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The Chemistry of the Collagen Cross-Links

CHARACTERIZATION OF THE PRODUCTS OF REDUCTION OF SKIN, TENDON AND BONE WITH SODIUM CYANOBOROHYDRIDE

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Reduction of tissues with sodium cyanoborohydride at pH7.4 gave results identical with those obtained by KBH_4 treatment. On reduction with sodium cyanoborohydride at pH4.4, however, a previously undetected basic compound was formed and was identified by mass spectrometry and chemical degradation techniques as dihydrohydroxymerodesmosine. Histidino-hydroxymerodesmosine was not present, and further analysis confirmed that reduced aldol, a major product of reduction with KBH_4 at the lower pH, was also absent. These results, together with an analysis of the time course of the reduction, support previous assertions that histidino-hydroxymerodesmosine is an artifact [Robins & Bailey (1973) *Biochem. J.* **135**, 657–665] and suggests that the non-reduced form of hydroxymerodesmosine probably does not constitute a major intermolecular bond *in vivo*.

The technique of reduction with borohydride has been instrumental in the progress made in recent years in determining the structure of intermolecular collagen cross-links (for reviews see Tanzer, 1973; Bailey *et al.*, 1974). Certain assumptions as to the mode of action of this reducing agent must be made, however, in assigning structures to the compounds present in the native fibre before reduction. Thus stabilization by reduction with borohydride of labile aldimine compounds derived by condensation of lysine aldehydes with ε-amino groups is consistent with the changes in properties of the tissue produced by this modification (Bailey, 1968). Conversely, reduction with NaB^2H_4 and KB^3H_4 of compounds derived from hydroxylysine aldehyde has shown them to be present *in vivo* as keto amines (Robins & Bailey, 1973*a*, 1975; Eyre & Glimcher, 1973), a fact that appears to account for the observed thermal and chemical stability of these components. That keto amine forms of the cross-link do in fact exist *in vivo* has been demonstrated by isolating a peptide containing this cross-link from cartilage without prior reduction with borohydride (Miller & Robertson, 1973; Balian & Bailey, 1975).

The multifunctional amino acids histidino-hydroxymerodesmosine and aldol-histidine have been isolated from borohydride-reduced tissue and appear to result from interactions of the intramolecular aldol-condensation product with histidine and hydroxylysine residues (Tanzer *et al.*, 1973*a*). These compounds were not present when the reduction with borohydride was carried out at slightly acid pH values (below pK_a of the imidazole group of histidine), leading to the conclusion that the involvement of histidine is a base-catalysed reaction promoted by the reduction procedure (Robins & Bailey, 1973*b*).

Sodium cyanoborohydride ($NaBH_3CN$) is a selective reducing agent which readily reduces aldehydes and ketones at acid pH (Borch *et al.*, 1971; Walker, 1976). The relative stability of this reagent under acid conditions prompted a re-investigation of the apparent acid lability of histidino-hydroxymerodesmosine. The results are presented here and their implications for the nature of the bonds occurring *in vivo* are discussed.

Materials and Methods

Materials

$NaBH_3CN$ supplied by Aldrich Chemical Co., Wembley, Middx., U.K., was recrystallized from dioxan (Borch *et al.*, 1971). KB^3H_4 (300mCi/mmol) and ^3H_2O (5Ci/ml) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Materials for scintillation fluid (Bray, 1960) were supplied by Nuclear Enterprises (G.B.) Ltd., Edinburgh, Scotland, U.K.

Preparation of tissues and reduction with $NaBH_3CN$

Tail tendons and skin from 3-month-old rats and bovine skin and achilles tendon from a 1-year-old animal were prepared as described previously (Bailey *et al.*, 1970; Robins *et al.*, 1973). Samples were equilibrated for at least 4h in 0.1M-sodium phosphate buffers at several pH values between 4.4 and 8.0, and, after addition of solid $NaBH_3CN$ (30:1 collagen/

$NaBH_3CN$ dry-weight ratio), reduction was allowed to proceed for various periods of time at 20°C. The samples were then rinsed with five changes of water (100ml of water/g wet wt. of tissue) and were freeze-dried.

