Biochem. J. (1976) 153, 211–215
Printed in Great Britain

Chemistry of the Collagen Cross-Links

NATURE OF THE CROSS-LINKS IN THE POLYMORPHIC FORMS OF DERMAL COLLAGEN DURING DEVELOPMENT

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(*Received* 6 *August* 1975)

Both the type I and type III collagens present in embryonic dermis are stabilized by the intermolecular cross-link, hydroxylysino-5-oxonorleucine, derived from hydroxylysine-aldehyde, although the type I collagen possesses a significant proportion of dehydrohydroxylysinonorleucine. However, concurrent with the change in the proportion of the two types of collagen during postnatal development there is a change-over with both type I and III collagens to the labile cross-link, dehydrohydroxylysinonorleucine, derived from lysine aldehyde. The results indicate that the change in the nature of the cross-link with development is determined primarily by the change in the extent of hydroxylation of the lysine residues in the terminal non-helical regions rather than being due to the change in the type of collagen.

The studies of Chung & Miller (1974) and of Epstein (1974) have confirmed their original observations that embryonic collagen contains two genetically distinct collagens, designated types III and I. The newly discovered type III is composed of three chemically identical α chains $[\alpha(III)]_3$ (Chung *et al.*, 1974), in contrast with the $[\alpha(I)]_2\alpha 2$ composition of the well characterized type I. Type III collagen appears to be unique in possessing intramolecular disulphide bonds at the *C*-terminal end of the molecule. The ratio of type I to type III collagen changes from 0.6:1 in embryonic skin to 4:1 in postnatal skin (Epstein, 1974). Our own studies (Bailey & Robins, 1972) have demonstrated a corresponding change-over in the nature of the cross-links during the early growth period from a stable intermolecular cross-link, derived from hydroxyallysine, to a chemically labile cross-link, derived from allysine. Therefore we decided to demonstrate whether type III collagen also possesses lysine-derived cross-links in addition to its intramolecular disulphide bonds, and if so, whether there is a change in type of cross-link during development.

In the present paper we report that such a change does occur in the nature of the intermolecular cross-links of both types of collagen during the early growth period, thus confirming the previous preliminary results (Bailey, 1975).

Materials and Methods

Materials

Pepsin was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. (3250units/mg); 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, Scotland, U.K. All other chemicals were of analytical grade and supplied by BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Skins were obtained from a 3-month foetal calf and a 9-month-old steer and were scraped clean of adhering fat, muscular tissue and the epidermis. The tissue was minced, and then extracted with 1M-NaCl/0.05M-Tris/HCl, pH7.5, for 2 days at 4°C.

Separation of collagen types. Pepsin digestion and separation of type I and type III collagens were carried out as described by Chung & Miller (1974). Briefly, the insoluble residue from the salt (1M-NaCl, pH7.4, 4°C for 2 days) and acid (0.5M-acetic acid, 4°C for 2 days) extractions was digested with pepsin at a substrate/enzyme ratio of 10:1 (w/w) for 19h at 5°C. After inactivation of the pepsin by raising the pH, the solubilized collagen was purified by precipitation with 5% (w/v) NaCl at 20°C, redissolved in 1M-NaCl at 4°C, and then subjected to fractional precipitation to obtain type III collagen at 1.6M-NaCl and type I collagen at 2.5M-NaCl.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The type I and type III collagens were redissolved, denatured in sodium dodecyl sulphate (2%, w/v) at 38°C and analysed for subunit composition by polyacrylamide-gel electrophoresis. The electrophoresis was carried out by using a flat-bed technique with 6.5% (w/v) polyacrylamide gel,

with both gel and electrode Tris/borate buffers at pH8.5, as described previously (Sykes & Bailey, 1971). The gel was sliced to remove the top and bottom faces; the centre section was stained with Coomassie Blue and destained in 5% (v/v) acetic acid containing 40% (v/v) methanol.

Amino acid analysis. Samples were hydrolysed in glass-distilled constant-boiling HCl (6M) for 24h. The HCl was removed by evaporation *in vacuo* at 60°C. Amino acid analyses were carried out on a Jeol 6AH-DK analyser. Cysteine was determined by oxidation to cysteic acid with performic acid (Moore, 1963).

CM-cellulose chromatography. The collagen precipitates were dissolved in 0.5% acetic acid and dialysed against 0.04M-sodium acetate, heated to 50°C for 10min and then applied to a CM-cellulose column (2.5cm×20cm) equilibrated with 0.04M-sodium acetate/1M-urea and eluted with a linear salt gradient of 0–0.1M-NaCl over a total volume of 500ml at 45°C (Bellamy & Bornstein, 1971).

