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The Chemistry of the Collagen Cross-Links

AGE-RELATED CHANGES IN THE REDUCIBLE COMPONENTS OF INTACT BOVINE COLLAGEN FIBRES

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The change in the amounts of the three major reducible cross-links was followed throughout the bovine-life span. The major reducible cross-link in embryonic skin is 6,7-dehydro-N^{ε}-(2-hydroxy-5-amino-5-carboxypentyl)hydroxylysine, but this is gradually replaced in the latter stages of gestation or early postnatal growth period by two other Schiff bases, 6,7-dehydro-N^{ε}-(5-amino-5-carboxypentyl)hydroxylysine and a component not yet identified, designated Fraction C. These latter two Schiff bases increase in amount during the rapid growth period to a maximum, after which they then slowly decrease until at maturity they are virtually absent. The proportion of these Schiff bases closely reflects the rate of growth, i.e. the amount of newly synthesized collagen present at any one time. Similarly, the three Schiff bases present in tendon and the one in cartilage slowly decrease during maturation. No evidence for the possible stabilization of these aldimine bonds during maturation by reduction *in vivo* was found by three different analytical techniques. Concurrently with the decrease in the proportion of the Schiff bases some new reducible components increased during maturation, but their characterization as N^{ε}-glycosylamines demonstrated that they were not related to the lysine-derived aldehyde components. The significance of these components in the aging process cannot at present be assessed. As no evidence was obtained for any new reducible cross-links replacing the Schiff bases, it is probable that the latter are intermediate cross-links and that during maturation they are stabilized to some as yet unknown non-reducible cross-link as previously proposed (Bailey, 1968).

Following the suggestion that Schiff-base cross-links might be present in collagen (Fessler & Bailey, 1965) we embarked on a programme to isolate and identify these cross-links after stabilization by reduction. During the past few years significant advances have been made by a number of laboratories in the elucidation of the intermolecular cross-linking of collagen. It is now established that specific lysine (Bornstein & Piez, 1966) or hydroxylysine (Bailey *et al.*, 1969) residues at the *N*-terminal and possibly the *C*-terminal ends (Stark *et al.*, 1971) of the molecules are oxidatively deaminated and that the aldehydes produced then condense either with similar residues to give aldols or with lysine or hydroxylysine residues to give the Schiff-base compounds, dehydrohydroxylysinonorleucine:

$$HO_2C–CH(NH_2)–CH_2–CH_2–CH_2–CH{=}N–CH_2–CH(OH)–CH_2–CH_2–CH(NH_2)–CO_2H$$

(Bailey & Peach, 1968; Tanzer *et al.*, 1970) and dehydrohydroxylysinohydroxynorleucine:

$$HO_2C–CH(NH_2)–CH_2–CH_2–CH(OH)–CH{=}N–CH_2–CH(OH)–CH_2–CH_2–CH(NH_2)–CO_2H$$

(Bailey *et al.*, 1969; Mechanic *et al.*, 1971; Davis & Bailey, 1971).

The aldol products, so far only discovered in tropocollagen as intramolecular bonds, undergo further condensations to form a more complex cross-link not yet identified, designated 'Fraction C' (Bailey *et al.*, 1969) or 'post-histidine peak' (Kang *et al.*, 1970).

With the exception of dehydrodihydroxylysinonorleucine, these Schiff-base compounds are both heat- and acid-labile and therefore can in no way contribute to the observed decrease in solubility of the fibre with age. Moreover, recent studies of the changes with age in these cross-links (Bailey & Shimokomaki, 1971) show that their proportions relative to the total collagen begin to decrease soon after the initial growth spurt so that at the age of maturity these cross-links are virtually absent, thus supporting the original

suggestion that the Schiff-base cross-links act only as intermediates in the formation of the stable collagen fibre (Bailey, 1968). The question then arises as to the nature of the stable forms of cross-link that can account for the physical properties of the mature collagen fibre. One possibility is that the aldimine bonds are reduced *in vivo*. Although this has been shown by direct analysis for the reduced form not to be the case (Bailey & Peach, 1971), up to 25% or 50% reduction *in vivo* in dentine and bone collagen respectively has been inferred from mass-spectral data (Mechanic *et al.*, 1971).

The present paper describes in detail the changes in the reducible components of collagen over the whole life-span from foetus to old age. In addition, further results concerning the possibility of stabilization by reduction of the cross-links *in vivo* are presented in an attempt to clear some confusion which at present exists on this point.

Materials and Methods

Materials

NaB^2H_4 was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. KB^3H_4 (100mCi/mmol) and L-[4,5-^{3}H]lysine monohydrochloride (300mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Materials for Bray's (1960) scintillator solution were supplied by Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K. Aureomycin was obtained from Lederle Laboratories, London, U.K. All other chemicals were of analytical grade and were supplied by British Drug Houses Ltd., Poole, Dorset, U.K.

