The Chemistry of the Collagen Cross-Links

THE MECHANISM OF STABILIZATION OF THE REDUCIBLE INTERMEDIATE CROSS-LINKS

By SIMON P. ROBINS and ALLEN J. BAILEY

*Department of Animal Husbandry, University of Bristol, Langford, and
A.R.C. Meat Research Institute, Langford, Bristol BS18 7DY, U.K.*

(Received 28 February 1975)

The periodate-degradation technique was used to demonstrate the mechanism by which the reducible cross-links of collagen are stabilized. In all the tissues examined, Smith degradations of the ^3H -labelled cross-links indicated that dihydroxylysinoxonorleucine is derived solely from hydroxylysino-5-oxonorleucine, the Amadori-rearranged product of the original condensation reaction. Monohydroxylysinoxonorleucine exists in both keto and aldimine forms, the former being derived from hydroxyallysine and the latter from allysine. Their relative proportions are tissue-dependent and are related to the degree of hydroxylation of the specific lysine residues in both the telopeptides and the triple helix.

Newly synthesized collagen is stabilized *in vivo* by two intermolecular lysine-derived cross-links, dehydrodihydroxylysinoxonorleucine and dehydrodihydroxylysinoxonorleucine (Bailey *et al.*, 1974). Although both of these compounds were initially thought to be aldimines, the stability of the non-reduced form of dihydroxylysinoxonorleucine to the action of dilute acids and thermal denaturation is inconsistent with its existence in an aldimine form (Bailey *et al.*, 1970).

Following the suggestion that the aldimine form of dehydrodihydroxylysinoxonorleucine might undergo an Amadori rearrangement *in vivo* (Robins *et al.*, 1973), periodate-degradation and mass-spectral data on the products of the cross-link isolated from borohydride-reduced bone collagen showed that at least 86% of the cross-link was present as the rearranged product, hydroxylysino-5-oxonorleucine (Robins & Bailey, 1973). Independently, periodate-degradation studies on cross-linked peptides isolated from bone (Eyre & Glimcher, 1973) and cartilage (Miller & Robertson, 1973) have demonstrated the existence of keto forms of the cross-link. From periodate-degradation studies of the reduced cross-link, Davis (1973) concluded that less than one-third of dehydrodihydroxylysinoxonorleucine was present as the keto form in bone collagen, and a cyclic hydrogen-bonded structure was proposed to account for the stability of the cross-link.

Mechanic (1974) has used reduction with sodium cyanoborohydride at pH 3.1 to demonstrate the presence of acid-stable forms of both dehydrodihydroxylysinoxonorleucine and dehydrohydroxylysinoxonorleucine in bone and dentine. Using a more indirect method, Mechanic *et al.* (1974) determined high proportions of the stable form of the cross-links by acetylation of the collagen under acid conditions

before borohydride reduction: their stability was attributed to the presence of keto-amine forms of the cross-links.

The present paper describes determinations of the amounts of the keto forms of cross-links in various collagens by using the periodate-degradation technique. The results demonstrate that the mechanism of stabilization of the cross-links occurs through an Amadori rearrangement, which is essentially complete for dihydroxylysinoxonorleucine but shows considerable tissue variation for hydroxylysinoxonorleucine, depending primarily on the proportion of the cross-link derived from hydroxyallysine.

Materials and Methods

Materials

KB^3H_4 (270 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Materials for Bray's (1960) scintillator fluid were supplied by Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K. Zeo-Karb 225 (14–52 mesh size), as supplied by BDH, Poole, Dorset, U.K., was washed successively with 2M-NaOH and 2M-HCl before use.

Preparation of intact collagen fibres and reduction with KB^3H_4

Bovine collagen fibres from skin of 16-week foetuses and from skin and Achilles tendon of 3-month-old animals were prepared as described previously (Robins *et al.*, 1973). Calf bone was demineralized with 0.1 M-EDTA for 48 h at 5°C. After reduction with KB^3H_4 (Bailey *et al.*, 1970), the samples were dialysed against water (5 litres) and freeze-dried.

Isolation of cross-links

Initial separations of the reduced cross-links were carried out by using a Technicon analyser with pyridine-formate buffers (Bailey *et al.*, 1970). Purification was performed by rechromatography of the appropriate fractions on an extended basic column of either a Locarte or Beckman analyser.