Reduction of cross-links. The type I and type III collagen precipitates obtained during the fractional precipitation procedure described above were redissolved in 0.5M-acetic acid, dialysed against 0.5M-acetic acid, and then reprecipitated by dialysis against 0.9% NaCl, pH7.4, at 18°C to produce native-type fibres.

The fibres were then reduced with KB^3H_4, hydrolysed, and the acid hydrolysate was separated on a Technicon Auto Analyser by using volatile buffers as described previously (Bailey *et al.*, 1970). The reduced components were located by their ^3H radioactivity (Bray, 1960) with a Packard scintillation counter, and their identity was confirmed by comparison of their chromatographic properties with authentic standards on the long basic column of the Beckman amino acid analyser (Bailey *et al.*, 1970).

Periodate oxidation. The hydroxylysinonorleucine isolated from the various hydrolysates of reduced type I and type III precipitates from embryonic and steer skin was subjected to periodate reduction. The cross-link material in 0.1M-sodium citrate buffer (pH5.3, 1ml) was treated with 0.01M-$NaIO_4$ (1ml) at 20°C for 5min. The reaction was stopped, and the products were reduced by adding 4M-NaOH and KB^3H_4 (2.5mg). After 30min the solution was adjusted to pH2.0 by the addition of 2M-HCl and the solution analysed for [^3H]proline and [^3H]lysine on the Locarte amino acid analyser (Robins & Bailey, 1975).

Isolation of types I and III collagen from borohydride-reduced fibres. The saline-washed intact fibres from 3-month foetal calf skin and 9-month-old steer skin were immersed in 0.5M-NaH_2PO_4 overnight at 4°C. The fibres were centrifuged, suspended in 0.9% NaCl, pH7.4, and immediately reduced by the addition of KB^3H_4. After 1h the pH was lowered to 4.0 by the addition of acetic acid and the fibre suspension dialysed against water overnight. A small portion of the fibres was then hydrolysed in 6M-HCl and the cross-link content determined as described above in order to confirm the absence of histidinohydroxymerodesmosine after the acid phosphate treatment (Robins & Bailey, 1973).

The fibres were then treated with pepsin under the conditions described above, for the non-reduced fibres, except that the temperature was raised to 15°C. The isolation of types I and III collagen from the pepsin-solubilized collagen was carried out by fractional precipitation as described above for the non-reduced fibres. After separation of the two types of collagen the fibres were hydrolysed with 6M-HCl and analysed for reducible cross-links on the Technicon analyser as described in detail above.