Methods

Preparation of intact collagen fibres. Skin and achilles tendon were obtained from cattle of various ages from a 16-week foetus to a 15-year-old cow. The tissues were cleaned of adhering fat and muscular tissue, shredded in an MSE Ato-Mix homogenizer and washed with copious amounts of 0.9% NaCl, pH7.4. Samples for comparative analysis of amounts of reducible cross-links were dialysed and freeze-dried.

Articular cartilages were dissected from cattle of various ages, homogenized in a Polytron (Northern Media Supply Co., Hull, U.K.) and then prepared for extraction of collagen by the method described by Miller (1971). Calf bone was similarly homogenized and then decalcified (Miller *et al.*, 1969) before analysis.

Reduction of collagen fibres. Weighed samples of the freeze-dried tissues suspended in 0.9% NaCl (pH7.4) were reduced with KB^3H_4 diluted with non-radioactive KBH_4 to 10mCi/mmol (30:1 weight ratio collagen:KBH_4). For comparison of tissues of different ages, all samples were reduced concurrently, the KB^3H_4 being dissolved in 1mM-NaOH and equal portions (1ml) of the solution being used for the reduction. The reaction was allowed to proceed for 1h, after which acetic acid was added to a final pH of 4; the mixture was then dialysed against several changes (5 litres) of water.

For reduction with NaB^2H_4, the sodium salt was mixed with KB^3H_4 (100mCi/mmol) to approx. 10mCi/mmol and the reduction was carried out 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.

Determination of hydroxyproline. Hydroxyproline analyses for the estimation of the collagen content of tissues were carried out by using the Locarte amino acid analyser, or the method of Stegeman (1958) as adapted for the Technicon autoanalyser (Grant, 1964).

Isolation of cross-links. The acid hydrolysates of tissue containing 5g of collagen were evaporated to dryness, dissolved in water (20ml) and adjusted to pH3 by the addition of 4M-NaOH. This solution was fractionated on a column (16cm^2 × 90cm) of Sephadex G-10 and the higher-molecular-weight fraction, which contained over 90% of the ^{3}H radioactivity in the hydrolysate, was then submitted to ion-exchange chromatography by using the Technicon analyser with pyridine–formate buffers (Bailey *et al.*, 1970). Relevant fractions were re-chromatographed on an extended basic column of the Locarte amino acid analyser. Fractions containing the cross-links were desalted on a short column (0.5cm^2 × 5cm) of Zeo-Karb 225 (H^+ form). The identity of the reduced cross-links was confirmed by comparison with the chromatographic properties of known standards by employing electrophoresis, paper chromatography and the extended basic column of the Beckman amino acid analyser.

Determination of colour yields. From accurately weighed samples of the isolated cross-links obtained from bovine tendon, the colour yields relative to that of leucine (1.00) were determined by using the Beckman amino acid analyser. The values obtained are given in Table 1. The value for Fraction C is based on an approximate molecular weight of 590 determined by vapour-pressure osmometry. The concentrations of the cross-links present in a known weight of collagen were then determined directly in hydrolysates of the reduced tissue by using the analyser (Table 1).

Isotope-dilution method. After hydrolysis of the non-reduced collagen a known amount of ^{3}H-labelled

Table 1. *Colour yields and amounts of the three Schiff-base cross-links present in bovine achilles tendon*

Colour yields are expressed relative to that of leucine (1.00). The number of residues per tropocollagen molecule (mol.wt. 300000) was determined for 18-month-old bovine achilles tendon. For details see the Materials and Methods section.

	Colour yield	No. of residues per tropocollagen molecule
Hydroxylysinonorleucine	1.75	0.78
Dihydroxylysinonorleucine	1.84	0.71
Fraction C	3.5	1.12

cross-link was added and the cross-link re-isolated by the technique described above.

Analysis of non-reduced collagen. Direct analysis for the presence of Schiff bases reduced *in vivo* was carried out by chromatography of acid hydrolysates of weighed amounts of tendon or bone, all of the material eluting between tyrosine and hydroxylysine from the volatile-buffer columns then being analysed on the extended basic column of the Beckman 120C analyser. Equivalent amounts of reduced collagen were analysed under the same conditions and comparisons made of the peak heights at the known positions of the reduced Schiff bases.

Mass spectrometry of deuterated cross-links. For mass-spectral analysis, the isolated compounds were further purified by chromatography on a column (0.4cm^2 × 35cm) of Sephadex G-10 by using 0.5% acetic acid as eluent. Trifluoroacetyl methyl ester derivatives of the borodeuteride-reduced cross-links were prepared as described by Bailey *et al.* (1970). Spectra were recorded on an LKB g.l.c.–mass spectrometer instrument by using the direct-insertion probe.