Calf and rat bone was decalcified with 0.2M-EDTA at 4°C and the washed residue reduced with KB^3H_4 as described by Robins *et al.* (1973) and with $NaBH_3CN$ at pH4.4 as described above.

Preparation of and reduction with NaB^3H_3CN

To 10mg of $NaBH_3CN$ dissolved in 0.1M-sodium citrate buffer, pH3.1 (0.1ml), was added 0.1ml of ^3H_2O (5Ci/ml) and the ^3H exchange allowed to proceed for 30min. The solution was then added to a stirred suspension of rat tail tendons previously equilibrated in 0.1M-NaH_2PO_4, pH4.4, and the reduction carried out for 24h as described above.

Hydrolysis procedure

Hydrolyses of the reduced materials were carried out in boiling 6M-HCl for 24h. The HCl was removed by evaporation *in vacuo* at 60°C.

Chromatographic analysis of the reduction products

Acid hydrolysates of the $NaBH_3CN$-reduced samples were analysed directly either on an extended basic column of the Locarte analyser or on a Technicon analyser by using pyridine/formate buffers (Bailey *et al.*, 1970). For preparative purposes, the hydrolysates were initially fractionated on a column (16cm^2×85cm) of Sephadex G-10 with 0.1M-acetic acid as eluent (Robins, 1976).

Chemical degradation of isolated compounds

(i) *Smith degradation.* Oxidation with periodate (10min reaction time) followed by reduction with KBH_4 was carried out as previously described (Robins & Bailey, 1975). The products were analysed directly by using the Locarte analyser.

(ii) *Periodate/permanganate oxidation followed by reduction.* The oxidation procedure was essentially that of Lemieux & von Rudloff (1955) used in degradative studies of the reduced aldol (Lent *et al.*, 1969). To the compound dissolved in 0.5ml of 0.1M-K_2CO_3 (pH7.5) was added 0.02M-$NaIO_4$ (0.5ml) and 5mM-$KMnO_4$ (0.05ml). Oxidation was allowed to continue for 15 or 45min at room temperature, whereon the products and excess of reagents were reduced by the addition of KBH_4 (4mg). After 30min, 6M-HCl was added to pH2 and portions (0.2ml) of the solution were analysed directly on the Locarte amino acid analyser.

Mass spectrometry

Final purification of the isolated compounds was achieved by chromatography on a column (0.4cm^2× 35cm) of Sephadex G-10 with 0.1M-acetic acid. Relevant fractions were evaporated to dryness at 40°C, and trifluoroacetylated methyl ester derivatives were prepared as described by Bailey *et al.* (1970). Spectra were recorded on an LKB g.l.c.–mass spectrometer or an AEI MS30 instrument by using direct-insertion probes.

Results

Reduction of rat tail tendon with $NaBH_3CN$

Analysis of hydrolysates of tendons reduced with $NaBH_3CN$ for 24h at pH7.4 showed that hydroxylysinonorleucine and histidino-hydroxymerodesmosine were present (Fig. 1): the chromatogram was identical with that obtained by analysis of KBH_4-treated tissue. On reduction at pH4.4, however, a major peak appeared (denoted X) that was completely absent from the sample reduced at pH7.4 (Fig. 1). Reduction of tendons at intermediate pH values resulted in a progressive decrease in the amount of peak X with increasing pH (Fig. 2), together with a concomitant increase in the amount of histidino-hydroxymerodesmosine, although accurate quantification of the latter in this system is hampered by the presence of galactosamine (Fig. 1). On reduction with NaB^3H_3CN at pH4.4, radioactivity was incorporated into peak X, and analysis of an alkaline hydrolysate of the reduced tendons in the pyridine/