Results

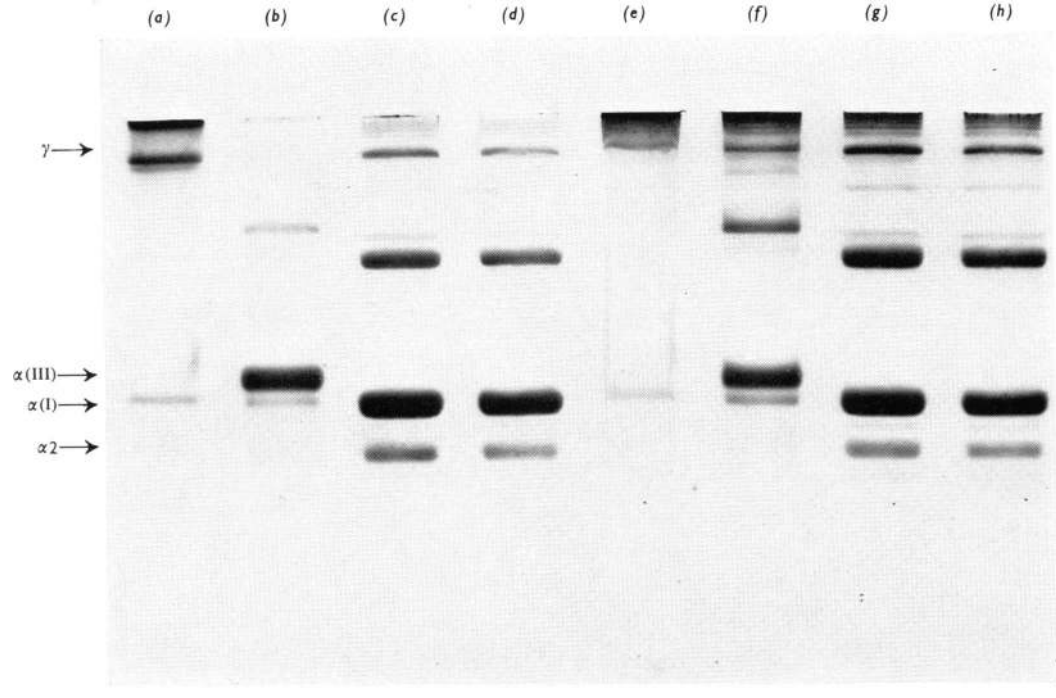
Type of collagen

Foetal calf skin. Only a small proportion of the foetal collagen was soluble in neutral salt (2%, w/v) or acetic acid (3%, v/v), but the insoluble residue was completely solubilized by the pepsin digestion.

The precipitate formed at 1.6M-NaCl during the fractional precipitation process was demonstrated to contain predominantly type III collagen by: (i) its mobility on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as a γ-component and conversion into α-components after treatment with mercaptoethanol; the use of the Tris/borate buffer permits separation of the α(III) chain from the α(I) chain (Plate 1); (ii) its elution position on CM-cellulose immediately before the $\alpha 2$ position and the slightly earlier elution position after cleavage and carboxymethylation of the disulphide bonds (Fig. 1); (iii) its amino acid composition (Table 1), showing the high content of hydroxyproline and glycine and the presence of cysteine.

The proportions of types I and III collagens were determined as 30 and 60% respectively from the weight of the freeze-dried precipitates. Since only a small proportion was solubilized in the salt and acid extractions the values are representative of the total tissue.

9-month-old steer skin. A high proportion (50%) of the collagen from the 9-month-old steer was extracted in 0.5M-acetic acid, and the insoluble residue was completely solubilized by the pepsin treatment. The proportions of types I and III collagen, based on the weights of the precipitates, were 60 and 40% respectively. Since a fractional precipitation carried out on the acetic acid-extracted material failed to reveal significant quantities of type III collagen, the proportion of type III collagen present



EXPLANATION OF PLATE 1

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis patterns of the fractional precipitates obtained after pepsin digestion of the collagen fibres

Samples were run with and without incubation with mercaptoethanol to demonstrate conversion of γ into α chain. (*a*) Embryonic calf skin, type III collagen; (*b*) embryonic calf skin, type III collagen plus mercaptoethanol; (*c*) embryonic calf skin, type I collagen; (*d*) embryonic calf skin, type I collagen plus mercaptoethanol; (*e*) 9-month old steer skin, type III collagen; (*f*) 9-month-old steer skin, type III collagen plus mercaptoethanol; (*g*) 9-month-old steer skin, type I collagen; (*h*) 9-month-old steer skin, type I collagen plus mercaptoethanol. Note that the use of Tris/borate buffer permits separation of α(I) and α(III) collagens.

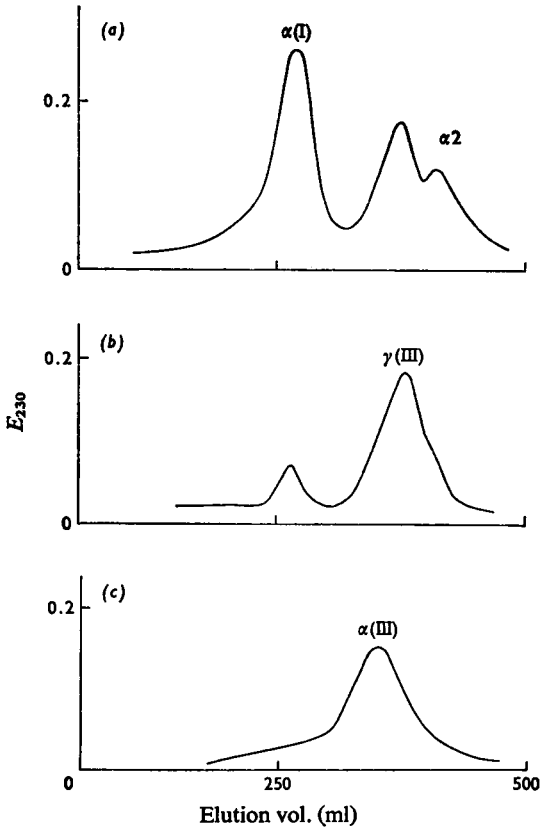


Fig. 1. *CM-cellulose chromatography of pepsin-solubilized embryonic calf skin*

(*a*) Total solubilized collagen; (*b*) type III collagen obtained by precipitation at 1.6M-NaCl; (*c*) type III collagen rechromatographed after reduction and alkylation.

in the initial skin collagen was calculated to be 20%. Similar results were obtained for 1-month-old calf skin. The proportion of type III collagen therefore changes rapidly from 60% in 3-month foetal calf skin to 20% in 1-month-old calf skin.

