cross-links prior to h.p.l.c. separation. (**a**) Glucose incubation showing pentosidine and Cmpd K1 and K2; (**b**) ribose incubation showing pentosidine and Cmpd K3 and K4; (**c**) combined elution profile of glucose and ribose incubations, showing clear separation of K1 and K2 formed from glucose, and K3 and K4 formed from ribose. P, pentosidine.

and K2 (Figure 2), whereas Cmpd K from the ribose incubation produced two different components, designated K3 and K4 (Figure 2). The pentosidine derived from the glucose and ribose respectively did not separate using this technique, indicating that the same product is formed from both sources, as previously reported (Grandee and Monnier 1991; Dyer et al., 1991).

Relative fluorescence yields of Cmpd K 1–4 and pentosidine

The fluorescence yields of the glucose-derived CmpdK 1 + 2 were five times greater than the those of K3 + K4 derived from ribose. The fluorescent yield of K1 + K2 was five times, and that of K3 + K4 was equivalent to, that of pentosidine respectively.

Ion-exchange chromatography of non-fluorescent cross-links

The peaks from the Fractogel column were also analysed on the extended gradient of the Alpha Plus automatic amino acid analyser.

In the short-term incubations of both ribose (3 days) and glucose (1 month), a major peak was obtained on the amino acid analyser at 52 min [termed non-fluorescent component (NFC)] for both ribose and glucose incubations. The peak increased in quantity on incubation with ribose for 1, 6 and 10 days (Figure 3), and similarly for glucose for 1 week, 3 months and 6 months. Longer-term incubations increased the complexity of the elution pattern, at least four additional peaks appearing in the glucose incubations and a larger number in the ribose incubations in the 20–30 min elution period of the chromatogram. The chromatograms were unaffected by prior reduction with borohydride,

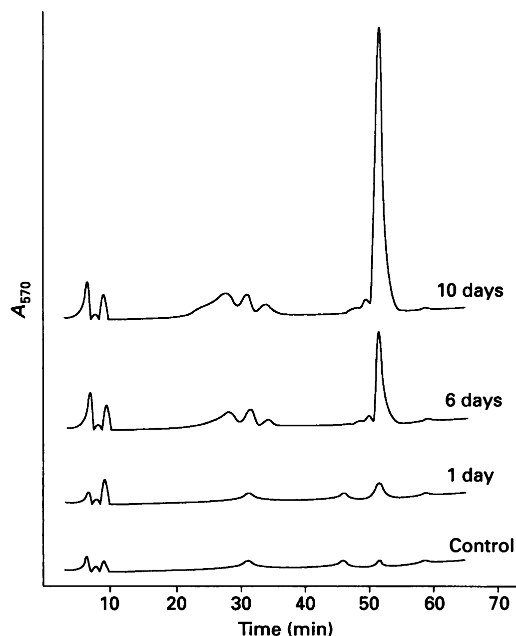

Figure 3 Elution profile of an extended amino-acid-analyser gradient showing increasing amounts of the NFC component with increasing incubation times (0–10 days) for rat tail tendon collagen and ribose (2 mg/ml)

The NFC compound and other cross-linking amino acids were separated on the amino acid analyser and detected with ninhydrin.

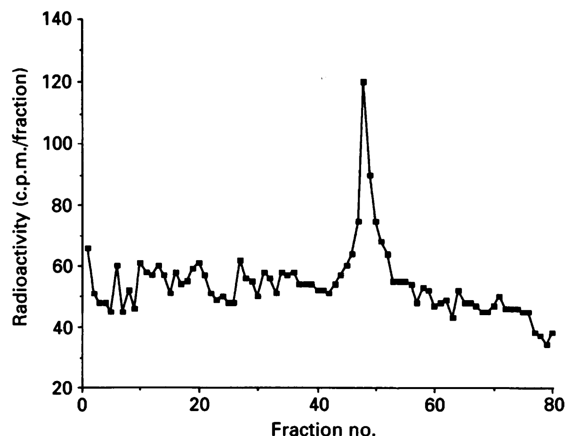

Figure 4 Elution profile from the amino acid analyser, following prefractionation on CF1 columns, of the radioactive components from the acid hydrolysate of tendon collagen incubated with 250 mM glucose spiked with [^{14}C]glucose (10 μCi) for 30 days

Fractions were collected after elution from the column and counted for radioactivity in a liquid-scintillation counter.

apart from the appearance of the known lysinaldehyde-derived cross-links.

