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# The Chemistry of the Collagen Cross-Links

## THE CHARACTERIZATION OF FRACTION C, A POSSIBLE ARTIFACT PRODUCED DURING THE REDUCTION OF COLLAGEN FIBRES WITH BOROHYDRIDE

By SIMON P. ROBINS* and ALLEN J. BAILEY†
**Department of Animal Husbandry, University of Bristol, Langford, and*
*†Agricultural Research Council Meat Research Institute, Langford, Bristol, BS18 7DY, U.K.*

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The present paper describes the isolation and identification of a major radioactive component of borotritide-reduced collagen, previously designated Fraction C. The derived structure for the compound confirms that it is identical with the 'post-histidine' component described by Tanzer *et al.* (1973) and given the trivial name histidino-hydroxymerodesmosine. Detailed studies of the effects of acid pH on the formation of Fraction C after borohydride reduction demonstrated the apparent lability of the non-reduced form, thus confirming our previous findings (Bailey & Lister, 1968). Inhibition of the formation of this component by the acid treatment appears to be due to protonation of the histidine imidazole group. Since the only new component formed on reduction of the acid-treated fibres was the reduced aldol condensation product, these results indicate that neither the histidine nor the hydroxylysine residues can be involved in covalent linkage with the aldol condensation product in the native fibre. It is suggested therefore that the proposed non-reduced aldimine form of Fraction C does not exist as an intermolecular cross-link *in vivo*. Thus the presence of histidino-hydroxymerodesmosine as a tetrafunctional cross-link in reduced collagen fibres is a result of a base-catalysed reaction promoted by the borohydride-reduction procedure and this component must therefore be considered as an artifact.

Reduction of collagen fibres with borohydride has provided a technique for investigating the nature of the aldimine-type cross-links (Bailey, 1968). This is partly due to the fact that the cross-links can be radioactively labelled by using $KB^3H_4$ for the reduction but also because these components are thereby rendered stable to the hydrolytic procedures necessary for their isolation.

Thus, of the two major reducible components present in skin, one was identified in its reduced form as 6-*N*-(5-amino-5-carboxy-*n*-pentyl)-5-hydroxy-L-lysine (Bailey & Peach, 1968). The compound is therefore presumably present *in vivo* as the 6,7-dehydro derivative, this aldimine structure being consistent with the observed lability of the component to the action of acids, 2-amino-3-thiols and thermal denaturation (Bailey, 1968; Bailey & Lister, 1968). The other major reducible component in skin, Fraction C, was shown by these earlier studies to be similarly labile in its non-reduced form, a fact that suggested this component is also of an aldimine type. Evidence that the intramolecular aldol condensation product (Bornstein & Piez, 1966) was involved in the formation of this component was provided by Kang *et al.* (1970). Further studies revealed that the compound was a tetrafunctional amino acid derived from the reaction of hydroxylysine and some other amino acid residue with the aldol condensation product (Bailey & Robins, 1972). The stability studies of the non-reduced compound indicated that the aldol condensation product must be bound to the other two residues by labile bonds. However, during the course of these investigations Tanzer *et al.* (1973) proposed a more stable structure for this compound based on mass-spectral and n.m.r. data. The present paper reports on the structure of the reduced compound, Fraction C, and discusses its implications in the light of our results concerning the apparent lability of the non-reduced component.

### Materials and Methods

#### *Materials*

$KB^3H_4$ (100mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Materials for Bray's (1960) scintillation fluid were supplied by Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K. All other chemicals, which were of analytical grade, were supplied by British Drug Houses Ltd., Poole, Dorset, U.K.

#### *Methods*

*Preparation of collagen fibres.* Skin from a 14-month-old calf was cleaned of fat and muscular tissue

and the hair was removed. The tissue was shredded in an MSE Ato-Mix homogenizer and washed with copious amounts of 0.9% NaCl, adjusted to pH7.4 with $NaHCO_3$. Freshly dissected rat tail tendons from 5-month-old animals were washed with 0.9% NaCl, pH7.4.

*Isolation and purification of Fraction C*

The calf skin (approx. 2kg wet wt.) suspended in 0.9% NaCl, pH7.4 (4 litres), was reduced with $KBH_4$ (20g). To the acid hydrolysate of the reduced tissue was added a small amount of $^3H$-labelled Fraction C of high specific radioactivity and the solution submitted to displacement chromatography on cation-exchange columns as described by Bailey *et al.* (1970). The band containing the $^3H$ radioactivity was evaporated to dryness and chromatographed on a column ($16cm^2 \times 90cm$) of Sephadex G-10 with 0.5% acetic acid as eluent. The excluded material from this chromatogram, which contained 95% of the $^3H$ radioactivity, was fractionated by using the Technicon analyser with pyridine–formate buffers (Bailey *et al.*, 1970). As a final purification step, the relevant fractions were pooled and chromatographed on a column ($8cm^2 \times 80cm$) of Bio-Gel P-2 with 0.5% acetic acid as eluent. The $^3H$-containing band was freeze-dried, yielding Fraction C in the form of a white fluffy powder. The compound was found to exhibit a blue phosphorescence of short duration. It migrated as a single component on high-voltage electrophoresis at pH2.0, 6.5 and 8.9. From 2kg wet wt. of calf skin the yield of purified Fraction C was 566mg.