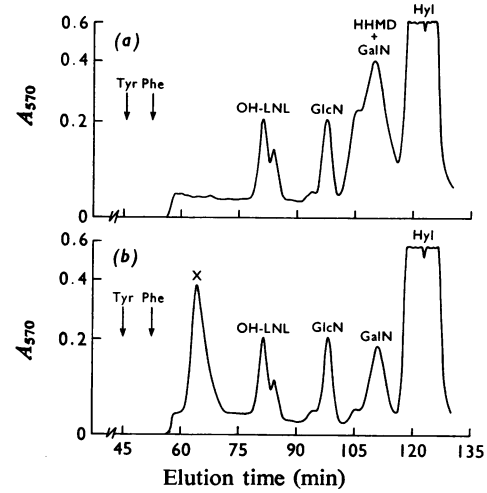


Fig. 1. *Chromatography of acid hydrolysates of rat tail tendon reduced with $NaBH_3CN$*

For each chromatogram, 5mg of material was applied to an extended basic column of the Locarte analyser and the effluent analysed directly with ninhydrin after the elution of phenylalanine: (*a*) reduced at pH7.4; (*b*) reduced at pH4.4. OH-LNL, 5-hydroxylysinonorleucine; HHMD, histidino-hydroxymerodesmosine.

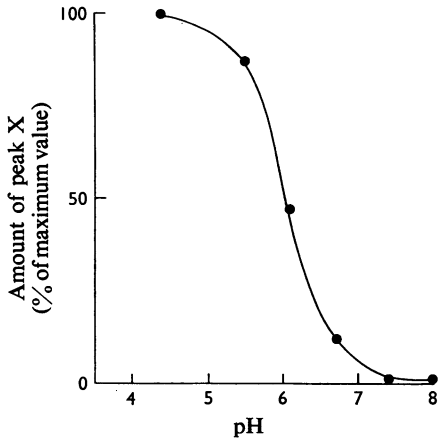


Fig. 2. *Variation with pH in the amount of peak X formed on reduction with $NaBH_3CN$*
Rat tail tendons (5mg) equilibrated at various pH values were reduced with $NaBH_3CN$ for 24h and after acid hydrolysis were analysed directly by using the Locarte analyser.

formate buffer system indicated that no reduced aldol was formed during the reduction. In the pyridine/formate system, peak X co-chromatographed with hydroxylysinonorleucine.

Characterization of the isolated reduction product

(i) *Chemical degradation.* A Smith degradation of the isolated compound (peak X) gave rise to a basic component, which was eluted 5min before hydroxylysine, and a 440nm-absorbing peak, which was identified by further chromatography as proline (Fig. 3). The amount of the basic component was only slightly diminished after periodate/permanganate oxidation followed by reduction, and the further decrease in amount seen with the prolonged oxidation time (Fig. 3) is probably a result of oxidation of the α-amino groups by periodate (Clamp & Hough, 1966). The basic component was not fully characterized.

The relationship of the isolated compound (peak X) to histidino-hydroxymerodesmosine, together with the known derivation of the latter from the aldol-condensation product, suggested that the unknown component might be hydroxymerodesmosine formed by reduction of the Schiff base between the aldol and hydroxylysine. This supposition was consistent with the production by Smith degradation of proline and a basic component with properties that might be expected for the 'amino-aldol' (Scheme 1). However, the resistance of the isolated basic component to periodate/permanganate oxidation, despite its presumed structural homology with the reduced aldol (Scheme 1), which is fully cleaved within 15min by this procedure (Lent *et al.*, 1969), indicated that no carbon–carbon double bond is present in the compound.

(ii) *Mass spectrometry.* Mass-spectral analysis of the trifluoracetylated methyl ester derivative produced a clearly defined molecular-ion peak at *m/e* 942 (Fig. 4), together with a fragmentation pattern consistent with the compound before derivative formation being dihydrohydroxymerodesmosine. These data therefore confirm the chemical evidence for the absence of an olefinic double bond.