Periodate degradation of cross-links

(i) *Reaction time-course studies.* To solutions of the reduced cross-links in 0.1 M-sodium citrate buffer, pH 5.3, was added an aqueous solution of NaIO_4 to a final concentration of 0.01 M, and the reaction was allowed to proceed in the dark at 20°C. At various time-intervals portions (0.2 ml) of the reaction mixture were removed and immediately applied to a column (0.25 cm² × 5 cm) of Zeo-Karb 225 (H^+ form). The column was eluted with water and then with 2 M- NH_3 , and the ^3H radioactivities in the aqueous and ammoniacal solutions were measured to determine the proportion of the ^3H label initially adsorbed on the ion-exchanger. After evaporation to dryness *in vacuo* at 40°C, the material eluted with NH_3 was analysed on the extended basic column of the Locarte analyser to determine the amount of undegraded cross-link present. This was confirmed by direct analysis of portions of the original periodate reaction mixture.

(ii) *Isolation of products after periodate degradation followed by reduction (Smith degradation).* The cross-link in citrate buffer, pH 5.3 (1 ml), was treated with 0.01 M- NaIO_4 at room temperature. After 5 min the pH was adjusted to 7.5 with 2 M- NaOH , and KBH_4 (2.5 mg) was added both to reduce the reaction products and destroy the excess of periodate. The reaction was continued for 30 min, at which time the solution was adjusted to pH 2 by the addition of 6 M-HCl. Portions of this solution were chromatographed on the Locarte analyser and the amounts of [^3H]proline or [^3H]lysine determined.

Hydrolysis

Acid hydrolyses were done in constant-boiling HCl under reflux for 24 h, and the HCl was removed by evaporation *in vacuo* at 60°C. Alkaline hydrolyses in 2 M- NaOH were carried out in polypropylene bottles at 108°C for 24 h. The hydrolysates were diluted with water and desalted on a column (1 cm² × 20 cm) of Zeo-Karb 225 (H^+ form).

Results

Reaction of the cross-links with periodate

The action of periodate on dihydroxylysino-norleucine purified from an acid hydrolysate of KB^3H_4 -reduced collagen resulted in an increase with time of

reaction in the ^3H radioactivity that was eluted with water from the Zeo-Karb 225 ion-exchange column. After 5 min reaction, over 90% of the ^3H was adsorbed and could not be eluted with water, but could subsequently be eluted with NH_3 . Analysis of the ammoniacal eluate showed that 98% of the cross-link had been degraded, and this was confirmed by direct analysis of a second portion of the periodate reaction solution. With increasing time of reaction with periodate, the proportion of the radioactivity adsorbed to the ion-exchanger decreased, until after 2 h reaction all of the ^3H was eluted from the column with water. Repeated evaporation to dryness of this aqueous solution *in vacuo* at 40°C revealed that less than 10% of the radioactivity was volatile under these conditions.

Amino acid analysis of the products of periodate-degraded dihydroxylysino-norleucine reduced with KBH_4 showed a similar decrease with time of periodate reaction in the yields of [^3H]proline and [^3H]hydroxynorvaline. Thus after 5 min almost 90% recovery was obtained, whereas after 1 h reaction with periodate only 5% of the theoretical yield was obtained.

The initial degradation of dihydroxylysino-norleucine with periodate is rapid, being virtually complete within 5 min. Thereafter, some further reaction occurs, resulting in the loss of the α -amino group and consequent non-absorption on the Zeo-Karb ion-exchange column. A similar pattern of over-oxidation leading to a decrease in the yields of the expected products was experienced not only with monohydroxylysino-norleucine but also with all other lysine- and hydroxylysine-derived compounds examined, including histidinohydroxymerodesmosine. In the light of these results, a periodate reaction time of 5 min was used in all studies of the proportions of keto cross-links.