For the preparation of the free base necessary for titration studies, an aqueous solution of the isolated material was adsorbed to a column ($0.5cm^2 \times 5cm$) of Zeo-Karb 225 ($H^+$ form), the column washed with water and then eluted with 0.1M-KOH. This final step was carried out under $N_2$.

*Vapour-pressure osmometry.* The model 301A osmometer (Mechrolab Inc., Calif., U.S.A.) was calibrated with known sugars, amino acids and peptides covering a range of molecular weights from 75 to 595. The calibration constant, $\Delta R/C$ (where $R$ is the resistance in ohms and $C$ is the molar concentration) for solutes containing a single molecular species (e.g. raffinose, sucrose, γ-glutamyl-α-*N*-lysine) was 66.0±2.0 and that for di-ionic solutes (e.g. lysine hydrochloride, NaCl) was 117.5±3.0. Fraction C was dried to constant weight *in vacuo* over $P_2O_5$ at 70°C and aqueous solutions containing from 5 to 30mg/ml were used to determine by extrapolation the value of $\Delta R$ at zero concentration for the calculation of molecular weight.

*High-voltage electrophoresis.* This was carried out on Whatman no. 1 paper by using a water-cooled, flat-bed-type apparatus (Camag, Muttenz, Switzerland). The buffer systems were 0.13M-$(NH_4)_2CO_3$, pH8.9, pyridine–acetic acid–water (25:1:225, by vol.), pH6.5, and 0.75M-formic acid, pH2.0. Endosmotic flow at pH6.5 was measured by reference to Dns-arginine added as a marker.

*Gel filtration.* Gel filtration on Bio-Gel P-2 (200–400 mesh) was performed by using a column ($0.7cm^2 \times 140cm$) with 0.5% acetic acid as eluent at a flow rate of 10ml/h. The column was calibrated with known amino acid and peptide standards.

*Periodate degradation followed by reduction.* To a solution of Fraction C (5mg) in 0.1M-sodium citrate buffer, pH5.5 (3ml) was added a solution (1ml) of $NaIO_4$ (5mg) in the same buffer. After 5min the solution was adjusted to pH7.5 with 4M-NaOH and the products were reduced (and the excess of periodate was destroyed) by the addition of either $KB^3H_4$ or $KBH_4$ (7mg). The reaction was allowed to continue for 30min, after which the solution was adjusted to pH2.2 by the addition of 6M-HCl. This solution was applied directly to the column ($0.8cm^2 \times 26cm$) of the Locarte amino acid analyser and was eluted with a single-column stepwise buffer system from 0.2M-sodium citrate, pH2.90, to 1.0M-sodium citrate, pH6.65. The distribution of $^3H$ radioactivity was measured in portions (0.2ml) of the collected fractions (2ml).

*Mass spectrometry of the reduced aldol.* The trifluoroacetyl methyl ester derivative was prepared as described by Bailey *et al.* (1970). Spectra were recorded on an LKB g.l.c.–mass spectrometer by using the direct-insertion probe.

*Hydrolysis procedures.* Acid hydrolyses were carried out in boiling 6M-HCl for 24h. The HCl was removed by evaporation *in vacuo* at 60°C. Alkaline hydrolyses in 2M-NaOH were performed in polypropylene bottles heated at 108°C for 24h. The hydrolysates were diluted tenfold with water and desalted on a column ($1cm^2 \times 5cm$) of Zeo-Karb 225 ($H^+$ form).

*Reduction of collagen fibres after phosphate treatment.* Rat tail tendons were immersed in 0.5M-$NaH_2PO_4$ (pH4.3) for 2h at room temperature. The fibres were then removed, resuspended in 0.9% NaCl, pH7.4, and immediately reduced with $KB^3H_4$ (collagen/$KB^3H_4$, 30:1, w/w). After 1h, the excess of borohydride was destroyed by the addition of acetic acid to final pH4, and fibres were dialysed against several changes of distilled water (each 1 litre) and freeze-dried.

*Reduction of collagen fibres at different pH values.* Tendon samples were equilibrated in 0.9% NaCl at a series of pH values ranging from 4.3 to 7.9. The pH was adjusted by the addition of 0.1M-HCl or 0.1M-NaOH to stirred suspensions of the fibres and equilibration was judged to be complete when the pH remained constant over a period of 2h. All of the samples were reduced concurrently by the addition

of equal portions (0.5ml) of a solution of $KB^3H_4$ in 1mM-NaOH. This procedure carried out the reduction of the fibres at their equilibrium pH value.