The ninhydrin yield, assuming a leucine equivalent of 2, indicated a yield of NFC to be one molecule per molecule of collagen after 3 days incubation with ribose. The yield of NFC for 1 month's incubation with glucose was calculated to be one molecule per five collagen molecules, and one molecule per one collagen molecule after 6 months.

Identification of ^{14}C-labelled glycation products

Elution of the radioactive amino acids following incubation with [^{14}C]glucose revealed several radioactive peaks in the normal cross-link region (20–30 min period) analogous to the peaks observed using ninhydrin as the detection system. More importantly, the major radioactive component was a peak at 54 min (Figure 4). Allowing for the 2 min dead time between the Alpha Plus flow cell and the fraction collector, this peak corresponds to the ninhydrin peak observed at an elution time of 52 min (Figure 3).

In the long-term incubations, the radioactive peaks observed in the elution region of 20–30 min were considerably increased following incubation with ^{14}C-labelled glucose.

Similar incubations with [^{14}C]ribose revealed the major radioactive peak at 50 min and a multiplicity of radioactive peaks in the 20–30 min elution period.

Molecular mass of the NFC

The molecular mass of the major NFC was estimated from the calibrated molecular-sieve columns at 520 Da.

Analysis of human diabetic skin

The levels of both pentosidine and Cmpd K were determined in samples of skin from human diabetic subjects. Both components were found to be present and increased curvilinearly with age, although the concentration of pentosidine was ten times that of Cmpd K. Despite the fact that the structure of the Cmpd K species are still unknown, their higher fluorescent yield compared with pentosidine indicates that, on a weight basis, pentosidine is the major fluorescent cross-link in diabetic skin. A plot of the levels of these components against the estimated duration of the diabetes failed to give any relationship whatsoever.

More importantly we identified the presence of NFC in the skin of diabetic patients (Figure 5). The levels of these compounds were found to be consistent with the *in vitro* studies, indicating that they are not artifacts of the *in vitro* system and must play a role in the changes in the physical properties of the collagen fibres *in vivo*. The relationship with age and duration of the diabetes remains to be evaluated.

DISCUSSION

The present studies have revealed an additional fluorescent component (Cmpd K) that increases with time and is present in greater yield than pentosidine following *in vitro* incubation with glucose. This component is formed at about the same rate as pentosidine during incubation with ribose and appeared to be identical with the component formed during glucose incubation, based on the elution times from the C_{18} h.p.l.c. column system. However, using the Hypercarb h.p.l.c. column the Cmpd K from glucose incubations was resolved into two peaks, K1 and K2, whereas the Cmpd K from the ribose incubations was resolved into two different peaks, K3 and K4 (Figure 2). Clearly the reactions of ribose and glucose with collagen are different.

In accounting for the level of cross-linking both pentosidine and Cmpd K must be taken into account. Determination of the yields of pentosidine from ribose or glucose incubated collagens at the stage of virtual insolubility revealed surprisingly low values, essentially one pentosidine molecule per 300 collagen molecules after 1 day's incubation with ribose and one per 200 after 6 months incubation with glucose. Similar levels for pentosidine were obtained on incubation of glucose with lens-capsule collagen (Bailey et al., 1993) and fibrous collagen (Sell and Monnier 1989; Dyer et al., 1991) . Unfortunately attempts to determine the structure of the isolated and purified Cmpd K species using electrospray m.s. were unsuccessful. However, on the basis of the fluorescence yield determined for both pentosidine and the Cmpd K species, the number of cross-links is still insufficient to account for the observed changes in physical properties. The pentosidine and Cmpd K account for about 60 % of the total fluorescence at 335/385 nm (Cmpd K, 45 %; pentosidine, 15 %) following incubation with glucose and 55 % in the ribose incubations (Cmpd K, 25 %; pentosidine, 30 %). It is possible that other fluorescent cross-links are still undetected, and further studies are required.