Time course of reduction

Direct measurement of hydroxylysinonorleucine and dihydrohydroxymerodesmosine concentrations in rat tail tendons after reduction with $NaBH_3CN$ for various times showed large differences in their rates of formation (Fig. 5). Thus reduction to hydroxylysinonorleucine was virtually complete within 1 h, whereas

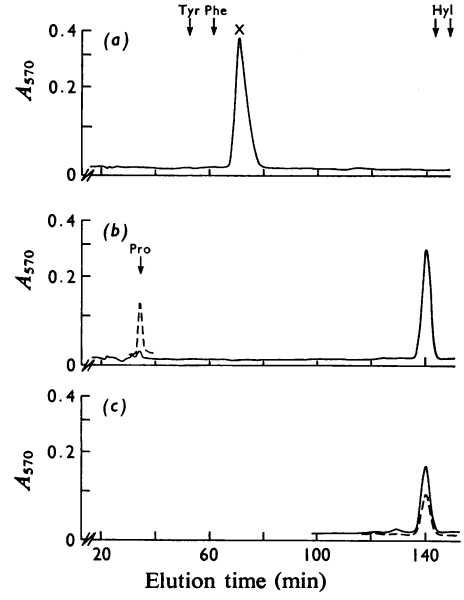
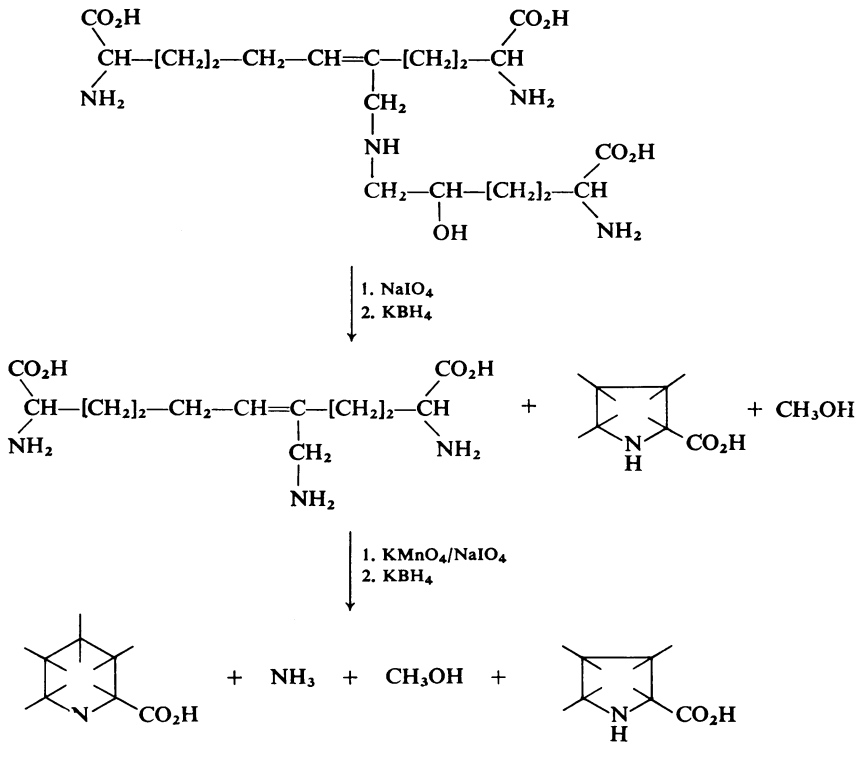


Fig. 3. *Oxidative degradation of peak X*
Chromatography on an extended basic column of the Locarte analyser showing (*a*) the isolated compound (peak X), (*b*) the products of periodate oxidation (10min reaction time) followed by reduction with KBH_4 where the basic compound which was eluted near hydroxylysine was detected at 570nm (——) and proline was detected at 440nm (----), and (*c*) the products of periodate/permanganate oxidation for 15min (——) or 45min (----) followed by reduction with KBH_4. For experimental details, see the Materials and Methods section.