The reduced tendons were dialysed against several changes of distilled water (each 1 litre) and then freeze-dried.

*Analysis of the reduced collagen fibres.* Weighed portions of the freeze-dried materials were hydrolysed and submitted to ion-exchange chromatography by using the Technicon analyser with pyridine–formate buffers (Bailey *et al.*, 1970).

## Results

### *Molecular-weight analysis of Fraction C*

*Vapour-pressure osmometry and pH-titration studies.* Analysis by vapour-pressure osmometry of aqueous solutions of the freeze-dried compound gave apparent molecular weights of 365, assuming that the compound was non-dissociable, and 650 if the compound was present as a fully dissociated salt yielding two ions. Subsequent pH titration of this material showed the presence of groups titrating at pH4.7, which, in view of the isolation procedure, suggested the presence of an acetate salt. On titration of the free base, the relative number of groups titrating at pH4.7 was decreased by approximately one-half. These results indicated that the free base of Fraction C contains a group with p$K$4.7 and that the isolated material was in the form of a monoacetate salt. The calculated value for the molecular weight of the free base was therefore 590.

*High-voltage electrophoresis.* An estimate of the molecular weight of Fraction C was made by comparison of its mobility at pH6.5 with a set of known amino acid standards. The relation (Offord, 1966):

$$m = k\epsilon M^{-\frac{2}{3}}$$

(where $m$ = mobility, $M$ = mass, $\epsilon$ = charge and $k$ = constant) was found to hold even for these relatively low-molecular-weight components. Thus a plot of log $m$ against log $M$ gave a straight line with a gradient of $-2/3$ (Fig. 1). The mobility of Fraction C indicated a molecular weight of approx. 550, assuming that the molecule has a net charge of +1 at pH6.5.

*Gel filtration.* On chromatography of Fraction C on Sephadex G-10, its elution position was indistinguishable from that of components completely excluded from the gel. Chromatography on a calibrated column of Bio-Gel P-2 indicated a molecular weight of approx 1100. This value is considerably higher than the values obtained by other methods, a fact that suggests the compound may chromatograph as a dimer under the conditions employed.

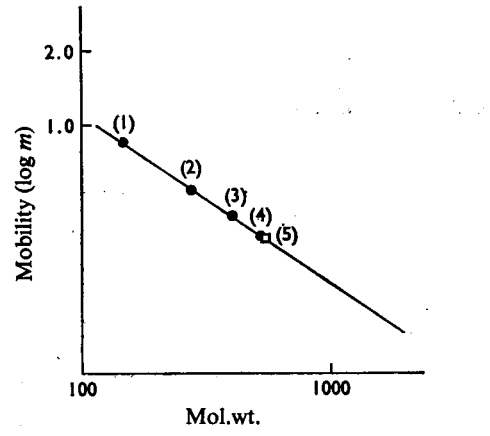



Fig. 1. *Logarithmic plot of the electrophoretic mobilities of some lysine-derived amino acids* (●) *and of Fraction C* (□) *as a function of the known molecular weights of the former compounds*

The mobilities were determined by high-voltage electrophoresis on Whatman no. 1 paper at pH6.5 with reference to those of aspartic acid (1.00) and Dns-arginine (0.00). (1) Lysine, (2) lysinonorleucine, (3) merodesmosine, (4) desmosine, (5) Fraction C.

### *Determination of colour yield*

Chromatography of Fraction C on an extended basic column of the Beckman analyser gave a very broad asymmetric peak, the elution time of which was extremely sensitive to small changes in pH. The colour yield was therefore determined by using the short basic column of the analyser. A value of 3.5 relative to that of leucine (1.00) was obtained, based on the molecular weight of 590 determined by vapour-pressure osmometry. This suggested that the molecule contained four $\alpha$-amino groups.

### *Treatment with acid phosphate buffer before reduction*

The effect was examined of treating rat tail tendons with 0.5M-$NaH_2PO_4$ (pH4.3) at room temperature for 2h before reduction with $KB^3H_4$. Although normally present as a major constituent in the reduced tendons, Fraction C was virtually absent from the phosphate-treated material. The relative proportions of the other reducible components were unaltered except for a large increase in the phosphate-treated samples in the amount of a $^3H$-labelled component eluting before phenylalanine. After isolation of this component from alkaline hydrolysates of phosphate-treated and reduced collagen, the chromatographic and electrophoretic properties were found to be identical with those of the dehydrated aldol isolated from elastin.