Proportion of keto form of cross-links in various collagens

After periodate degradation followed by reduction with KBH_4 of isolated ^3H -labelled dihydroxylysino-norleucine, the amount of radioactivity recovered as [^3H]proline and [^3H]hydroxynorvaline is a measure of the proportion of cross-link in the Amadori-rearranged form in the fibre, since degradation of the aldimine-derived cross-link would result in the formation of [^3H]methanol (Robins & Bailey, 1973). Dehydromonohydroxylysino-norleucine may be derived either by condensation of allysine with hydroxylysine or by reaction of hydroxyallysine with lysine (Scheme 1). The latter form could undergo an Amadori rearrangement, giving rise to a presumably more stable cross-link, lysino-5-oxonorleucine. After isolation of the cross-link from KB^3H_4 -reduced collagen, the two forms may be distinguished by their

Scheme 1. Products of Smith degradation of [^3H]hydroxylysinonorleucine derived from either hydroxyallysine or allysine
* and ‡ denote ^3H .

Table 1. Proportions of bovine collagen cross-links in aldimine and keto forms

The ^3H -labelled cross-links were treated with periodate and reduced with KBH_4 , and the proportions of aldimine and keto forms were calculated from the recoveries of the ^3H -labelled amino acids. For experimental details see the Materials and Methods section.

	Monohydroxylysinonorleucine					Dihydroxylysinonorleucine		
	$10^{-5} \times \text{Radioactivity (c.p.m.)}$					$10^{-4} \times \text{Radioactivity (c.p.m.)}$		
	Initial ^3H	[^3H]Lysine	[^3H]Proline+ [^3H]hydroxy- norvaline	Aldimine form (%)	Keto form (%)	Initial ^3H	[^3H]Proline+ [^3H]hydroxy- norvaline	Keto form (%)
Dermis	17.02	14.41	0.97	85	5.7			
Achilles tendon	12.41	11.30	0.59	91	4.8	5.85	5.23	89
Bone	13.86	3.68	9.26	27	67	12.37	10.63	86
Cartilage	2.34	0.32	1.79	14	76	20.42	18.62	91
Embryonic skin	1.11	0.97	0.09	88	8.3	9.50	8.25	87

products of the Smith degradation: the aldimine-derived form would yield [^3H]lysine, whereas the degradation products of the keto-derived cross-link would be [^3H]proline and [^3H]hydroxynorvaline (Scheme 1).

The results of these Smith degradations of KB^3H_4 -reduced cross-links isolated from various tissues are shown in Table 1. The proportions of the cross-links in the aldimine and keto forms are calculated from

the actual recoveries of ^3H -labelled amino acids and therefore represent minimal values.

Discussion

Analysis of the products of periodate degradation has confirmed that the aldimine cross-links derived from hydroxyallysine are stabilized by a mechanism involving the Amadori rearrangement (Scheme 1) as

originally proposed (Robins & Bailey, 1973). Quantification of the proline yield permits determination of the extent of the rearrangement. The major source of error with the periodate technique is over-oxidation, and in the later studies the reaction time was restricted to 5 min (Robins & Bailey, 1973).

Our present detailed analyses have shown that the reaction time is indeed critical. Under the conditions used complete cleavage of the theoretically susceptible bonds occurs within 5 min, but a slower secondary reaction also takes place, resulting in the loss of the α -NH₂ group as judged by its non-adsorption on a sulphonic acid ion-exchange resin. In a study of the reaction of amino compounds with periodate, Clamp & Hough (1966) found that oxidation of amino acids occurs according to the general equation:

and a mechanism was proposed involving electrophilic attack at the amino group by IO₃⁺. This mechanism provides a probable explanation for the observed results of over-oxidation of the cross-links. The demonstration that inhibition of the decarboxylation reaction occurs after esterification of the carboxyl group (Clamp & Hough, 1966) suggests the possible use of this form of modification as a prelude to structural investigations of amino acid derivatives by using periodate.

The conditions of periodate degradation of dihydroxylysinoxonorleucine used by Davis (1973) were similar to those described in the Materials and Methods section, except that the reaction time was 2 h. Over-oxidation of the initial reaction products to aliphatic aldehydes probably contributed to erroneously high values for 'volatile' ³H, thus vitiating Davis's (1973) conclusions about the proportions of aldimine and keto forms of cross-link. These results were based on the assumptions that the volatile radioactivity was solely [³H]HCHO derived from the aldimine form and that the non-volatile residue contained [³H]proline derived from the keto form.

However, with a 5 min reaction time the oxidation is complete and the extent of the over-oxidation is only about 8%, which largely accounts for the incomplete recoveries of ³H from the degraded cross-links as labelled amino acids. It should be emphasized that in the present study, where the periodate-degradation products were fully characterized, determination of the ratio between aldimine- and keto-derived cross-links is unaffected, since the reduced forms are equally susceptible to the over-oxidation reaction. The technique therefore provides an accurate assessment of the proportion of the cross-links in the keto form.