'Aging' in vitro. Both freshly dissected intact rat tail tendons and carefully purified re-precipitated fibres were incubated in physiological saline (0.9% NaCl, pH7.4, containing 5μg of aureomycin/l) at 37°C. Samples were removed at pre-determined intervals, a portion being analysed for solubility and the remainder being reduced with NaB^3H_4 to determine the reducible cross-links as described previously (Bailey *et al.*, 1970). The solubility was determined by weighing the amount insoluble after gelatinizing a known amount in water at 80°C for 2h.

Results

Changes with age in the reducible aldimine cross-links

The proportions of the three major reducible cross-links are plotted in Fig. 1 on a time-scale to include the period of prenatal growth. The first striking feature is that the major reducible cross-link in foetal skin is dehydrodihydroxylysinonorleucine. During the later stages of gestation and early post-

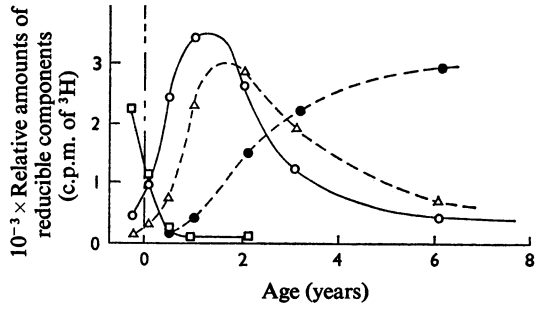


Fig. 1. *Variation in the amounts relative to the total collagen of the major reducible components present in bovine skin with age of the animal*

○, Hydroxylysinonorleucine; □, dihydroxylysinonorleucine; △, Fraction C; ●, Fraction A. The values were obtained by determination of the radioactivity after separation of the components by ion-exchange chromatography with pyridine–formate buffers.

natal growth, the proportion of dehydrodihydroxylysinonorleucine decreases, while there is a corresponding increase in the amounts of the labile cross-links, dehydrohydroxylysinonorleucine and Fraction C.

The second important feature of these changes is that the proportion of the latter Schiff-base cross-links reaches a maximum after approximately the first year of life, corresponding to the period of maximum rate of growth. The amounts of these components relative to the total amount of collagen then decreases until at the age of maturity (4–5 years) they are virtually absent and thereafter remain at a low value.

Fig. 2 shows that the solubility of the tissue at first increases during the rapid postnatal growth period, i.e. during the initial rise in the amounts of the labile reducible cross-links, and then slowly decreases as the latter decrease, thus confirming the fact that the proportions of these components bear some relationship to the solubility. An approximate growth curve

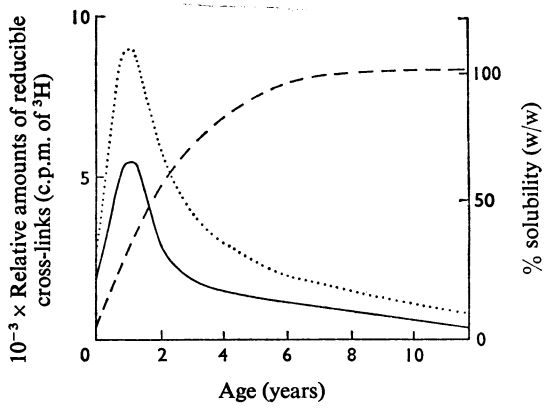


Fig. 2. *Variation in the solubility of bovine skin collagen and in the amounts relative to the total collagen of the reducible cross-links with increasing age*

——, Solubility; ····, reducible cross-links (hydroxylysinonorleucine plus Fraction C); – – – –, growth curve. For experimental details see the Materials and Methods section.

is included in Fig. 2, showing that the maxima in both the amount of Schiff-base cross-links and solubility correspond to the maximum growth rate.

Changes with age in Fractions A

The analyses of aged tissue after reduction with KB^3H_4 showed a marked increase in the amounts of the radioactive components, designated Fractions A1 and A2, which eluted close to tyrosine in the pyridine–formate buffer system (Fig. 3). The variations with age in the total amounts of these components in bovine skin are presented graphically in Fig. 1 in comparison with the results for the aldimine-type cross-links. Similar results were obtained for other animal species and man and for most other types of soft tissue including cartilage, but these components were not present in bone collagen.

Labelling of rat skin collagen with [^{3}H]lysine followed by reduction of the tissue with non-radioactive borohydride indicated that Fractions A were derived from either lysine or hydroxylysine. This fact, together with the observed increase with age in these components in contrast to the age-related changes in the Schiff-base cross-links, initially suggested that these compounds might have some bearing on the stabilization of the tissues. However, recent studies have demonstrated that these components are N^{ε}-glycosylamines and are therefore clearly not involved in the cross-linking process (Robins & Bailey, 1972).