*Confirmation of the identity of the reduced aldol.* The identification of the collagen-derived compound was confirmed by mass spectrometry of the trifluoroacetyl methyl ester derivative. A molecular ion at $m/e = 590$ was recorded together with a fragmentation pattern consistent with the structure shown in Fig. 2. This result confirmed the fact that the aldol condensation product is a precursor of Fraction C.

*Periodate degradation followed by reduction*

Fig. 3 shows the products obtained after reaction of the isolated Fraction C with periodate at pH5.5 for 5min followed by reduction with borohydride. After reduction with $KB^3H_4$, the major radioactive products were [$^3$H]proline and $^3$H-labelled material that was slightly more basic than undegraded Fraction C. The $^3$H label in the latter was derived from the original material, since the $^3$H label was present in this component in an amount similar to that present in the sample reduced with non-radioactive $KBH_4$ (Fig. 3).

With periodate reaction times of longer than 5min there was a progressive decrease in the amounts of proline formed after reduction. Instead, a multiplicity of acidic, neutral and basic ninhydrin-positive components were produced, some of which showed absorption maxima at around 250nm. This result was probably due either to over-oxidation by the periodate or to the condensation of the reaction products before reduction.

The formation of proline after periodate treatment and reduction showed that hydroxylysine was involved in the formation of Fraction C. The possible involvement of hydroxyallysine could be eliminated since, if this were the case, a considerable proportion of the $^3$H-radioactivity originally present in the reduced Fraction C would not have been retained in the basic degradation product, but would have been present as $^3$H-labelled methanol. The basic degradation product was not characterized.

*Analysis of Fraction C by $^1$H n.m.r. and mass spectrometry*

The investigations described above established that the compound, Fraction C, was the reduced product of condensations of the $\alpha\beta$-unsaturated aldehyde with hydroxylysine and some other amino acid residue. These facts greatly aided the interpretation of $^1$H n.m.r., $^{13}$C n.m.r. and mass-spectral analyses of Fraction C, the results of which have been reported in detail elsewhere (Hunt & Morris, 1973).

The $^1$H n.m.r. spectrum of Fraction C indicated the presence of an imidazole group and comparisons of the $^{13}$C chemical shifts of $^2H_2O$ solutions of Fraction C, 1-methyl- and 3-methyl-histidine suggested that the imidazole moiety was linked through N-3 rather than N-1. These analyses together with the mass spectra of fluoroacetyl ester derivatives were consistent with the structure (I) for the reduced compound, Fraction C.

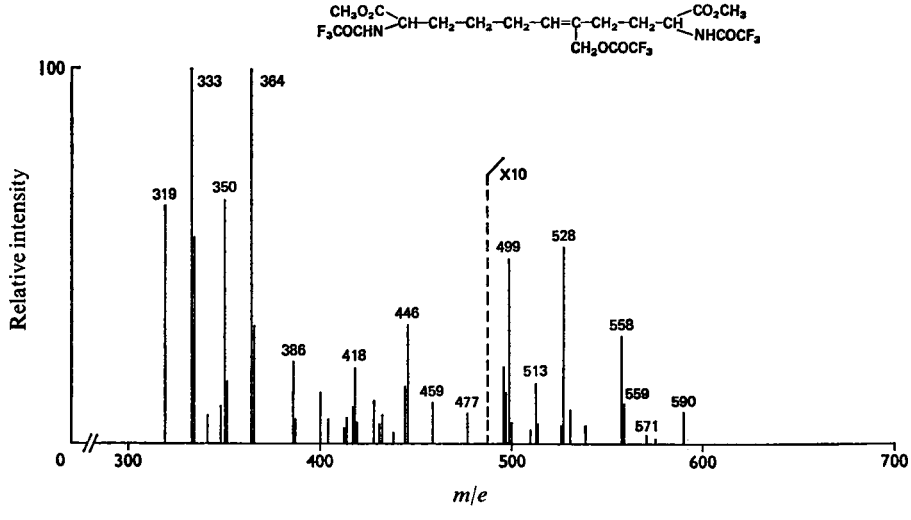



Fig. 2. *Partial mass spectrum of the trifluoroacetyl methyl ester derivative of the reduced aldol isolated from phosphate-treated collagen fibres reduced with $KB^3H_4$*

For details see the Materials and Methods section.

