A Possible Role for Dehydrodihydroxylysinoisonorleucine in Collagen Fibre and Bundle Formation

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The concentrations of NaB\(^3\)H\(_4\)-reducible collagen cross-links were determined at the time when collagen fibres and bundles are observed in electron micrographs of connective tissue developing around the implanted Ivalon sponge in adult male rats. The highest radioactivity occurs with hydroxylysinoisonorleucine and histidinohydroxymerodesmosine, and the lowest with lysisonorleucine, the reducible amounts of these cross-links remaining relatively constant as fibres and bundles appear. On the other hand, dihydroxylysinoisonorleucine amounts are low during the initial stages of connective-tissue formation and rise sharply as collagen fibres and bundles develop and collagen matures, as shown by increased resistance of insoluble collagen to digestion with bacterial collagenase. The bulk of hydroxylysinoisonorleucine and dihydroxylysinoisonorleucine is glycosylated, the former with galactosyl or glucosylgalactosyl residues and the latter with glucosylgalactosyl residues. The changing relationships between the amounts of \(^3\)H-labelled hydroxylysinoisonorleucine, glucosylgalactosyl dihydroxylysinoisonorleucine and non-glycosylated dihydroxylysinoisonorleucine as fibres and bundles appear suggest three post-translational steps involving lysyl-derived cross-links in the organization of collagen into fibres and bundles.

After secretion from the cell into the extracellular space, monomeric collagen is aligned into microfibrils, which then aggregate into the fibres that subsequently form bundles as collagen organizes in developing connective tissue. An important biochemical process contributing to the stability of monomeric collagen and the microfibrils is the enzymic oxidative deamination of specific lysyl and hydroxylysyl residues into reactive aldehydes, allysine and hydroxyallysine (Bornstein & Piez, 1966; Pinnell & Martin, 1968; Siegel & Martin, 1970). These aldehydes may condense to produce intramolecular cross-links or may react with amino acid side chains from neighbouring monomers to form Schiff-base intermolecular cross-links.

The allysine- and hydroxyallysine-containing cross-links are unsaturated labile compounds that after reduction with borohydride are stabilized against acid hydrolysis (Bailey & Peach, 1968; Tanzer, 1968). When the reduction is carried out with NaB\(^3\)H\(_4\), the cross-links are rendered radioactive and can be isolated by appropriate chromatographic procedures (Mechanic, 1974). The cross-links identified by these procedures are lysisonorleucine, hydroxylysinoisonorleucine, dihydroxylysinoisonorleucine, hydroxymerodesmosine, aldonhistidine and histidinohydroxymerodesmosine (Tanzer, 1973; Bailey et al., 1974). Uncertainties remain, however, as to whether histidinohydroxymerodesmosine arises from a cross-link in vivo or is an artifact of the reduction procedure (Robins & Bailey, 1973a, 1977).

As part of our studies on the biology of collagen cross-links, we examined the lysyl-derived NaB\(^3\)H\(_4\)-reducible cross-links as fibres and bundles appear in developing connective tissue of the Ivalon-sponge implant in the adult male rat. High concentrations of hydroxylysinoisonorleucine and histidinohydroxymerodesmosine are found during the initial stages of connective-tissue formation and remain essentially unchanged as collagen fibres and bundles develop. Dihydroxylysinoisonorleucine amounts, on the other hand, are low initially and then increase as fibres and bundles form. Approx. 45% of hydroxylysinoisonorleucine and 80% of dihydroxylysinoisonorleucine are in the glucosylgalactosyl from during the period of tissue development. The striking increases in glucosylgalactosyl dihydroxylysinoisonorleucine (140%) and non-glycosylated dihydroxylysinoisonorleucine (270%) as collagen fibres and bundles appear and collagen matures suggest a role for dehydrodihydroxylysinoisonorleucine in the higher organization of collagen in developing connective tissue in adult male rats.

**Experimental**

Ivalon-sponge implantation and tissue harvesting in 3-month-old adult male Sprague–Dawley rats
(400 g body wt.) were carried out as previously described (Boucek & Noble, 1955). The animals were killed by cervical dislocation, and the tissue capsule surrounding the implant was harvested after 2–6 days of growth.