Scheme 1. *Theoretical degradative pathway of hydroxymerodesmosine*

A Smith degradation yields a basic component, 'amino-aldol', together with 1 mol each of proline and methanol. Oxidation of the 'amino-aldol' with periodate/permanganate followed by reduction would yield equimolar proportions of pipecolic acid, proline, methanol and ammonia.

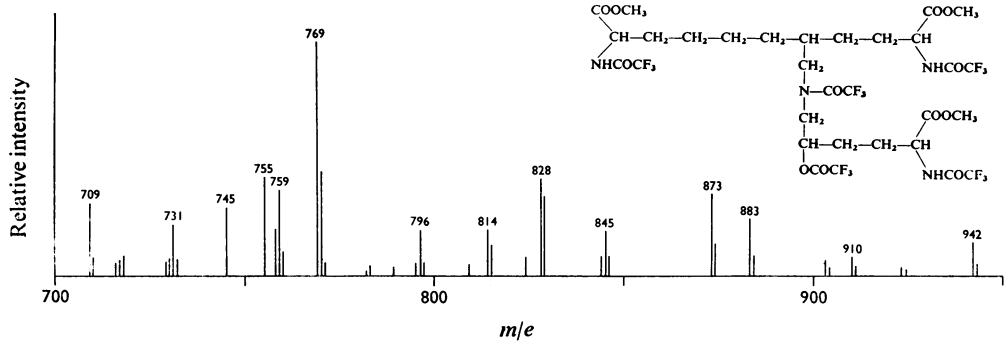


Fig. 4. *Partial mass spectrum of the trifluoracetylated methyl ester derivative of dihydrohydroxymerodesmosine isolated from $NaBH_3CN$-reduced rat tail tendon*

The spectrum shows a peak at *m/e* 942, which is confirmed as the molecular ion (*M*) by the presence of ions derived from the parent by losses characteristic of this type of compound, namely: *m/e* 883, $M-CH_3CO_2^{\bullet}$; 873, $M-CF_3^{\bullet}$; 828, $M-CH_3CO_2H$; 769, $M-(CF_3CO_2H+CH_3CO_2^{\bullet})$.

only about 10% of dihydrohydroxymerodesmosine was formed during that time, complete reduction occurring between 10 and 24h.

When tissue which had been reduced for 1h at pH4.4 with $NaBH_3CN$ was subsequently treated with KB^3H_4 as carried out previously (Robins & Bailey, 1973*b*), reduced aldol was the sole major radioactive peak detected after alkaline hydrolysis.

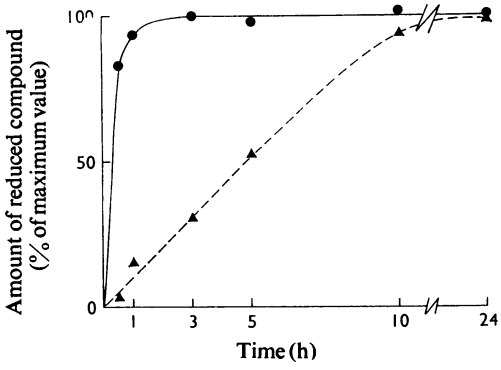


Fig. 5. *Time course of reduction of rat tail tendon with $NaBH_3CN$ at pH4.4*
The reduced samples (5mg) were hydrolysed in acid and the amounts of hydroxylysinonorleucine (●) and dihydrohydroxymerodesmosine (▲) measured directly by using the Locarte analyser.

Analysis of other tissues

In addition to rat tail tendon, rat skin and bovine skin and achilles tendon were analysed after reduction with $NaBH_3CN$ at pH4.4 for 24h, and in all cases dihydrohydroxymerodesmosine was present. Its formation therefore appears to be common to those tissues in which histidino-hydroxymerodesmosine is formed on reduction at pH7.4.

Analysis of bone collagen, which contains only a small proportion of histidino-hydroxymerodesmosine, after reduction with KBH_4 at pH7.4 (Fowler & Bailey, 1972) revealed only a very small amount of dihydrohydroxymerodesmosine on reduction with $NaBH_3CN$ at pH 4.4, the major components being dihydroxylysinonorleucine and hydroxylysinonorleucine (Fig. 6).