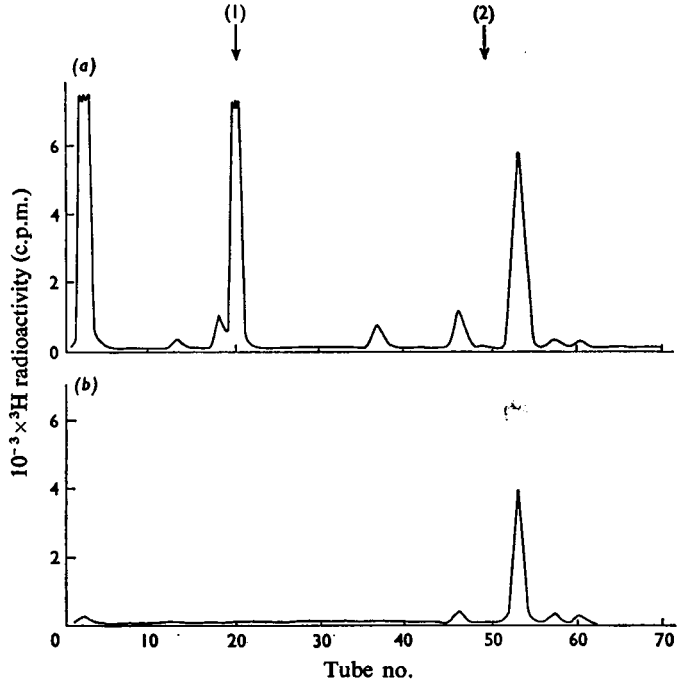
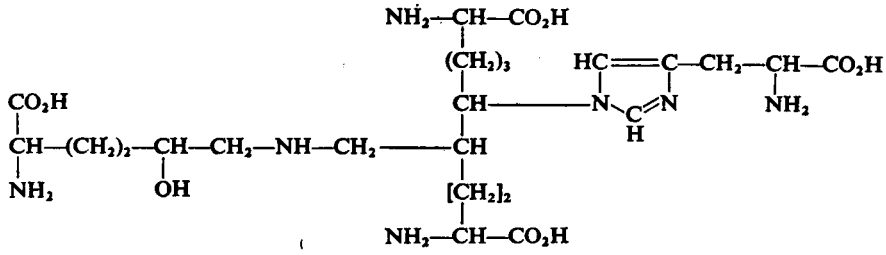



Fig. 3. *Products of periodate treatment followed by reduction with borohydride of the isolated compound, Fraction C*

After the periodate degradation, the products were reduced with either (*a*) $KB^3H_4$ or (*b*) $KBH_4$. The elution positions of proline (1) and Fraction C (2) are indicated. The radioactivity at peak (1) was $29.8 \times 10^3$ c.p.m. For experimental details see the Materials and Methods section.



(I) Histidino-hydroxymerodesmosine

*Apparent lability of the non-reduced form of Fraction C*

Since the formation of Fraction C appears to involve a Michael addition of histidine to the aldol condensation product, the effect of phosphate treatment described above became difficult to interpret. Further studies were therefore carried out to determine the nature and specificity of this effect.

After treatment of collagen fibres with phosphate buffer ($I = 0.5$) at pH7.5 under the conditions used above, analysis of the acid hydrolysate of the reduced material showed that Fraction C was present in the normal amount. To determine the effect of pH on the formation of Fraction C, collagen fibres were equilibrated in 0.9% NaCl solutions at a series of pH values ranging from 4.3 to 7.9. The results of analyses of Fraction C and the reduced aldol after reduction of fibres with $KB^3H_4$ are shown in Fig. 4. Fraction C was determined in acid hydrolysates of the fibres and the reduced aldol was measured after alkaline hydrolysis. The amount of Fraction C formed after reduction decreased sharply below about pH6.5. Concurrently the amount of

Table 1. *Amounts of the $^3H$-labelled cross-links in both acid and alkaline hydrolysates of rat tail tendons reduced with $KB^3H_4$ after equilibration at either pH 7.5 or 4.3*

The tendons were equilibrated in 0.9% NaCl at the required pH for 2h, after which the reductions were carried out with equal portions of a solution of $KB^3H_4$ in 1mM-NaOH and the components isolated as described in the Materials and Methods section.

| Equilibration pH | Hydrolysis procedure | $^3H$ radioactivity (c.p.m./mg of collagen) | | |
|---|---|---|---|---|
| | | Hydroxylysino-norleucine | Reduced aldol | Fraction C |
| 7.5 | Acid | 1873 | – | 3021 |
| | Alkaline | 1360 | 221 | 1543 |
| 4.3 | Acid | 1910 | – | 159 |
| | Alkaline | 1382 | 2711 | 116 |

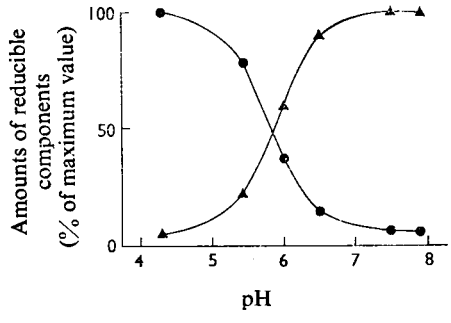



Fig. 4. *Variation with pH of the amounts of Fraction C (▲) and the reduced aldol (●) formed after reduction of collagen fibres with $KB^3H_4$*

Fraction C was determined after acid hydrolysis and the reduced aldol after alkaline hydrolysis of fibres equilibrated in 0.9% NaCl at different pH values before the reduction.

reduced aldol increased, reaching a maximum value at about pH 4.3, at which point Fraction C was virtually absent from the reduced fibres.