A series of 12 animals was used for the studies on collagen synthesis and NaB\(^3\)H\(_4\)-reducible cross-links of acid-hydrolysed collagen. For the former, collagen was labelled with L-[\(^{14}\)C]proline (290 mCi/mmole; Amersham/Searle, Arlington Heights, IL, U.S.A.) by an intraperitoneal injection (2.5 \(\mu\)Ci/100 g body wt.) 4h before harvesting. A portion of the tissue was hydrolysed (6 M-HCl, 110°C, 16h) and samples of the hydrolysate were taken for hydroxyproline determination (Woessner, 1961) and for isolation of hydroxy-\(^{14}\)Cproline by cation-exchange-column chromatography (Mechanic, 1974) and determination of its radioactivity. The \(^{14}\)C and total hydroxyproline values were expressed on the basis of the dry weight of the sponge implant (100 mg) to give the content of newly formed and of accumulated collagen respectively.

For the studies on collagen cross-links, the remainder of the tissue was homogenized in 10 vol. of cold 0.02 M-NH\(_4\)HCO\(_3\), pH 7.8, and extracted with this solution for two 1.5h periods at 4°C to remove unbound carbohydrates. The extracts were removed by centrifugation (2000rev./min, 4°C, 30 min) and the pellet was reduced with NaB\(^3\)H\(_4\) (186 mCi/mmole; New England Nuclear, Boston, MA, U.S.A.), by using 2.7 mg of NaBH\(_4\)/40 mg of collagen, by the procedure of Tanzer & Mechanic (1968). The 12 tissues were reduced at the same time. After exhaustive dialysis against water at 4°C, the reduced tissue pellet was freeze-dried and hydrolysed in 3 M-toluene-p-sulphonic acid (110°C, 24 h). The \(^3\)H-labelled cross-links in the hydrolysate were separated on a cation-exchange column (62 cm \(\times\) 0.9 cm) packed with resin (MR 201; Mark Instrument Co., Villanova, PA, U.S.A.) to a height of 58 cm. To determine the purity of the radioactive compounds, the eluate fractions of each cross-link peak were pooled and rechromatographed on a second column (30 cm \(\times\) 0.9 cm) packed with resin (MR 208; Mark Instrument Co.) to a height of 26.5 cm (Mechanic, 1974). The \(^3\)H-labelled hydroxyproline, hydroxylysinoonorleucine, lysinoonorleucine and histidinodihydroxymerodesmosine peaks from the first column were shown to be homogeneous and to elute in the appropriate areas on the second column with respect to marker amino acids. The dihydroxylysinoonorleucine peak on the first column, however, was contaminated with other labelled material, and consequently dihydroxylysinoonorleucine tissue amounts were calculated after sequential separation on the two columns. The radioactivity in each cross-link was expressed in c.p.m./mg of collagen, with collagen being 7.46 times (Neuman & Logan, 1950) the amount of hydroxyproline applied to the column.

Another series of 15 animals was implanted for comparison of cross-link profiles in acid and alkaline hydrolysates. The tissues were homogenized in water, freeze-dried and reduced with NaB\(^3\)H\(_4\) (228 mCi/mmole). One portion of the reduced tissue was hydrolysed in 3 M-toluene-p-sulphonic acid and a second in 2 M-NaOH (105°C, 22h) in propylene tubes. The alkaline hydrolysates were desalted on AG-50 columns (H\(^+\) form, 100–200 mesh) before application to the cation-exchange column for resolution of the collagen cross-links.

The glycosylated cross-links present in the alkaline hydrolysates were tentatively identified as glucosylgalactosyl dihydroxylysinoonorleucine and hydroxylysinoonorleucine and galactosylhydroxylysinoonorleucine by their elution characteristics on the first column (close to reduced aldol and between methionine and phenylalanine) (Robins & Bailey, 1974). Chromatography of the individual peaks of glycosylated cross-links on the second column indicated that the glucosylgalactosyl dihydroxylysinoonorleucine and galactosylhydroxylysinoonorleucine peaks were 90–95% homogeneous, whereas glucosylgalactosylhydroxylysinoonorleucine was approx. 73% pure, being contaminated by reduced aldol-condensation product.

The percentages of hydroxylysinoonorleucine and dihydroxylysinoonorleucine present in the keto-amine and aldime forms were determined by identifying the products formed by periodic acid oxidation followed by reduction with NaBH\(_4\) by the procedure described by Robins & Bailey (1973b).