'Aging' in vitro

Both the intact and reprecipitated fibres showed a gradual decrease in the solubility and in the reducible cross-links over a 6-month period (Fig. 4). Hydroxylysinonorleucine tended to decrease faster than Fraction C and both much faster than in aging *in vivo*. During this 'accelerated' aging process *in vitro* it was significant that the Fractions A1 and A2 did not increase with 'age' as the Schiff bases decreased, a feature normally observed with aging *in vivo*, again indicating that they were not involved in the stabilization of the Schiff bases.

Reduction in vivo

To confirm the previous studies (Bailey & Peach, 1971) that the decrease in the amounts of the reducible cross-links was not a result of their reduction *in vivo* several methods have been used.

(*a*) Isotope dilution. With this method, ^{3}H-labelled samples of the reduced cross-links of known specific radioactivity are added to the total hydrolysates of the non-reduced tissues. The cross-links are then isolated chromatographically and their radioactivity is determined. The presence in the hydrolysates of any cross-links reduced *in vivo* resulting in the same final product as borohydride reduction would cause a drastic decrease in the specific radioactivity of the recovered material.

Table 2 shows the specific radioactivities of the cross-links isolated from non-reduced calf tendon and 15-year-old bovine tendon in relation to the radioactivities of these compounds that were added to the hydrolysates. The results for hydroxylysinonorleucine and Fraction C show conclusively that the degree of reduction of these compounds *in vivo* is negligible. Taking the hydroxylysinonorleucine in 15-year-old bovine tendon as an example, the observed decrease in specific radioactivity corresponds to 0.007 μmol of this cross-link derived from the 5 g of non-reduced collagen. From the value for the number of hydroxylysinonorleucine cross-links per tropocollagen molecule given in Table 1, it can be calculated that the apparent degree of reduction *in vivo* is of the order of 0.05%. Further, the values for the old bovine tendon were not significantly different from those for calf tendon, the latter being taken through the procedure as a control. With dihydroxylysinonorleucine it was not possible to determine accurately the specific radioactivity in the 15-year-old tendon, since its presence was masked by other ninhydrin-positive compounds close to its elution position on the amino acid analyser. However, it was calculated that the maximum amount of dihydroxylysinonorleucine present represented an apparent reduction *in vivo* of less than 0.4%. This was supported by analysis of calf tendon, where dihydroxylysinonorleucine can be