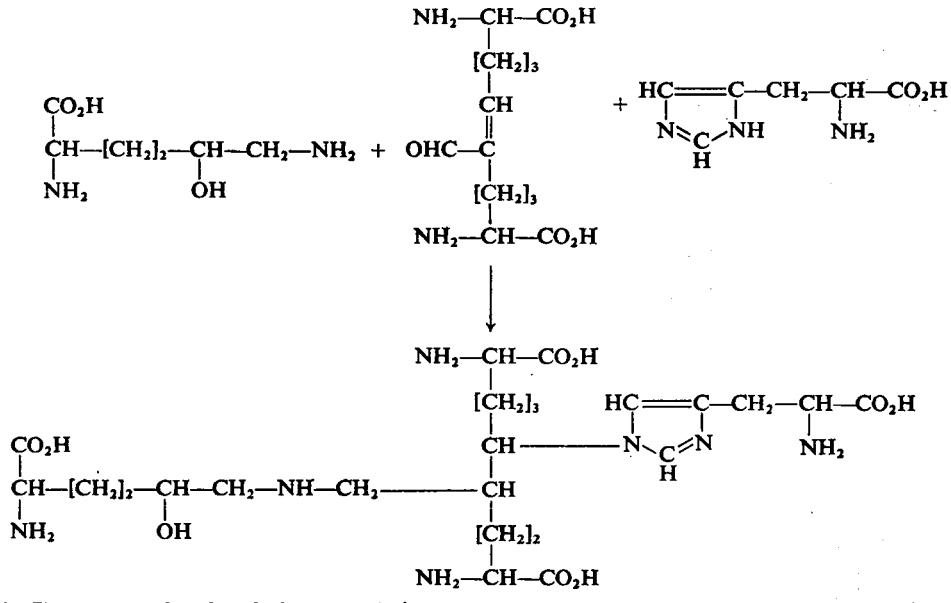
Table 1 gives the quantitative data for the major reducible components in both the acid and alkaline hydrolysates of collagen fibres reduced at pH 7.5 and at 4.3. Both hydroxylysinonorleucine and Fraction C appear to be partially degraded during alkaline hydrolysis, whereas the reduced aldol is stable under these conditions, but is extremely labile to acid hydrolysis. On reduction of the fibres at pH 4.3, the amount of $^3H$ radioactivity recovered as the reduced aldol was approximately equivalent to that recovered as Fraction C after reduction at pH 7.5 (Table 1). This result confirms the precursor relationship of the aldol and indicates that only one $^3H$ atom is incorporated into Fraction C on reduction with $KB^3H_4$. The amounts of $^3H$-labelled hydroxylysinonorleucine obtained after the reductions at pH 7.5 and 4.3 were very similar, showing that the non-reduced, aldimine form of this component is stable within the pH range investigated.

## Discussion

It has been shown that the reduced component, Fraction C, is histidino-hydroxymerodesmosine, thus confirming that the structure of this compound is identical with that of the 'post-histidine' component described by Tanzer *et al.* (1973). The compound appears to be formed by a Michael addition of a histidine residue to the β-carbon atom of the αβ-unsaturated aldol condensation product together with the formation and subsequent reduction by borohydride of an aldimine bond between the aldehyde function of the aldol condensation product and the ε-amino group of a hydroxylysine residue. This component might be expected to exist *in vivo* as the non-reduced aldimine bond form, dehydro-histidino-hydroxymerodesmosine, thus acting as a cross-link potentially linking four chains (Scheme 1).

In view of the labile nature of aldimine bonds, this interpretation would be consistent with the observed lability of non-reduced Fraction C to thermal denaturation and to the action of 2-amino-3-thiols and dilute acids (Bailey *et al.*, 1970). However, the fact that fibres pretreated with the above reagents not only failed to form Fraction C on borohydride reduction but also resulted in the formation of an increased amount of the reduced aldol casts considerable doubt on the existence of the non-reduced form of this component as an intermolecular cross-link *in vivo*.