A biochemical index of collagen maturation and cross-link density was provided by the resistance of insoluble collagen to bacterial-collagenase (clostridial collagenase, CLSPA; Worthington Biochemical Corp., Freehold, NJ, U.S.A.) digestion by the method used by Kohn & Rollerson (1960) and Harris & Farrell (1972). In a third series of animals, the capsule tissue was homogenized in 10 vol. of cold 1 M-NaCl/0.02M-Tris/HCl, pH 7.4, and shaken for two 24h periods at 4°C, first with the NaCl solution and then with 10 vol. of 0.5 M-acetic acid, to remove soluble collagens. The pellet of insoluble collagen obtained on centrifugation (41200g, 1h, rotor 40, Beckman model L ultracentrifuge) was freeze-dried and a 1 mg sample hydrolysed (6 M-HCl, 110°C, 16h) for collagen (hydroxyproline) determination. A series of tubes containing the amount of insoluble collagen required to give an enzyme/substrate ratio of 1:350 were incubated at 37°C in 2 ml of 0.05M-Tris/HCl buffer, pH 7.5, containing 0.36 mM-CaCl\(_2\), 250 \(\mu\)g of streptomycin and 142 \(\mu\)g of penicillin G, and the kinetics of collagenolysis determined by measuring hydroxyproline in the supernatant and pellet as a function of time. The reaction followed first-order kinetics, and the rate of collagenolysis was expressed as \(k\) (the time in minutes required to digest 50% of the collagen).
The data on collagen (hydroxyproline) content in the developing connective tissue were obtained from the different series of rats.

Portions of radioactive samples were analysed in a Packard 3003 Tri-Carb liquid-scintillation spectrometer, in the solvent system Triton X-100/toluene (1:2, v/v) containing 5.0 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/litre. To keep in solution the buffer salts in the 0.1–0.3 ml samples from the column fractions, the addition of 1 ml of water to 14 ml of the scintillation liquid was necessary. Results were expressed as c.p.m. or d.p.m., calculated by using computer-generated equations developed with the automatic external standard of the instrument, and corrected for background.

**Results**

A review of the electron-microscopic images of collagen organization in developing connective tissue in the 100–250 μm-wide area adjacent to the implanted Ivalon sponge serves to introduce the collagen cross-link studies (Boucek, 1977). By 4 days (Plate 1a), bundles (2–3 μm in width) of collagen fibres (20–25 nm in diameter) develop in restricted areas near the fibroblast surface, with the intervening intercellular space occupied by apparently amorphous ground substance. From 4 to 6 days, the extracellular spaces become filled by collagen bundles arranged in plies (Plate 1b). Within the bundles, the collagen fibres appear graduated in thickness from 25 nm at the periphery to 50 nm at the centre.

Biochemical measurements of collagen synthesis (hydroxy[14C]proline) and accumulation (hydroxyproline) were made on tissues at the 2–6 day period. The resistance of collagen in the acid-insoluble fraction to digestion with bacterial collagenase was measured at each time period as an assessment of collagen maturation and overall collagen cross-link density (Fig. 1). Although the amount of hydroxy[14C]proline in the tissues increases progressively from 2 to 6 days, the total tissue hydroxyproline is similar at 2 and 4 days, and increases between days 4 and 6 with the test of significance giving a probability (P) of <0.001. The resistance of insoluble collagen to digestion with bacterial collagenase is identical at days 2 and 4, but at day 6 there appears to be an increased resistance, and the time required to degrade 50% of the collagen (t4) is significantly (P < 0.05) increased.

The changes in the profile of acid-hydrolysed boro-hydride-reducible cross-links and precursor allysine during the period of collagen synthesis and organization (3–6 days) are highlighted by the chromatograms shown in Fig. 2 and the data in Table 1. Positions of radioactive peaks relative to marker amino acids identify reduced allysine, hydroxylysinoonorleucine, lysinonorleucine and histidinohydroxymeros desmosine in the column-chromatographic system (Mechanic, 1974). The most striking change in the profile occurs in the fractions designated peak D, which is eluted as a doublet in the 3-day preparation and as a large broad-based peak in the 6-day preparation. Peak D is resolved into multiple peaks when redchromatographed on the short column (Fig. 2, insets), the first of which is co-eluted with authentic dihydroxylysinoonorleucine (provided by G. L. Mechanic). Dihydroxylysinoonorleucine is 24% of the total radioactivity in peak D at 3 days and 85% at 6 days.