Dihydroxylysinoxonorleucine

In all of the tissues examined in the present study, the proportion of dihydroxylysinoxonorleucine origin-

ally present in the keto form was calculated to be about 90% (Table 1). Taking into account the experimental error, these results indicate that conversion *in vivo* of the aldimine form into hydroxylysino-5-oxonorleucine is complete, thus endorsing our previous observations for bone collagen (Robins & Bailey, 1973). The fact that no volatile ³H-labelled components were detected after periodate degradation confirms that no aldimine-derived form of this cross-link is present in the tissues examined. These results suggest that the Amadori rearrangement is probably spontaneous and complete in all cases.

Hydroxylysinoxonorleucine

Previous periodate-degradation studies of hydroxylysinoxonorleucine isolated from rat skin and tail tendon (Bailey & Peach, 1968) have confirmed its existence *in vivo* as an aldimine bond. In other tissues the telopeptide lysine residue is hydroxylated to a significant extent, and the possibility arises that the cross-link may also be formed by the condensation of hydroxyallysine with lysine (Scheme 1). Only the latter form of the cross-link is capable of undergoing an Amadori rearrangement, and thus, if both forms exist together in a tissue, periodate oxidation provides a direct method for the determination of their relative proportions.

The analyses of monohydroxylysinoxonorleucine in bone collagen showed that almost 70% was present as lysino-5-oxonorleucine before reduction, thus contributing to the stability of this tissue. By contrast, in bovine skin and tendon only about 5% of this cross-link was present in the keto form (Table 1).

Although there is some evidence of lysine-derived aldehydes in the helical part of the molecule (Deshmukh & Nimni, 1971; Kuboki *et al.*, 1973), it is generally accepted, on the basis of amino acid-sequence data (Hulmes *et al.*, 1973) and isolated cross-linked CNBr peptides (Kang, 1972; Miller, 1971), that the aldehydes are confined to the non-helical terminal regions. Thus the formation of lysino-5-oxonorleucine depends on the presence of available hydroxyallysine in the non-helical region and lysine in the helical portion of an adjacent molecule. The extent of hydroxylation of the non-helical lysine residues has been shown to vary considerably, from about 50% in cartilage and bone, about 25% in tendon, and very low in skin (Barnes *et al.*, 1971a,b; Miller *et al.*, 1969). It might be expected therefore that a correlation would exist between the degree of hydroxylation and the proportion of dehydrohydroxylysinoxonorleucine in the keto form. However, no such correlation exists because the proportion of the keto form also depends on the availability of a lysine residue in the helix, and consequently on the extent of hydroxylation of these residues. For example, in bone the telopeptide

lysine residue is extensively hydroxylated, whereas specific lysine residues that are present in the triple helix apparently are not completely hydroxylated, leading to a high proportion of the keto form. A similar situation obtains for cartilage collagen, despite the higher overall extent of hydroxylation in this tissue (Miller & Lunde, 1973). In tendon, although a significant proportion of the telopeptide lysine is hydroxylated (Barnes *et al.*, 1974), lysine residues in the helix are apparently not available, resulting in the formation predominantly of the aldimine cross-link, dehydrohydroxylysino-norleucine. In skin, the low hydroxylation of the telopeptide lysine residue precludes formation of a significant proportion of the keto form. On the other hand, the telopeptide lysine residue of embryonic skin is extensively hydroxylated, yet the cross-link is mainly in the aldimine form, indicating almost complete hydroxylation of the specific lysine residues in the helix.

Both these cross-links are partially glycosylated on the hydroxylysine moiety in the helix. Since lysino-5-oxonorleucine clearly cannot form a glycosylated derivative, its presence in bone collagen is consistent with the previous observation of a lower degree of glycosylation of monohydroxylysino-norleucine relative to the dihydroxylated derivative (Robins & Bailey, 1974). The fact that carbohydrate derivatives of this cross-link are present to a small extent provides additional evidence that monohydroxylysino-norleucine in bone collagen also exists partially in the aldimine-bond form.