Reducible cross-links

Native fibres. The reduced fibres from types I and III collagen isolated from embryonic skin both possessed the same stable reduced cross-links, dihydroxylysinonorleucine and hydroxylysinonorleucine. However, a higher proportion of the latter was present in the type I collagen precipitate, together with a significant amount of histidino-hydroxymerodesmosine (Table 2).

Analysis of the native fibres from the adult steer revealed only traces of the di- and mono-hydroxy

Table 1. *Amino acid composition of collagens isolated from bovine skin*

	Composition (residues/1000 residues)			
	3-month embryonic skin		9-month-old steer skin	
	Type I	Type III	Type I	Type III
Hydroxyproline	100	122	93	118
Aspartic acid	43	41	45	45
Threonine	17	15	17	15
Serine	33	38	33	37
Glutamic acid	74	72	74	70
Proline	130	112	132	105
Glycine	324	348	323	344
Alanine	109	95	107	90
Valine	22	14	21	14
Methionine	5	6	5	5
Isoleucine	12	13	13	12
Leucine	23	22	24	18
Tyrosine	1.4	2	2	2
Phenylalanine	12	7	12	8
Hydroxylysine	9	7	7	6
Lysine	27	30	29	28
Histidine	5	6	5	8
Arginine	51	48	52	46
Cystine	0	1.7	0	1.5

cross-links in both precipitates. The major reduced components were found to be the hexosyl-lysine derivatives.

Borohydride-reduced fibres. Preliminary cross-link analysis of the intact borohydride-reduced fibres before pepsin treatment confirmed that the reduced intermolecular cross-links were present and that after the acid phosphate treatment the formation of histidino-hydroxymerodesmosine had been inhibited (Table 2).

Analysis of the type I and III collagen precipitates from the pepsin digest, as expected, revealed a higher proportion of hydroxylysinonorleucine in both types of collagen. The relative proportions of dihydroxylysinonorleucine and hydroxylysinonorleucine in the 3-month embryonic calf collagen were reversed in the type I and type III collagen precipitates, the hydroxylysinonorleucine being highest in the former. On the other hand, only hydroxylysinonorleucine was present to any significant extent in both the type I and type III collagens from the 9-month-old steer (Table 2).

Periodate oxidation of intact fibres

Insufficient hydroxylysinonorleucine was present in the type III collagen precipitate for analysis. The larger amount present in the type I collagen precipitate was oxidized and 60% was found to be in the

Table 2. *Relative distribution of ^3H radioactivity in the intermolecular cross-links isolated from acid hydrolysates of various types of collagen after reduction with KB^3H_4*

		Dihydroxy-lysinonorleucine (c.p.m.)	Hydroxylysino-norleucine (c.p.m.)	Histidino-hydroxy-merodesmosine (c.p.m.)
3-month embryonic skin	Total collagen	3500	1600	800
	Type III	2000	Trace	600
	Type I	1500	1200	1200
Reduced 3-month embryonic skin	Phosphate-treated total collagen	3000	1200	Nil
	Type III	1000	200	Nil
	Type I	500	1400	Nil
9-month-old steer skin	Total collagen	Trace	1800	2000
	Type III	Trace	Trace	Trace
	Type I	Trace	Trace	Trace
Reduced 9-month-old steer skin	Phosphate-treated total collagen	Trace	3000	Nil
	Type III	Trace	700	Nil
	Type I	Trace	850	Nil

Table 3. *Proportion of hydroxylysinonorleucine in the keto form*

The ^3H-labelled cross-links were subjected to a Smith degradation and the proportion of the keto form was calculated from the recoveries of the ^3H-labelled amino acids (see the Materials and Methods section for details).

	Keto form (%)			
	3-month embryonic skin		9-month-old steer skin	
Whole skin	8.3		5.7	
Intact pepsin-digested fibres	Type III	*	Type III	†
	Type I	60	Type I	†
Reduced pepsin-digested fibres	Type III	14.0	Type III	8.5
	Type I	3.5	Type I	4.5

* Insufficient cross-link present in these precipitates for periodate analysis.