Concentrations of radioactivity present as hydroxylysinoonorleucine and histidinohydroxymerosdesmosine are high during the early stages of collagen synthesis (2 days) and remain essentially unchanged as collagen fibres and bundles develop (4–6 days) (Table 1). The concentration of lysinonorleucine remains at a low value throughout the period of connective-tissue formation. In sharp contrast, dihydroxylysinoonorleucine amounts are low at 2 and 3 days and increase markedly as collagen fibres and bundles appear.
Fig. 2. Radioactivity elution profile of NaB\(^3\)H\(_4\)-reduced (a) 3-day and (b) 6-day capsule tissue

The column (see the Experimental section) was eluted by using a nine-chamber complex gradient of sodium citrate buffers (0.25 M-sodium citrate, pH 2.93, and 0.4 M-sodium citrate) at 54.5°C. The flow rate was maintained at 70 ml/h and 1.05 ml fractions were collected. The amounts of collagen applied to the columns were 7.1 mg (a) and 8.1 mg (b). The abbreviations for the lysyl-derived compounds on the chromatograms are: HNL, hydroxylysinonorleucine; HLN L, hydroxylysinosynorleucine; LNL, lysinosynorleucine; HHMD, histidinohydroxymerodesmosine. The elution profiles on the second column (see the Experimental section) of the pooled fractions of peak D (indicated by horizontal bar in main Figure) are shown in the insets, the arrow indicating the positions of dihydroxylysinosynorleucine. On the second column, eluting buffer was 0.35 M-sodium citrate, pH 5.25; the flow rate was 80 ml/h, and 1.33 ml fractions were collected. For radioactivity determination, 0.1 ml and 0.3 ml were taken from fractions of the first and second columns respectively.

Fig. 3. Comparison of radioactivity elution profiles of (a) acid and (b) alkaline hydrolysates of NaB\(^3\)H\(_4\)-reduced 6-day capsule tissue

The elution of the first column was as described in Fig. 2. The amounts of collagen applied to the columns were (a) 1.0 mg and (b) 1.8 mg. Radioactivity determinations were made on 0.1 ml fractions. The abbreviations for cross-links are given in the legend to Fig. 2; in addition: ACP, aldol condensation product; DHLNL, dihydroxylysinosynorleucine.

and chromatographic separation on the first cation-exchange column. Partial hydrolysis released predominantly the monosaccharide derivative of the cross-links, and complete acid hydrolysis yielded \(^3\)H-labelled compounds eluted only as dihydroxylysinosynorleucine and dihydroxylysinosynorleucine. The carbohydrates obtained on complete hydrolysis were characterized by g.l.c. (Griggs et al., 1971) and by t.l.c. (Moczar et al., 1967) as glucose and galactose.

Of the dihydroxylysinosynorleucine present during the 2–6-day period (Table 2), approximately 45% is in the disaccharide and 20% in the monoglycosylated form. On the other hand, an average of 80% of the total dihydroxylysinosynorleucine is glucosylgalactosydihydroxylysinosynorleucine, the remainder being non-glycosylated. Between 3 and 6 days, glucosylgalactosydihydroxylysinosynorleucine increases by 140% and non-glycosylated dihydroxylysinosynorleucine increases by 270%, whereas the total dihydroxylysinosynorleucine, glucosylgalactosyldihydroxylysinosynorleucine and galactosylhydroxylysinosynorleucine remain constant as fibres and bundles form.