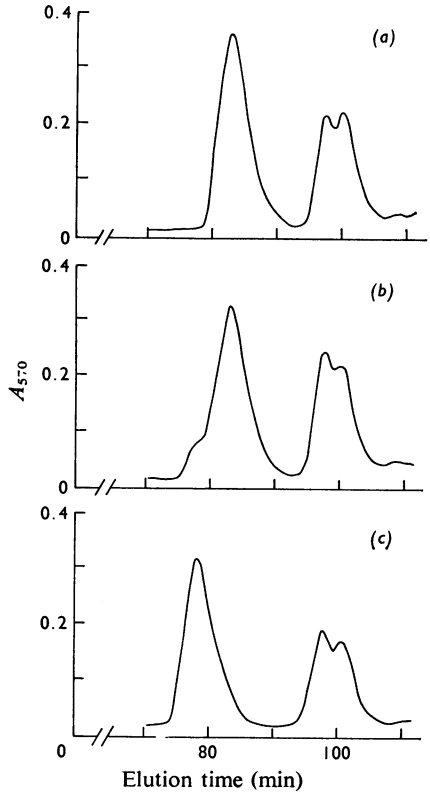
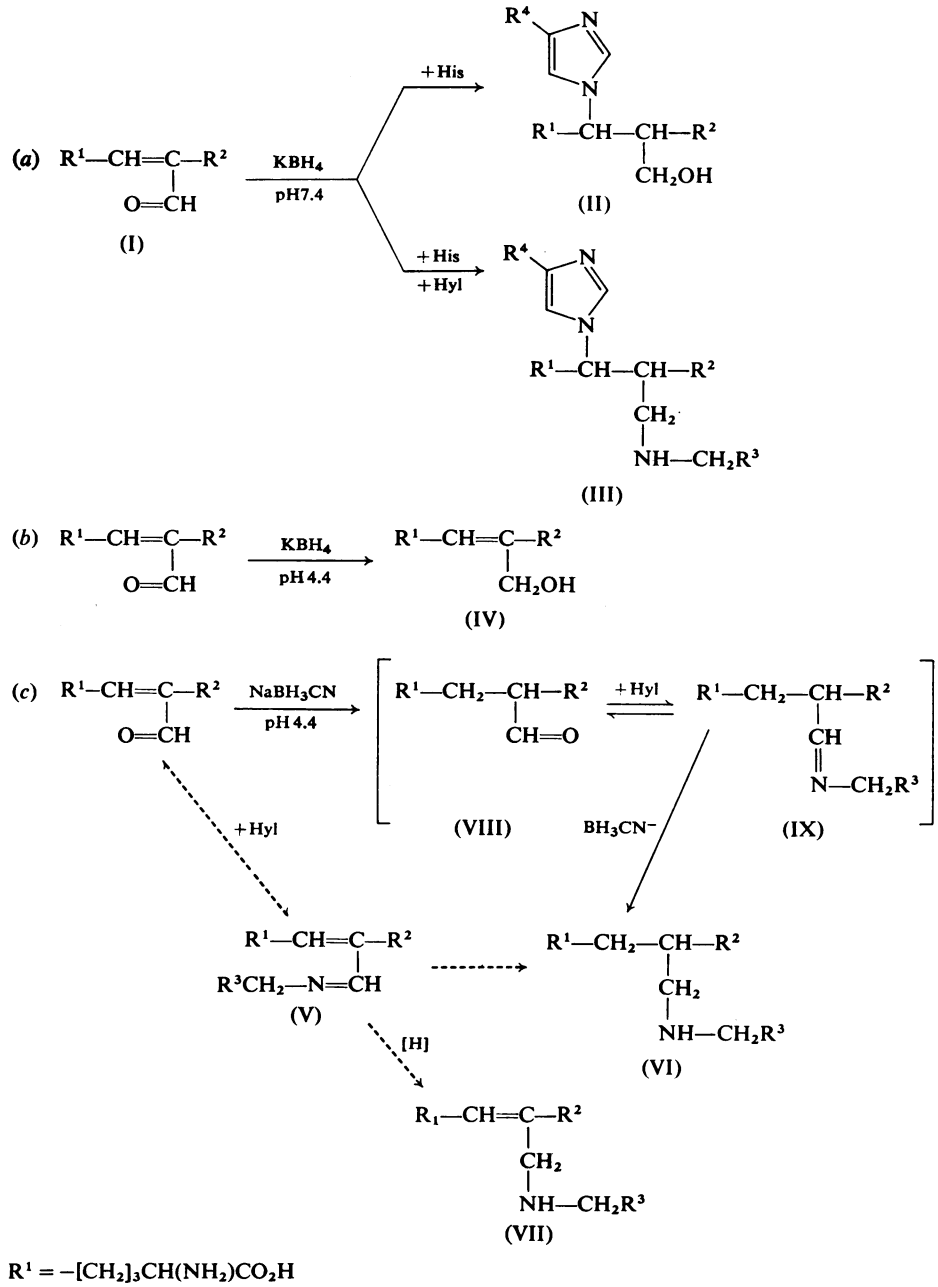