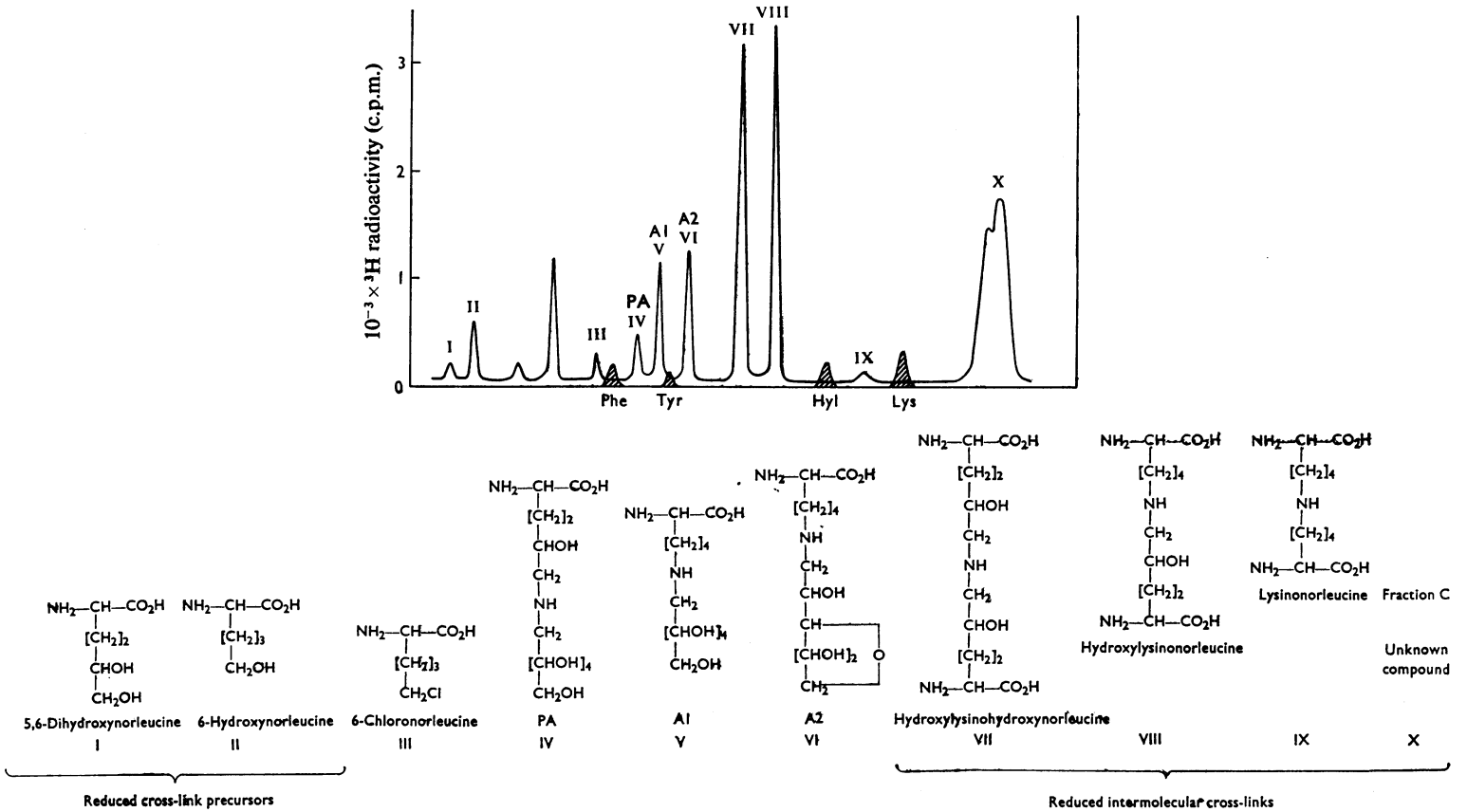


Fig. 3. *Diagrammatic representation of a typical elution chromatograph of a hydrolysate of KB^3H_4-reduced collagen depicting the location of all the reducible components*

The diagram includes the structures of the reduced cross-link precursors, the cross-links, the hexitol–lysine derivatives and one possible structure for their anhydro-derivatives. PA comprises the hexitol–hydroxylysine derivatives.

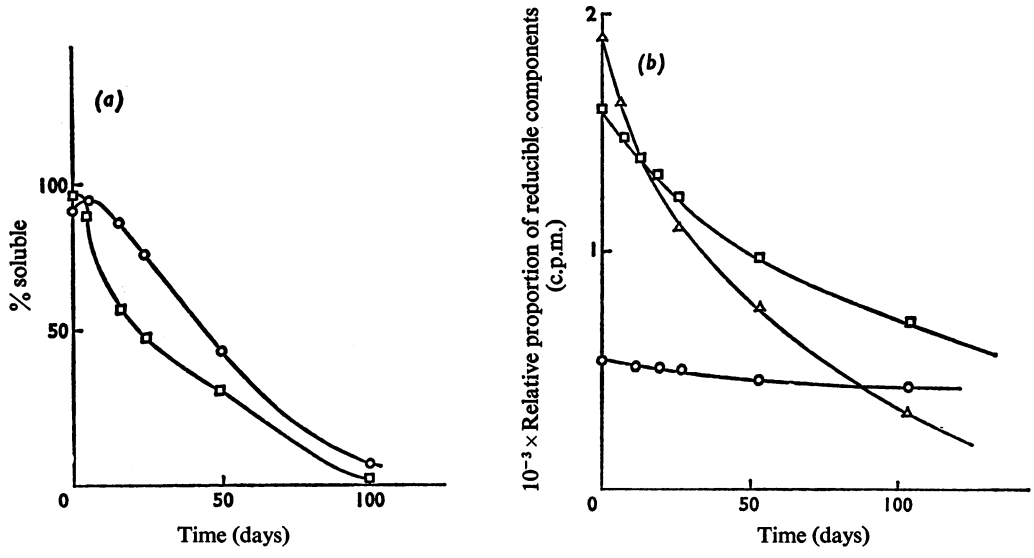


Fig. 4. *Aging of intact and highly purified reprecipitated rat tail tendon fibres in vitro*

(*a*) Solubility (2h, 80°C in water) changes during incubation at 38°C. ○, Intact fibres; □, reprecipitated fibres. (*b*) Changes in the proportion of the reducible borohydride-reduced components during incubation at 38°C. ○, Hexitol lysines; △, hydroxylysinonorleucine; □, Fraction C.

Table 2. *Specific radioactivities of reduced cross-links added to and recovered from hydrolysates of non-borohydride-reduced tendons totalling 5g of collagen*

For experimental details see the Materials and Methods section. The values are not corrected for hydrolysis losses.