The periodate-oxidation/NaB\(^3\)H\(_4\)-reduction procedure degrades radioactively labelled dihydroxylysinosynorleucine and dihydroxylysinosynorleucine to \(^3\)H-
EXPLANATION OF PLATE 1

Electron micrographs of (a) 4-day and (b) 6-day tissues in the 100–250 μm-wide area adjacent to the implanted Ivalon sponge in adult male rats

After removal of the sponge implant and the overlying skin, the tissue block was fixed briefly in 2.5% glutaraldehyde and then a thin section (approx. 1–2 mm) was made through the entire block and placed in glutaraldehyde for longer periods. The section was post-fixed in 1% OsO₄ and embedded in Araldite. Thin sections on grids were stained with uranyl acetate. Observations were made with a Siemens electron microscope. Abbreviations used: F, fibroblast; ECM, extracellular matrix; CF, collagen fibres; CB, collagen bundles.
hydroxynorvaline and [3H]proline when the cross-links are in the keto-amine form and to [3H]methanol and [3H]lysine when the cross-links are in the aldime form (Robins & Bailey, 1975). The hydroxylysinonorleucine isolated from 3- and 6-day tissues yielded only [3H]lysine after oxidation-reduction procedure, indicating that only the aldime form is present. However, the yield of [3H]proline and [3H]hydroxynorvaline from non-glycosylated dihydroxylysinonorleucine obtained from the same tissues (Table 3).

Table 1. Distribution of radioactivity in acid hydrolysates of NaB3H4-reduced developing connective tissue (adult male rat)

<table>
<thead>
<tr>
<th>Tissue age (days)</th>
<th>Hydroxy-norleucine</th>
<th>Dihydroxy-lysino-norleucine</th>
<th>Hydroxylysino-norleucine</th>
<th>Lysino-norleucine</th>
<th>Histidino-hydroxymero-desmosine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early collagen synthesis</td>
<td>2</td>
<td>7.6 ± 0.9</td>
<td>1.1 ± 0.2</td>
<td>25.9 ± 1.9</td>
<td>4.3 ± 0.4</td>
<td>22.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.7 ± 1.0</td>
<td>1.8 ± 0.1</td>
<td>29.7 ± 2.4</td>
<td>2.2 ± 0.2</td>
<td>21.6 ± 0.9</td>
</tr>
<tr>
<td>Fibre and bundle organization</td>
<td>4</td>
<td>10.3 ± 0.3</td>
<td>5.5 ± 0.7</td>
<td>32.1 ± 0.7</td>
<td>3.9 ± 0.5</td>
<td>21.9 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9.9 ± 1.1</td>
<td>12.9 ± 0.2</td>
<td>31.0 ± 1.3</td>
<td>2.5 ± 0.2</td>
<td>18.4 ± 0.4</td>
</tr>
</tbody>
</table>

Table 2. Distribution of radioactivity in dihydroxylysinonorleucine and hydroxylysinonorleucine cross-links of alkaline hydrolysates of NaB3H4-reduced developing connective tissue (adult male rat)

Each cross-link peak was collected from the first column and rechromatographed on a shorter second column (for details see the Experimental section). The percentage of the total radioactivity for the particular cross-link obtained from the second column was used to quantify the cross-link from the first column. Results are mean values ± S.E.M. for tissues from three animals. The alkaline-hydrolysate studies were made on a different group of animals from the one used for the acid-hydrolysate studies (Table 1), and the specific radioactivities of NaB3H4 used to reduce the preparations from the two groups of animals were different (see the Experimental section).

Table 3. Proportions of non-glycosylated and glucosylgalactosyldehydrodihydroxylysinonorleucine in the aldime and keto forms

NaB3H4-reduced non-glycosylated and glucosylgalactosyldihydroxylysinonorleucine from tissue hydrolysates of three animals at 3 and 6 days were treated with periodate and reduced with NaBH4. The 3H-labelled amino acids were then quantified by cation-exchange column chromatography (Robins & Bailey, 1975) (for details, see the Experimental section).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>10^-4 x Radioactivity (c.p.m.)</th>
<th>[3H]Proline +</th>
<th>Keto form (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-glycosylated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-day</td>
<td>3.78</td>
<td>1.85</td>
<td>49</td>
</tr>
<tr>
<td>6-day</td>
<td>10.0</td>
<td>7.03</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 3. Proportions of non-glycosylated and glucosylgalactosyldehydrodihydroxylysinonorleucine in the aldime and keto forms

<table>
<thead>
<tr>
<th>Tissue</th>
<th>10^-4 x Radioactivity (c.p.m.)</th>
<th>[3H]Proline +</th>
<th>Keto form (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diglycosylated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-day</td>
<td>10.2</td>
<td>5.81</td>
<td>57</td>
</tr>
<tr>
<td>6-day</td>
<td>27.6</td>
<td>15.8</td>
<td>57</td>
</tr>
</tbody>
</table>
shows an increasing amount of keto form as collagen fibres and bundles form and collagen maturation occurs. In contrast, the percentage of keto form in glucosylgalactosyldihydroxylysinonorleucine remains constant.