Fig. 6. *Reduction of collagens with $NaBH_3CN$*
Acid hydrolysates were analysed directly with a JEOL amino acid analyser: (*a*) bone collagen reduced at pH7.4 (an identical pattern was obtained after reduction with KBH_4); (*b*) bone collagen reduced at pH4.4; (*c*) rat tail tendon reduced at pH4.4 shown for comparison. The peak eluted at 78min is dihydrohydroxymerodesmosine, that at 83min is dihydroxylysinonorleucine and those at 97 and 100min are isomers of hydroxylysinonorleucine.

Discussion

The experiments described above show that reduction with cyanoborohydride at pH7.4 appears to give rise to products identical with those obtained by reduction with borohydride. The isolation and characterization of dihydrohydroxymerodesmosine from tendon and skin reduced at acid pH with cyanoborohydride clearly indicates differences in specificity of the $NaBH_3CN$ and KBH_4 reagents, since the latter results in the formation of reduced aldol under these conditions. The important question to be resolved, however, is whether these differences shed any light on the nature of the bonds present before reduction.

Previous studies have shown that, during reduction with KBH_4 at neutral pH, reactions of the intramolecular $\alpha\beta$-unsaturated aldehyde (structure I; Scheme 2) with histidine and hydroxylysine residues give rise to aldol-histidine (II) and histidino-hydroxymerodesmosine (III) (Tanzer *et al.*, 1973*a*). Our contention that addition of the histine residue is a base-catalysed reaction promoted by the reduction procedure was based on the fact that reduction with KBH_4 at pH4.4 (at which the imidazole ring is protonated) inhibited the reaction, resulting in the formation of reduced aldol (IV): if this histidine–aldol bond were present before reduction, it is unlikely to have been cleaved under such mildly acid conditions. The possibility exists, however, that condensation of the unsaturated aldehyde with hydroxylysine occurs and that the cross-link present *in vivo* is the Schiff base, dehydrohydroxymerodesmosine (V).



$R^1 = -[CH_2]_3CH(NH_2)CO_2H$

$R^2 = -[CH_2]_2CH(NH_2)CO_2H$

$R^3 = -CH(OH)[CH_2]_2CH(NH_2)CO_2H$

$R^4 = -CH_2CH(NH_2)CO_2H$

Scheme 2. *Reactions of the aldol-condensation product in collagen during reduction with borohydride and cyanoborohydride*
For explanatory details, see the Discussion section.