Cross-link	Tissue	Amount added (μmol)	10^{-7} × Specific radioactivity (c.p.m./μmol)		Apparent maximum amount reduced (μmol)	Apparent % reduced
			Added	Recovered		
Hydroxylysinonor-leucine	Calf tendon	0.023	9.6	8.1	0.004	0.02
	15-year-old bovine tendon	0.023	9.6	7.3	0.007	0.04
Fraction C*	Calf tendon	0.14	6.1	5.3	0.021	0.03
	15-year-old bovine tendon	0.14	6.1	4.6	0.045	0.07
Hydroxylysino-hydroxynorleucine	Calf tendon	0.048	8.3	7.5	0.005	0.04
	15-year-old bovine tendon	0.048	8.3	4.2	0.047	0.35

* Values for Fraction C are expressed as μmol of leucine equiv.

determined with less interference from contaminants, and again no apparent reduction was recorded. For Fraction C the specific radioactivities are expressed as leucine equivalents, and again less than 0.1% of the form reduced *in vivo* could be detected in 15-year-old tendon.

(*b*) Reduction with NaB^2H_4. The tendons were reduced with NaB^2H_4 and hydroxylysinonorleucine was isolated from the acid hydrolysates. For the 15-year-old tendon, which contains very little of the reducible cross-link, it was necessary to reduce several grams of tissue to obtain sufficient material

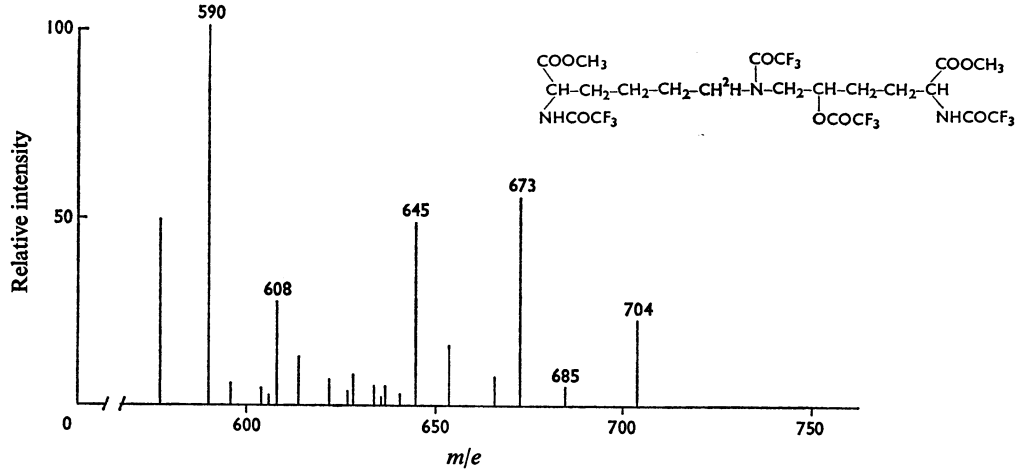


Fig. 5. *Partial mass spectrum of the trifluoroacetyl methyl ester of hydroxylysinonorleucine isolated from bovine tendon reduced with* NaB^2H_4

for analysis. The trifluoroacetylated methyl ester derivatives of the compounds from both calf tendon and 15-year-old bovine tendon gave identical mass spectra (Fig. 5), showing molecular ion peaks only at $m/e = 704$ corresponding to the monodeuterated derivatives. Any material reduced *in vivo* would have given rise to a molecular ion peak at $m/e = 703$, but no such peak was detected.

(*c*) Isolation from non-reduced collagen. After reduction of bovine achilles tendon with KB^3H_4, sufficient material was analysed to give large ninhydrin-positive peaks for the cross-link components on the Beckman analyser. A similar amount of non-reduced collagen was analysed, but no peaks were observed corresponding to any of the three reduced Schiff bases. Reduced calf bone collagen contains a high proportion of hydroxylysinohydroxynorleucine, but no corresponding peak from non-reduced bone was detected.

Analysis of other tissues

In addition to bovine tendon, other tissues were examined for cross-links reduced *in vivo*, either by one of the isotope methods or by the simpler but no-less effective technique of attempting to isolate the reduced cross-links from large quantities of non-reduced tissues by using their known chromatographic behaviour. The tissues examined included skin, intramuscular collagen, cartilage and dentine. In no case was any evidence for a significant degree of reduction *in vivo* obtained.

Discussion

The results presented above show that both the nature and type of the aldehyde-derived cross-links stabilizing the intact collagen fibres change with age of the tissue.