Discussion

Several observations emerge from these studies relating collagen cross-links to fibre and bundle formation in developing connective tissue. High concentrations of NaB\(^3\)H\(_4\)-reducible hydroxylsinoisonorleucine and histidinohydroxymerodesmosine develop early as connective-tissue forms, and these high concentrations remain essentially unchanged as collagen fibres and bundles develop. Radioactive dihydroxylysinoisonorleucine, on the other hand, present initially in low concentration, increases sharply as collagen fibres and bundles appear. During the 2–6-day period, approx. 45% of the total hydroxylsinoisonorleucine and 80% of the total dihydroxylysinoisonorleucine are glycosylated with glucosylgalactosyl residues. All of the hydroxylsinoisonorleucine is in the aldime form. Increases in the non-glycosylated dihydroxylysinoisonorleucine represent the largest percentage change in the cross-link profile as collagen fibres and bundles appear, and the non-glycosylated dehydrodihydroxylysinoisonorleucine is the only cross-link that undergoes a progressive internal rearrangement from an aldime to a keto form as collagen fibres thicken and bundles develop.

Previously Bailey et al. (1973), using the Ivalon-implant model in rats, found the major cross-link in 1–8-month-old tissue to be \(^{3}H\)dihydroxylysinoisonorleucine, and they related the amount of this cross-link to collagen organization (solubility). In our studies only the capsule surrounding the implant was used, because this tissue represents a more compact and dense collagen than the tissue within the interstices of the sponge (Boucek & Noble, 1955), and the fibroblast is the dominant cell type in the capsule, whereas within the sponge it is the neutrophil (Weiner et al., 1975). Furthermore, the profile of reducible collagen cross-links in our studies was temporally related to fibre and bundle formation, as shown by electron micrographs, and to biochemical indices of collagen accumulation and maturation in developing connective tissue.

There are limitations in the use of the resistance of insoluble polymeric collagen to digestion with bacterial collagenase. But, as pointed out by Steven (1976), the digestion of intermolecularly cross-linked reconstituted collagen by bacterial collagenase may not be directly related to the digestion of insoluble polymeric collagen fibrils, since fibre thickening may influence the interaction between enzyme and substrate. Furthermore differing amounts of protein-bound glycosaminoglycans in the acetic acid-insoluble protein might affect enzyme–substrate interactions and thereby alter the rate of collagen digestion. Nevertheless both the resistance to digestion of polymeric collagen by bacterial collagenase and the collagen content increase between 4 and 6 days in the sponge-implant tissue (Fig. 1), suggesting a maturation of collagen during that interval.

Relating the cross-link profile to collagen metabolism in the Ivalon-capsule tissue during the 2–4-day period shows that amounts of radioactive hydroxylsinoisonorleucine and histidinohydroxymerodesmosine remain constant (Table 1). During this period collagen synthesis and degradation are in near-balance, as judged by the constant tissue-collagen content (Fig. 1). These temporal associations suggest a relationship between hydroxylsinoisonorleucine and histidinohydroxymerodesmosine and the organization of newly synthesized collagen. Furthermore, the similarity of the 9:1:7 ratio for hydroxylsinoisonorleucine/lysinosonorleucine/histidinohydroxymerodesmosine in the collagen of the 2–6-day tissues with that of 8:1:5 for these cross-links reported by Kang et al. (1970) for reconstituted native-type fibrils from soluble chick skin collagen supports this idea.

Dihydroxylysinoisonorleucine concentration, on the other hand, is low initially and increases as fibres thicken and bundles appear. An increased amount of dihydroxylysinoisonorleucine suggests an acceleration of lysyl hydroxylase activity with selectivity for lysine residues involved in the formation of this cross-link. Barnes et al. (1974) found an increased lysyl hydroxylase activity in embryonic tissue selective for the telopeptidyl lysine residue, possibly involved in dehydrodihydroxylysinoisonorleucine formation.