If indeed dehydrohydroxymerodesmosine does exist at pH7.4, then its apparent disruption with very slight lowering of pH such that cleavage of the compound is 50% complete at pH6 (Robins & Bailey, 1973*b*) would not be expected, particularly in view of the fact that dehydrohydroxylysinonorleucine has been shown to be present even at pH4.4 by reduction. Thus formation of dihydrohydroxymerodesmosine (VI) by reduction of the Schiff base (V) is unlikely, since reduction at pH4.4 with KBH_4, which is known to reduce Schiff bases at this pH, results in the formation not of hydroxymerodesmosine (VII) but of reduced aldol (III). Moreover, if reduction of the Schiff base did occur, then isolation of a mixture of the chromatographically similar compounds (VI) and (VII) would be expected from the relative reactivities of the double bonds (Paz *et al.*, 1971), but no peak at *m/e* 940 corresponding to the unsaturated compound was detected in the mass spectra (Fig. 4). The most likely mechanism for the formation of dihydrohydroxymerodesmosine (VI) is that initial reduction of the $>C=C<$ bond occurs by a Michael-type attack of the cyanoborohydride anion at the β-carbon atom, and the unconjugated aldehyde produced (VIII) condenses with hydroxylysine to form a Schiff-base intermediate (IX), which is reduced to compound (VI) (Scheme 2). The fact that reductive amination occurs in preference to reduction of the aldehyde is in agreement with studies on model compounds, even at relatively low pH values (Borch *et al.*, 1971). As shown in Fig. 5, reduction of the Schiff base dehydrohydroxylysinonorleucine by $NaBH_3CN$ at pH4.4 is relatively rapid, and slow reduction of the $>C=C<$ bond is therefore probably mainly responsible for the observed low rate of formation of dihydrohydroxymerodesmosine. On reduction of tendons with $NaBH_3CN$ at pH7.4, histidino-hydroxymerodesmosine formation was complete within 1h: the cyanoborohydride anion therefore appears to act as an efficient catalyst for imidazole addition at the β-carbon atom and reduction of the olefinic bond does not occur at this pH.

Thus these results strengthen our previous arguments that histidino-hydroxymerodesmosine is an artifact of reduction and that dehydrohydroxymerodesmosine does not constitute a major intermolecular cross-link in the tissues examined. Formation of this Schiff base appears to depend on disruption of the delocalized electron system of the $\alpha\beta$-unsaturated aldehyde, either by a base-catalysed addition of the imidazole group of histidine at pH7.4 (Robins & Bailey, 1973*b*) or by reduction of the $>C=C<$ bond by $NaBH_3CN$ at pH4.4.

Very small amounts of hydroxymerodesmosine have been isolated from insoluble bovine collagen after borohydride reduction, and the non-reduced compound was suggested as an intermediate in the formation of the histidine-containing derivative (Tanzer *et al.*, 1973*b*). The reason that this compound has not been detected by our chromatographic systems in any of the tissues examined is not at present clear.

The isolation and characterization by nuclear-magnetic-resonance and mass spectrometry of large amounts of hydroxymerodesmosine from borohydride-reduced rat bone was described (Masuda *et al.*, 1976). Indeed, Masuda *et al.* (1976) claim that this cross-link is present in a similar amount to that of dihydroxylysinonorleucine, previously reported as the major lysine-derived cross-link in hard tissue (Bailey *et al.*, 1969; Davis & Bailey, 1971; Mechanic *et al.*, 1971). The results of Masuda *et al.* (1976) are difficult to explain, since in bone collagen the high degree of hydroxylation of lysine residues in the non-helical regions (Barnes, 1973), which are generally accepted as the sites of oxidative deamination, is not consistent with the formation of dehydrohydroxymerodesmosine, which requires the participation of two lysine aldehyde residues. In our hands, reduction of bone collagen with KBH_4 or $NaBH_3CN$ at pH7.4 failed to produce any detectable hydroxymerodesmosine, although after reduction with $NaBH_3CN$ at pH4.4 a very small amount of the dihydro derivative could be detected. In both cases the major cross-links present in reduced bone are dihydroxylysinonorleucine and hydroxylysinonorleucine.

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