Treatment of rat tail tendons with acid phosphate buffer has long been known to allow subsequent rapid dissolution in water at pH 7 (Dumitru &



Scheme 1. *Formation of reduced Fraction C from residues of hydroxylysine, the aldol condensation product and histidine*

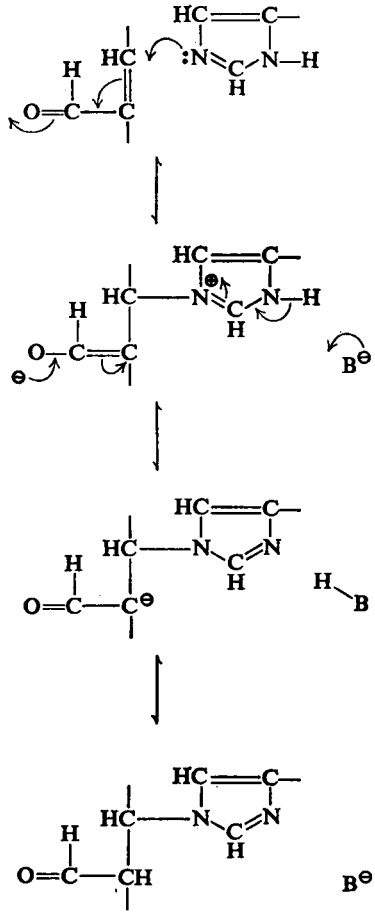
Garrett, 1957). An investigation into the possible effects of this treatment on the intermolecular cross-linking led to the discovery that Fraction C in its non-reduced form appeared to be cleaved by this reagent. However, the presence of Fraction C after treatment of tendons with phosphate at pH 7.5 followed by reduction showed that inhibition of the formation of this component was due to the acid pH of the phosphate. This conclusion was supported by the finding that such inhibition was a function only of pH and was independent of the type of buffer used.

The fact that treatment of the collagen with acid buffer before reduction allowed isolation of the reduced aldol implies that cleavage has occurred of both the aldimine and the Michael addition product. However, unlike the aldimine bond linking the hydroxylysine residue, the Michael adduct forms a covalent bond which is not stabilized on reduction with borohydride but is a stable entity *per se*, as evidenced by the resistance of the histidine–aldol bond to cleavage by acid or base hydrolysis during the isolation of Fraction C. Further evidence for the effect of acid treatment is provided by the observation that reduction of intact or reprecipitated collagen fibres results in the formation of Fraction C whereas reduction of the completely re-dissolved fibres results in the formation not of Fraction C but of a high yield of the reduced aldol. No evidence for the presence of the expected aldol-histidine was obtained. The most likely explanation therefore seems to be that the Michael addition does not occur under physiological conditions but is brought about during the reduction with borohydride.

The reaction must therefore be base catalysed by the borohydride ion possibly proceeding by the mechanism suggested in Scheme 2.

The critical dependence on pH of the formation of the Michael addition product is probably due to inhibition of this reaction by protonation of the imidazole ring. The results shown in Fig. 4 for the variation with pH in the amounts of Fraction C formed could therefore be considered as titration data for the imidazole group of the histidine residue involved, although it should be borne in mind that the actual pH within the fibre may differ from that in solution.

If, as seems likely, the Michael addition of histidine is a result of catalysis by the borohydride ion during reduction, it is probable that the cross-link present *in vivo* is the aldimine, dehydro-hydroxymerodesmosine. However, a significant aspect of these results is that no new components other than the reduced aldol were detected on reduction of fibres previously treated with acid buffers. The aldimine bond between hydroxylysine and the aldol is therefore not present, at least at pH values below 6.5, since reduction of this bond would result in the formation of the stable compound hydroxymerodesmosine. By analogy with the non-hydroxylated derivative isolated from elastin, this component would chromatograph close to hydroxylysine in the pyridine–formate buffer system. One obvious explanation for this result is that the aldimine bond is cleaved by the



Scheme 2. *Suggested mechanism for the base-catalysed Michael addition of the imidazole group of a histidine residue to αβ-unsaturated aldehyde*

treatment with acid buffer. Although aliphatic aldimines are known to vary considerably in their stability, the complete disruption of this bond under such mild conditions is perhaps unlikely, especially as the aldimine dehydro-hydroxylysinonorleucine is entirely unaffected by this treatment. An alternative explanation is that the Michael addition of histidine to the aldol condensation product and the consequent disruption of the delocalized system involving the aldehydic carbonyl group activates the latter for the formation of the aldimine bond with the hydroxylysine residue. Clearly, if this were the case it would suggest that Fraction C is entirely an artifact of the reduction procedure.