Fibrils of differing thickness are seen by electron microscopy and may reflect the presence of type-III and -I collagens (Wiedemann et al., 1975). Lapiere et al. (1977) reported that the addition of type-III to type-I collagen accelerates the formation of bundles in vitro, with the maximum effect observed with the mixture containing 30% type III. Furthermore, according to Bailey & Sims (1976), both type-I and type-III collagens from embryonic dermis are stabilized by dehydrodihydroxylysinoisonorleucine, although type-I collagen contains greater amounts of dehydrodihydroxylysinoisonorleucine and type-III collagen larger amounts of dehydrodihydroxylysinoisonorleucine. The high and constant amounts of hydroxylsinoisonorleucine from 2 to 6 days could reflect the
synthesis of predominantly type-I collagen, and an accelerated synthesis of type-III collagen after 3 days might explain the increasing amounts of dihydroxylysinoonorleucine. Perhaps when the mixture of type-I and -III collagens becomes optimal for fibril aggregation, i.e. at 5–6 days, collagen bundles rapidly appear.

If type-I and -III collagens are the predominant genotypes in the developing Ivalon capsule in rats, the α1(I), α2 and probably the α1(III) chains would have the bulk of glucosylgalactose on the hydroxylysyl residue 87 of the helical portion of the chains (Fietzek & Kühn, 1976). And the quarter-stagger alignment of collagen molecules in the Hodge–Petruska (1963) model would juxtapose this diglycosylated hydroxylysine near the N-terminal end of the helical region with the C-terminal telopeptide lysine residue of the adjacent polypeptide to accommodate the formation of the Schiff-base cross-link glucosylgalactosydihydroxylysinoonorleucine. The constant concentration of glucosylgalactosydihydroxylysinoonorleucine during the period when collagen synthesis and degradation are in near-balance suggests an involvement of this cross-link in an early stage of collagen organization.

The glucosylgalactosydihydroxylysinoonorleucine cross-link, making up 90% of the total dihydroxylysinoonorleucine of the capsule tissue at 2 days and approx. 75% at 4–6 days, might also involve helical hydroxylysine residue 87. Although the location in the polypeptide of the other hydroxylysine of the cross-link is at present unknown, it may involve the C-terminal telopeptide hydroxylysine, and, if so, would be similar to the glucosylgalactosydihydroxylysinoonorleucine reported by Eyre & Glimcher (1973) and by Henkel et al. (1976) for cartilage, tendon and skin collagens. Also, the steady rate of increase in glucosylgalactosydihydroxylysinoonorleucine from 2 days (about 0.9 g.p.m./μg of collagen/day; Table 2), with approx. 60% in the keto form, might suggest a participation of this intermolecular cross-link in a more stable (Miller & Robertson, 1973) organization of collagen molecules.

Of the reducible collagen cross-links, non-glycosylated radioactive dihydroxylysinoonorleucine undergoes the greatest percentage increase as collagen fibres form and bundles become organized (Table 2). The increase in the amount begins with a 4-fold rise between 2 and 3 days, followed by a doubling of the amount from 3–4 and from 5–6 days. During this period, the cross-link changes to a dominant keto form (from 49% at 3 days to 70% at 6 days) (Table 3), reflecting an internal rearrangement of the compound with the emergence of the more stable form (Miller & Robertson, 1973) as fibres and bundles appear. These features of the non-glycosylated radioactive dihydroxylysinoonorleucine are unique among the collagen cross-links, suggesting the possibility that the non-glycosylated dehydrodihydroxylysinoonorleucine might be a key cross-link in the organization and maturation of collagen fibres and bundles in developing connective tissue in adult male rats.

The near-constant amount of hydroxylysinoonorleucine, the steady rate of increase of glucosylgalactosydihydroxylysinoonorleucine and the stepwise rise in non-glycosylated dihydroxylysinoonorleucine amounts as collagen fibres thicken and bundles form suggest different rates of formation for these intermolecular collagen cross-links. The different ratios of hydroxylysinoonorleucine/glucosylgalactosydihydroxylysinoonorleucine/non-glycosylated dihydroxylysinoonorleucine from 3 to 6 days suggest three post-translational steps involving lysyl-derived cross-links as microfibrils appear, fibres and bundles organize and collagen matures in developing connective tissue.

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