To ensure that Cmpd K was of some significance in diabetes and not just an artefact of *in vitro* incubations, we analysed skin collagen from diabetic subjects and identified both pentosidine and Cmpd K. However, in contrast with the *in vitro* incubations with glucose, the concentration of Cmpd K was several times lower than that of pentosidine, and therefore, since it appears to possess a much higher fluorescence yield than pentosidine, the actual amount of Cmpd K could be lower and therefore may not make an important contribution to the accelerated aging of diabetic skin. However, the structures of the Cmpd K species remain to be elucidated and the fluorescent yield confirmed. In the skin of diabetic subjects these two compounds only account for 30 % of the fluorescence (Cmpd K, 8 %; pentosidine, 22 %), again suggesting that other fluorescent components may be important.

In view of the low yields of the fluorescent cross-links we turned our attention to analysis of the non-fluorescent high-

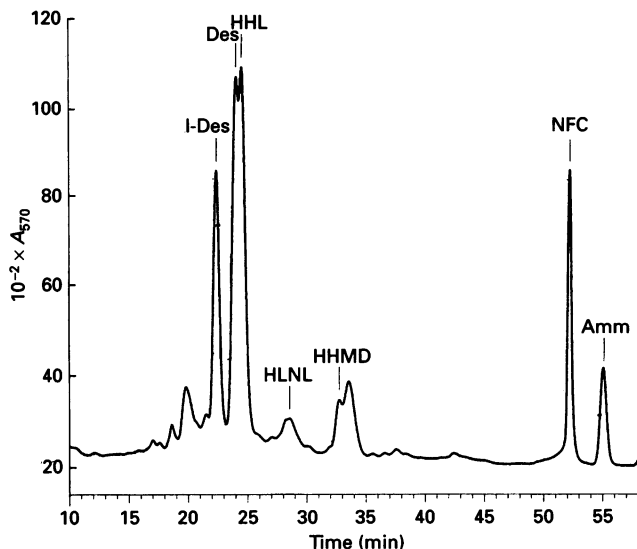

Figure 5 Detection of NFC in human diabetic tissue on the amino acid analyser, following prefractionation using the CF1 cellulose columns, using a ninhydrin detection system

Abbreviations: I-Des, isodesmosine; Des, desmosine; HHL, histidinohydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; HHMD, histidinohydroxymerodesmosine; Amm, ammonia.

molecular-mass components formed. Using the technique previously developed to concentrate and separate the lysyl-oxidase-derived cross-links (Sims and Bailey, 1992), we revealed the presence of a new peak that increased with increasing stability of the fibres as determined by their physical properties. A major ninhydrin peak occurred at 52 min following a short incubation with ribose and a similar peak at 52 min after longer incubation with glucose (Figure 3). Incubation in the presence of [^{14}C]glucose or [^{14}C]ribose revealed a major radioactive peak at this point, indicating their derivation from these sugars (Figure 4). The increase in the yield of the component with incubation time and the change in physical properties, such as increased insolubility and denaturation temperature, its high molecular mass (520 Da) and its derivation from glucose or ribose, suggests its potential as a cross-link. Assuming a leucine-equivalent ninhydrin colour yield of 2 (compared with a value of 1.8 for the tervalent cross-link pyridinoline), then, on the basis of the amount of collagen placed on the column, NFC can be estimated to be present at a concentration of one cross-link per one to five collagen molecules, depending on incubation time. The concentration of this putative cross-link in collagen glycosylated by both glucose and ribose is obviously sufficient to account for the changes in the physical properties observed during glycation. Investigations are continuing to determine its structure by electrospray m.s.

Analysis of human skin from control and diabetic patients revealed a similar profile of peaks in the chromatogram (Figure 5), NFC increasing with age and duration of diabetes. The component is therefore not an artefact of *in vitro* incubations and, on the basis of its estimated concentration, could be the major cross-link causing accelerated aging in diabetics.

The formation of pentosidine *in vitro* could be due to reaction with glucose or ribose, as shown by Grandee and Monnier (1991) and by Dyer et al. (1991), but we have shown here that other compounds are also formed that are derived specifically from either glucose or ribose, clearly indicating a different mechanism for each sugar. Although the products of the reaction with ribose are also present in the skin of diabetic subjects, their relative importance and their derivation from ribose or degraded glucose remains to be elucidated.

Clearly a more detailed knowledge of the chemical nature of the complexities of the glucose-mediated cross-links will assist our understanding of the mechanism of its synthesis, and once this has been achieved, enhance the possibility of inhibiting the excessive glycosylation and consequent accelerated aging, which has such a deleterious effect on collagenous tissues in diabetes and, to a lesser extent, in aging.

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