Embryonic collagen

Analysis of embryonic skin has revealed that, in contrast to young postnatal skin, the major reducible cross-link was identical with that found in bone and cartilage, dehydrodihydroxylysinonorleucine (Bailey & Robins, 1972). The proportion of this cross-link decreases during the later gestation period and the early postnatal growth period, to be replaced by the two Schiff bases normally found in young skin (Fig. 1). The presence of this cross-link indicates that hydroxylysine occurs in the *N*-terminal telopeptide of embryonic skin collagen, whereas it is known to be absent from adult tissues (Kang *et al.*, 1967; Rauterberg & Kuhn, 1971). Confirmation that hydroxylysine occurs in the *N*-terminal telopeptide of embryonic skin was provided by Barnes *et al.* (1971). It appears that embryonic skin must be chemically different from adult skin in being highly hydroxylated, and although the effect is most clearly demonstrated in skin it is highly probable that other embryonic tissues such as tendon and bone are similarly highly hydroxylated. Miller *et al.* (1971) suggested that newborn human skin contained, in addition to the normal $(\alpha 1)_2\alpha 2$-type (where $\alpha 1$ and $\alpha 2$ represent the two distinct types of subunit) collagen, a new $(\alpha 1)_3$ type. This highly hydroxylated

$(\alpha 1)_3$ type must be resorbed, or at least diluted out, during the rapid postnatal growth phase by the normal $(\alpha 1)_2\alpha 2$-type collagen. Barnes *et al.* (1971) reported that both the $\alpha 1$- and $\alpha 2$-chains were highly hydroxylated in embryonic tissues; thus it is possible that both the $(\alpha 1)_2\alpha 2$ and $(\alpha 1)_3$ types of collagen are present in embryonic skin and that both are highly hydroxylated. Either type would produce the cross-link observed and its gradual decrease provides a means of following its replacement by the adult-type skin collagen.

Postnatal collagen

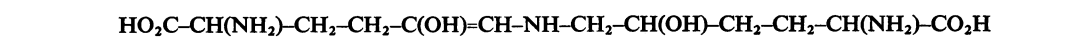
The results shown in Fig. 2 indicate that the reducible cross-links are only present in newly synthesized collagen. The proportion of the cross-links increases rapidly during the postnatal growth period to reach a maximum, which reflects the point at which the fastest rate of growth also occurs, i.e. when the maximum amount of newly synthesized collagen is present. As the growth rate slows down the proportion of reducible cross-links decreases until at maturity, i.e. when growth stops and the epiphyseal plates close, they are virtually absent. The proportion of reducible cross-links then remains at a low value, no further change being observed during senescence.

It is generally agreed that with increasing age the collagen fibre steadily increases in stability to external influences, e.g. thermal denaturation, swelling, solubility and enzymes. All these changes could be accounted for by a gradual increase in the number of covalent cross-links between the peptide chains as originally proposed by Verzár (1964).

It might have been expected that the number of reducible cross-links would remain constant after reaching the maximum, but they subsequently decrease with age. This decrease must therefore be due to some change in the reducible cross-link, since it is clear from the increased stability of collagen that there cannot be a decrease in the number of cross-links. We therefore propose that as the animal matures the labile Schiff bases are replaced by more thermally stable cross-links that are not reducible; the Schiff bases must therefore be considered as intermediate cross-links. This gradual stabilization would account for their decrease with increasing age and also the increased chemical and thermal stability of the bonds reflected in the decrease in solubility and swelling.

Solubility relationship

Clearly the relationship between the Schiff-base cross-links and solubility is not a direct one. The postnatal increase in the solubility of bovine skin (Fig. 2) has also been observed by Carmichael & Lawrie (1967), for sheep skin by Bowes & Raistrick (1965) and for rat skin by Wirtschafter & Bentley (1962). This relative effect can be related to the high proportion of acid-labile cross-links. However, the actual solubility may be more directly related to the proportion of stable cross-links. The rate at which the labile bonds are stabilized may vary considerably between tissues and particularly between species and thus account for the wide variation of solubility between rat, bovine, avian and human skin. On the other hand, cartilage and bone contain the more stable Schiff base, dehydrodihydroxylysinonorleucine, and the relative importance of the aldimine bond and the form stabilized *in vivo* is therefore more difficult to establish. The reason for the increased stability of dehydrodihydroxylysinonorleucine is not yet clear. It is possible that some rearrangement of the structure occurs; one possible such rearrangement could be movement of the double bond to give:



$$HO_2C\text{–}CH(NH_2)\text{–}CH_2\text{–}CH_2\text{–}C(OH)\text{=}CH\text{–}NH\text{–}CH_2\text{–}CH(OH)\text{–}CH_2\text{–}CH_2\text{–}CH(NH_2)\text{–}CO_2H$$

This structure could undergo keto–enol tautomerism and therefore still be reducible by borohydride.

Any attempted correlation of the known cross-links with solubility would at this time be premature; the nature of the thermally stable cross-links, whether they are derived from the Schiff bases or are based on an entirely different mechanism must be elucidated. In addition the contribution of the interaction with glycosaminoglycan to the solubility properties of collagen must also be determined before direct correlations between cross-links and solubility can be made.