The *N*-terminal telopeptide contains neither hydroxylysine nor histidine residues, suggesting that Fraction C is an intermolecular cross-link in reduced collagen. The histidine residues of the α1 chain are located at positions 105 (peptide α1CB5) and 945 (peptide α1CB6) along the chain (K. Kuhn, personal communication). At both locations these residues occur in the sequence — Hyl-Gly-His-Arg— (Butler, 1970; van der Mark *et al.*, 1970), and the hydroxylysine residue in the peptide α1CB5 sequence is *O*-glycosidically linked with the galactose-glucose disaccharide (Butler, 1970). It is noteworthy that the peptide α1CB6 sequence is at a position where the *N*-terminus of an adjacent molecule would be located assuming an overlap and quarter-stagger alignment of molecules (Hodge & Petruska, 1963) in the fibre (Fig. 5). Thus it is conceivable that the proximity of the hydroxylysine and histidine residues in the linear sequence sterically favours their reaction with the intramolecular aldol during the reduction with borohydride, although the possibility cannot be ruled out that four different chains are involved in the formation of Fraction C. Further, the histidine-containing sequence in peptide α1CB5 would be situated adjacent to the *C*-terminus of a neighbouring molecule (Fig. 5) and, although the presence of the aldol condensation product at this location is not proven, the *C*-terminal telopeptide does contain an allysine residue (Stark *et al.*, 1971) and cross-linking at this site is therefore feasible. It is possible that the histidine residues involved are activated; such histidines are a common feature of some enzymes, e.g. ribonuclease and aldolase, and in collagen these particular histidines may play a role in the binding of the transferases involved in the glycosylation of the nearby hydroxylysine. The presence of carbohydrate attached to this hydroxylysine moiety of the cross-links has recently been demonstrated (S. P. Robins & A. J. Bailey, unpublished work).

Any disruption of the precise three-dimensional arrangement of molecules in the fibre is likely to inhibit the formation of Fraction C on reduction. Indeed, freeze-drying and subsequent re-hydration of the tissue before reduction was found to cause an approx. 50% decrease in the amount of this component. Fairweather *et al.* (1972) have isolated from calf skin relatively small amounts of a component identified as aldol-histidine. The contention of these workers that this compound constitutes a cross-link *in vivo* is, however, subject to the same criticism as that for the Fraction C or 'post-histidine' component. As this compound was isolated from tissue which before reduction had been exhaustively extracted with acid, freeze-dried and then re-hydrated, the formation of aldol-histidine could be due to the absence of a sterically viable hydroxylysine residue. By similar reasoning, it is conceivable that in this treated tissue small amounts of hydroxymerodesmosine could be formed under the conditions of reduction.

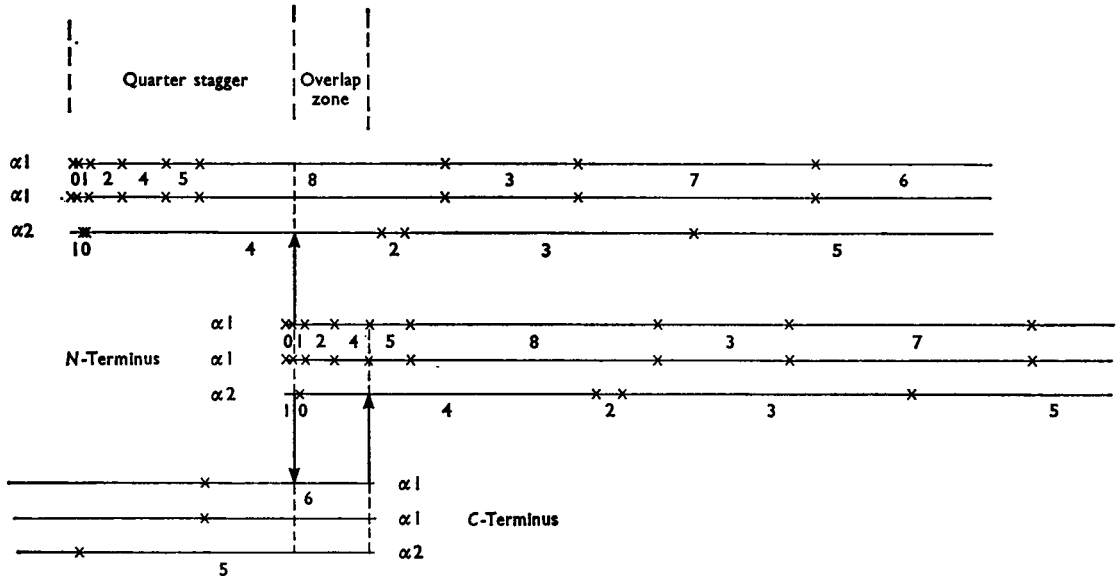



Fig. 5. *Schematic representation of collagen molecules in quarter-stagger alignment showing the possible location of cross-links*

× indicates points of cleavage by CNBr into the numbered peptides. Arrows indicate possible intermolecular cross-link positions.

The fact that the amounts of Fraction C obtained after reduction decreases with the age of tissue (Bailey & Shimokomaki, 1971) indicates that the intramolecular aldol cross-link does undergo further reaction during maturation of the fibre. A decrease in the amount of the intramolecular aldol has also been demonstrated during the aging of collagen *in vitro* (Deshmukh *et al.*, 1971). At present, we have no knowledge of the maturation processes, although formation of an intermediate and subsequent reduction *in vivo* to give histidino-hydroxymerodesmosine certainly does not occur (Robins *et al.*, 1973).

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