† No cross-link present in these precipitates.

keto form (Table 3). Analysis of the hydroxylysino-norleucine from the fibres reduced before pepsin digestion revealed a higher proportion of the cross-link and a smaller percentage in the keto form; 14% in the embryonic type III collagen and 4–8% in the 9-month-old steer dermal types I and III collagens.

Discussion

The finding that genetically distinct collagens occur at the same time in the same tissue is a relatively recent one. The high proportion of type III molecules in early foetal skin compared with adult skin suggested that it should be considered an embryonic collagen. However, although a relatively lower proportion is present in adult human skin (Epstein, 1974) and the adult bovine skin of the present studies, the total amount present is much greater than that present in the foetus. This indicates that continued synthesis of type III collagen occurs, at least up to maturity, albeit at a different rate.

The rapid postnatal replacement of the type III collagen molecules by type I closely parallels the change-over in the nature of the cross-links (Bailey & Robins, 1972), initially suggesting that the stable hydroxylysino-5-oxonorleucine was derived from the type III collagen. On the other hand, it was not inconceivable that the lysine-derived cross-link detected was due to the smaller amount of type I collagen present, the type III collagen being stabilized solely by disulphide bonds.

The isolation of the two polymorphic forms of collagen involves the cleavage of the non-triple-helical terminal region with pepsin. Under the acidic conditions used hydroxylysino-5-oxonorleucine would be stable (Bailey *et al.*, 1970; Robins & Bailey, 1973) and hence remain attached to the molecule donating the hydroxylysine residues. In contrast, the aldimine cross-link would be cleaved under the acid conditions and the lysine-derived aldehyde in the terminal region would be removed from the molecules as a small peptide and consequently not be detected on subsequent reduction of the reprecipitated fibres. To confirm the presence of the labile dehydrohydroxylysinonorleucine it was necessary to stabilize the bond by borohydride reduction before pepsin digestion. To prevent excessive cross-linking the formation of histidino-hydroxymero-desmosine was inhibited by acid phosphate treatment before borohydride reduction. Under these conditions the dehydrohydroxylysinonorleucine is unaffected (Robins & Bailey, 1973).

By using these techniques the type III collagen of embryonic collagen was shown to be stabilized by hydroxylysino-5-oxonorleucine in addition to its known intramolecular disulphide cross-link. Surprisingly the type I collagen of embryonic skin also possessed the stable keto cross-link, demonstrating the presence of hydroxyallysine in the non-helical regions. Although a higher proportion of the hydroxylysinonorleucine was present, periodate oxidation indicated that a significant proportion of this cross-link was in the keto form and was therefore derived from hydroxyallysine. The cross-link existing *in vivo* would be lysino-5-oxonorleucine rather than the aldimine form, thus accounting for its resistance to the acidic pepsin digestion conditions.

On the other hand, analysis of the steer skin demonstrated that both types of collagen were stabilized by the labile cross-link dehydrohydroxylysinonorleucine. These results were initially based on its absence from acid pepsin digests and confirmed by its presence in borohydride-reduced collagen digests. Confirmation that the cross-link was derived from allysine was obtained by periodate oxidation, over 80% being found to be in the aldimine bond form.

Thus both types of molecules possess hydroxylysine in the terminal telopeptides, when present in embryonic dermal tissue, but revert to lysine in adult tissues. Although sequence studies have not yet been carried out on type III collagen, these results are consistent with the overall biosynthetic and sequence studies on type I collagen showing a decrease in the extent of hydroxylation of the specific telopeptide lysine residues involved in cross-linking (Barnes *et al.*, 1971, 1974). Since the change occurs within a single type of molecule it is not a question of a different sequence in the *N*-terminal region. The post-ribosomal hydroxylation of the telopeptide lysine residues may be due to the increased activity of the enzymes involved. Indeed, it has been reported (Anttinen *et al.*, 1973) that the lysine hydroxylase activities in embryonic tissues are 200 times higher than in postnatal tissues. It is interesting that no significant increase in hydroxylation of the triple-helical body of the molecules could be demonstrated by amino acid analysis, although further studies of the CNBr peptides are necessary to confirm this. It is conceivable, therefore, that there is a specific lysine hydroxylase for the telopeptide region, and this could possibly be effective on the non-triple-helical region even after the triple helix had been formed.

We thank Dr. S. M. Partridge, F.R.S., for continued interest and support and Dr. S. P. Robins for carrying out the periodate oxidations.

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