The Amadori rearrangement is known to be an acid-base-catalysed reaction (Gottschalk, 1966). Thus studies using extremes of pH cannot constitute direct evidence for the existence of keto-imine forms of the cross-links, and the results obtained by sodium cyanoborohydride reduction at pH 3.1 (Mechanic, 1974) or acetylation of aldimines cleaved at low pH (Mechanic *et al.*, 1974) may therefore be equivocal. Clearly, if the Amadori rearrangement were already complete *in vivo* as in the case of dehydrodihydroxylysino-norleucine, then similar results to our direct periodate method would be obtained. However, for dehydrohydroxylysino-norleucine in bone collagen Mechanic *et al.* (1974) concluded that this cross-link is completely in the keto form *in vivo* compared with the 2:1 ratio of keto to aldimine form determined in the present study.

The results demonstrate therefore that dehydrodihydroxylysino-norleucine exists *in vivo* solely as hydroxylysino-5-oxonorleucine. On the other hand, dehydromonohydroxylysino-norleucine may be present in either an aldimine or a keto form (Scheme

1), depending on whether it is derived from allysine or hydroxyallysine respectively. The availability of the lysine in the helical region varies from tissue to tissue and there does not appear to be a correlation with the overall content of hydroxylysine. It is possible that hydroxylation of the telopeptide lysine residue is independent of the lysine residues in the triple helix, and may even involve a separate enzyme.

We thank Professor T. K. Ewer and Dr. S. M. Partridge for their continued interest. This work was supported in part by the Nuffield Foundation.

References

- Bailey, A. J. & Peach, C. M. (1968) *Biochem. Biophys. Res. Commun.* **33**, 812–819
- Bailey, A. J., Peach, C. M. & Fowler, L. J. (1970) *Biochem. J.* **117**, 819–831
- Bailey, A. J., Robins, S. P. & Balian, G. (1974) *Nature (London)* **251**, 105–109
- Barnes, M. J., Constable, B. J., Morton, C. F. & Kodicek, E. (1971a) *Biochem. J.* **125**, 433–437
- Barnes, M. J., Constable, B. J., Morton, C. F. & Kodicek, E. (1971b) *Biochem. J.* **125**, 925–928
- Barnes, M. J., Constable, B. J., Morton, L. F. & Royce, P. M. (1974) *Biochem. J.* **139**, 461–468
- Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285
- Clamp, J. R. & Hough, L. (1966) *Biochem. J.* **101**, 120–126
- Davis, N. R. (1973) *Biochem. Biophys. Res. Commun.* **54**, 914–922
- Deshmukh, K. & Nimni, M. E. (1971) *Biochem. Biophys. Res. Commun.* **43**, 416–422
- Eyre, D. R. & Glimcher, M. J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 663–671
- Gottschalk, A. (ed.) (1966) *Glycoproteins*, vol. 5, pp. 98–99, Elsevier Publishing Co., Amsterdam
- Hulmes, D. J. S., Miller, A., Parry, D. A. D., Piez, K. A. & Woodhead-Galloway, J. (1973) *J. Mol. Biol.* **79**, 137–148
- Kang, A. H. (1972) *Biochemistry* **11**, 1828–1835
- Kuboki, Y., Tanzer, M. L. & Mechanic, G. L. (1973) *Arch. Biochem. Biophys.* **158**, 106–115
- Mechanic, G. L. (1974) *Biochem. Biophys. Res. Commun.* **56**, 923–927
- Mechanic, G. L., Kuboki, Y., Shimokawa, H., Nakamoto, K., Sasaki, S. & Kawanishi, Y. (1974) *Biochem. Biophys. Res. Commun.* **60**, 756–763
- Miller, E. J. (1971) *Biochem. Biophys. Res. Commun.* **45**, 444–451
- Miller, E. J. & Lunde, L. G. (1973) *Biochemistry* **12**, 3153–3159
- Miller, E. J. & Robertson, P. B. (1973) *Biochem. Biophys. Res. Commun.* **54**, 432–439
- Miller, E. J., Lane, J. M. & Piez, K. A. (1969) *Biochemistry* **8**, 30–39
- Robins, S. P. & Bailey, A. J. (1974) *FEBS Lett.* **33**, 167–171
- Robins, S. P., Shimokomaki, M. & Bailey, A. J. (1973) *Biochem. J.* **131**, 771–780