Absence of reduction in vivo

The mechanism by which these cross-links are stabilized is not at present known. The most obvious possibility is reduction *in vivo* of the aldimine bond to produce a stable non-reducible cross-link. Direct analysis for the reduced form of the Schiff base in old tissue failed to reveal any detectable quantities (Bailey & Peach, 1971). We have now confirmed by using three different methods that reduction *in vivo* does not take place.

These results are clearly in disagreement with the later studies of Mechanic *et al.* (1971) and Deshmukh & Nimni (1972). The latter workers incubated [^{14}C]-lysine-labelled collagen at 37°C *in vitro* and reported the formation of the reduced forms of the Schiff bases after 4 weeks. Using methods previously adopted for the determination of naturally reduced compounds in elastin (Paz *et al.*, 1970, 1971), Mechanic *et al.* (1971) have inferred from mass spectra

of deuterated dihydroxylysinonorleucine derivatives that 25% of the cross-link reduced *in vivo* is present in dentine and that up to 50% is present in calf bone collagen. The reason for the discrepancies in these findings is not yet clear. It would seem that such a high degree of reduction *in vivo* as proposed by these workers could readily have been confirmed by isolation of the reduced cross-link from non-borohydride-reduced collagen, and thus afford a convenient method of substantiating their claim.

The isotope-dilution method is an extremely sensitive technique and, under the conditions used, any significant degree of reduction *in vivo* would have given results far outside the experimental error in determining the specific radioactivities. This point is emphasized by considering as an example that if the dehydrohydroxylysinonorleucine in bovine tendon were, say, 25% reduced *in vivo*, this would have resulted in a 240-fold decrease in the specific radioactivity, i.e. from 9.6×10^7 to 4×10^5 c.p.m./μmol.

Our results from mass spectrometry with the borodeuteride reduction technique confirmed our previous results in failing to detect any of the cross-link reduced *in vivo* and thus it would appear that the technique is applicable under our experimental conditions. The discrepancy between this result and the mass-spectral studies of Gallop and his co-workers (Mechanic *et al.*, 1971) is difficult to account for, but the possibility of deuterium–hydrogen exchange occurring under different experimental conditions, thus resulting in an overestimate of the extent of reduction *in vivo*, cannot be discounted.

Direct analysis of non-borohydride-reduced collagen for the reduced form of the cross-links failed to reveal any detectable amounts, even in calf bone previously reported to contain 25–50% of the reduced form (Mechanic *et al.*, 1971).

Presence of glycosylamines

Parallel with the decrease in the reducible cross-links it was found that two of the minor components in young tissue, designated Fraction A, increased with age. This was apparent in all the tissues examined except bone and dentine. The characterization of these peaks as condensation products of lysine and hydroxylysine with mannose and glucose (Robins & Bailey, 1972) clearly indicates that these components play no role in the cross-linking process. They can neither constitute cross-links between collagen molecules, nor can they bind collagen to glycoprotein or proteoglycan since in the former case the reducing end group is no longer available, and in the latter case no free reducing end groups are present to facilitate such a reaction. On the other hand, Tanzer *et al.* (1972) reported the partial characterization of a hexitol–hydroxylysine, but their proposal that this type of compound could constitute a cross-link is clearly untenable. Although it is possible that these components are artifacts produced during the preparation of the tissues, the presence of glycosylamine under physiological conditions has previously been demonstrated for normal human haemoglobin (Holmquist & Schroeder, 1966; Bookchin & Gallop, 1968). The possibility that these compounds also exist in collagen cannot be dismissed. The observed increase in the amounts of these glycosylamines with age might indicate a systematic increase in the binding of free hexose, or the reducing end-group of a polysaccharide, to collagen, a fact that would have a considerable effect on the organization and stability of the fibres owing to the change in the charge profile.

Since the glycosylamines are not involved in the cross-linking and no mechanism exists in collagen for reduction *in vivo* of the Schiff base some non-reducible mode of cross-linking must be present. It is at present unknown whether the stable form of the cross-links is derived by modification of the reducible types or whether some completely different form of bonding is involved. If the latter alternative were the case then the function of the reducible cross-links is uncertain, although it could be that an important factor is the reversibility of the formation of aldimine bonds, thus allowing reorganization of the collagen molecules during fibril formation. This may be compared with the reorganization which takes place during the biosynthesis of some globular proteins through disulphide interchange (Givol *et al.*, 1964). However, although aging *in vitro* of normal collagen leads to insolubilization, this does not occur with lathyritic collagen *in vitro* (Gross, 1963), as might be expected if the formation of the stable cross-links involved residues other than the lysine-derived aldehydes. Further, aging of highly purified collagen *in vitro* results in a decrease in the reducible cross-links and a concomitant decrease in solubility, although the process is inhibited in the presence of β-aminopropionitrile (Deshmukh *et al.*, 1971). Thus at the present time the available evidence supports the view that the stable non-reducible cross-links are derived in some way from the reducible intermolecular bonds.

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