COMPARATIVE STUDIES ON THE NATURE OF THE CROSS-LINKS STABILIZING THE COLLAGEN FIBRES OF INVERTEBRATES, CYCLOSTOMES AND ELASMOBRANCHS

Allen J. BAILEY
Department of Biochemistry and Physiology, Agricultural Research Council Meat Research Institute, Langford, Bristol, England

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1. Introduction

The possession of collagen as a structural framework is by no means a prerogative of higher animals but is found in the simplest multicellular animals such as marine sponges and jelly fish [1]. Comparative studies on the nature of the cross-links are therefore of particular interest not only because of the obvious phyogenetic antiquity of these animals but also because of the finding that these primitive collagens comprise a single type of α-chain [2, 3].

Previous studies on collagen fibres from mammalian and avian tissues have demonstrated the presence of inter-molecular cross-links derived from lysine and hydroxylysine aldehydes [4, 5]. These cross-links have also been shown to be present in reprecipitated collagen from various tissues [6, 7]. The collagen of fish tissues have also recently been shown to be stabilized by the same type of cross-links [8], and in a continuation of these comparative studies a number of more primitive animals have now been analysed.

This paper reports that the stability of the collagen fibres of primitive invertebrates and cyclostomes are based on the same cross-linking system as the higher vertebrates.

2. Materials and methods

2.1. *Preparation of tissues*

(i) Elasmobranchs.

The skins of dogfish (*Scyllum canicula*) and of rayfish (*Raja clavata*) were scraped clean of adhering muscle tissue, cut into small pieces and shredded in an Ato-Mix homogenizer (M.S.E.). The fibres were washed with 0.9% NaCl (pH 7.4) and reduced as a suspension as described below.

(ii) Cyclostomes.

The lamprey was used as an example of this class of animal. The skin and the soft cartilagenous backbone were dissected out and washed in copious amounts of 0.9% NaCl (pH 7.4), homogenized and reduced as a suspension in buffered saline.

(iii) Invertebrates.

a) Sea anemone. The collagen fibres were obtained from three species of sea anemone. Calliactus contains a hard cartilagenous body wall, while Metridium has a soft body wall, both of which could be readily obtained by scraping away the softer tissues. The collagen fibres of *Actinia equina*, however, are distributed throughout the body and, in contrast to the above, the fibres are soft and very fine. The outer body was homogenized, centrifuged and washed extensively until the fine white collagenous fibres were obtained.

b) Sponge. A portion of the body of the sponge (*Suberites*) was homogenized in 0.9% saline and reduced as a suspension.

c) Earthworm cuticle. Common earthworms (*Lumbricus*) were washed, immersed in ether and the cuticles peeled off and scraped clean. After washing in 0.9% saline the cuticle was homogenized in a Polytron homogenzier (Northern Media Co., Hull, England) and reduced as a suspension of intact fibres.

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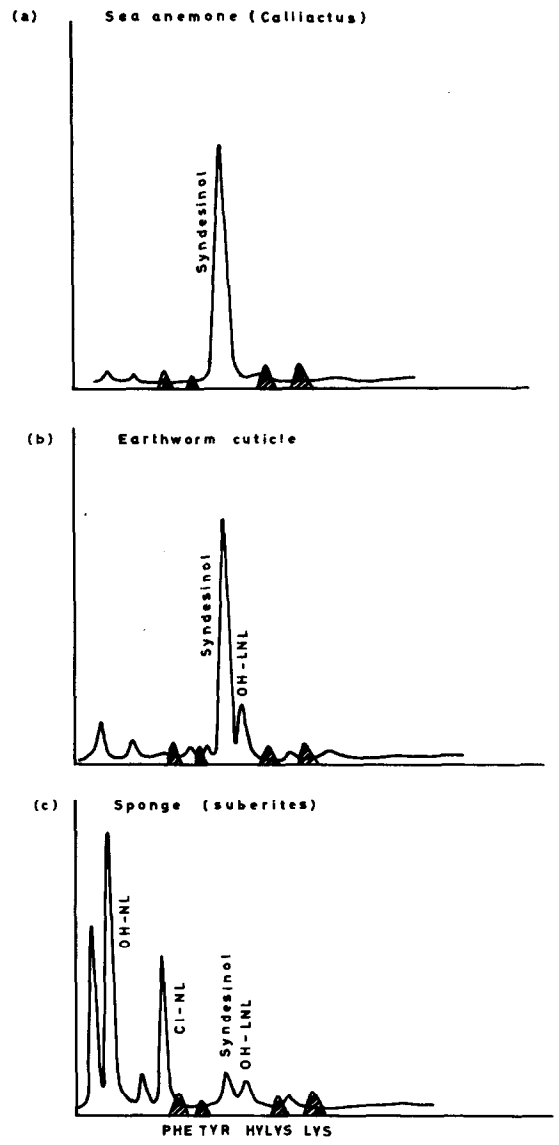

Fig. 1. Tritium distribution in acid hydrolysates of reduced invertebrate collagens. (a) Sea anemone (Calliactus), (b) Earthworm cuticle and (c) Sponge (Suberites). The diagram shows the position of the two reduced intermolecular cross-links, syndesinol and hydroxylysinonorleucine (OH-LNL).

d) Ascaris cuticle. *Ascaris lumbricoides* were obtained from porcine intestines. After freezing at -20° they were allowed to thaw slightly, allowing the cuticle to be easily peeled off as a clear membrane. The cuticle was washed and homogenized in 0.9% saline (pH 7.4) prior to reduction.

2.2. *Reduction and analysis of the reducible components*

The intact fibres were reduced as a suspension in buffered saline (pH 7.4) with tritiated potassium borohydride as previously described in detail [9]. The reduced collagen was hydrolysed (24 hr; 6 N HCl) and analysed on a Technicon Autoanalyzer using volatile buffers. The tritium activity was assayed in Bray's solution using a Packard 3375 liquid scintillation counter. The elution patterns obtained are shown in figs. 1 and 2.

2.3. *Identity of the radioactive components*

In addition to the elution position of the components on the volatile buffer system, confirmation of identity was achieved by analysis of the isolated component on the Beckman Amino Acid analyser. The mobilities were checked against authentic samples obtained either by chemical synthesis or isolation from reduced collagen.

2.4. *Acrylamide gel electrophoresis*

Denatured, solubilized collagens from the above sources were analysed by SDS-acrylamide gel electrophoresis using the technique described previously [10].

3. Results

3.1. *Reducible cross-links*

(i) Invertebrates.

The hard supporting body wall of the invertebrate sea anemone, Calliactus and Metridium, and the soft fibres of *Actinia equina* all contained syndesine as the only significant reducible cross-link (fig. 1a). The reduced sponge revealed a small amount of both syndesinol and hydroxylysinonorleucine, but the major peaks were the reduced cross-link precursors (fig. 1c).

The body wall of the earthworm contained syndesine as the major reducible component and a small amount of hydroxylysinonorleucine (fig. 1b).

Ascaris cuticle collagen on the other hand failed to reveal the presence of the reducible inter-molecular cross-links derived from lysine. The four reducible components present were not identified and may be degradation products of reduced cystine.

(ii) Cyclostomes and elasmobranchs.

The skins of the lamprey, dogfish and rayfish all

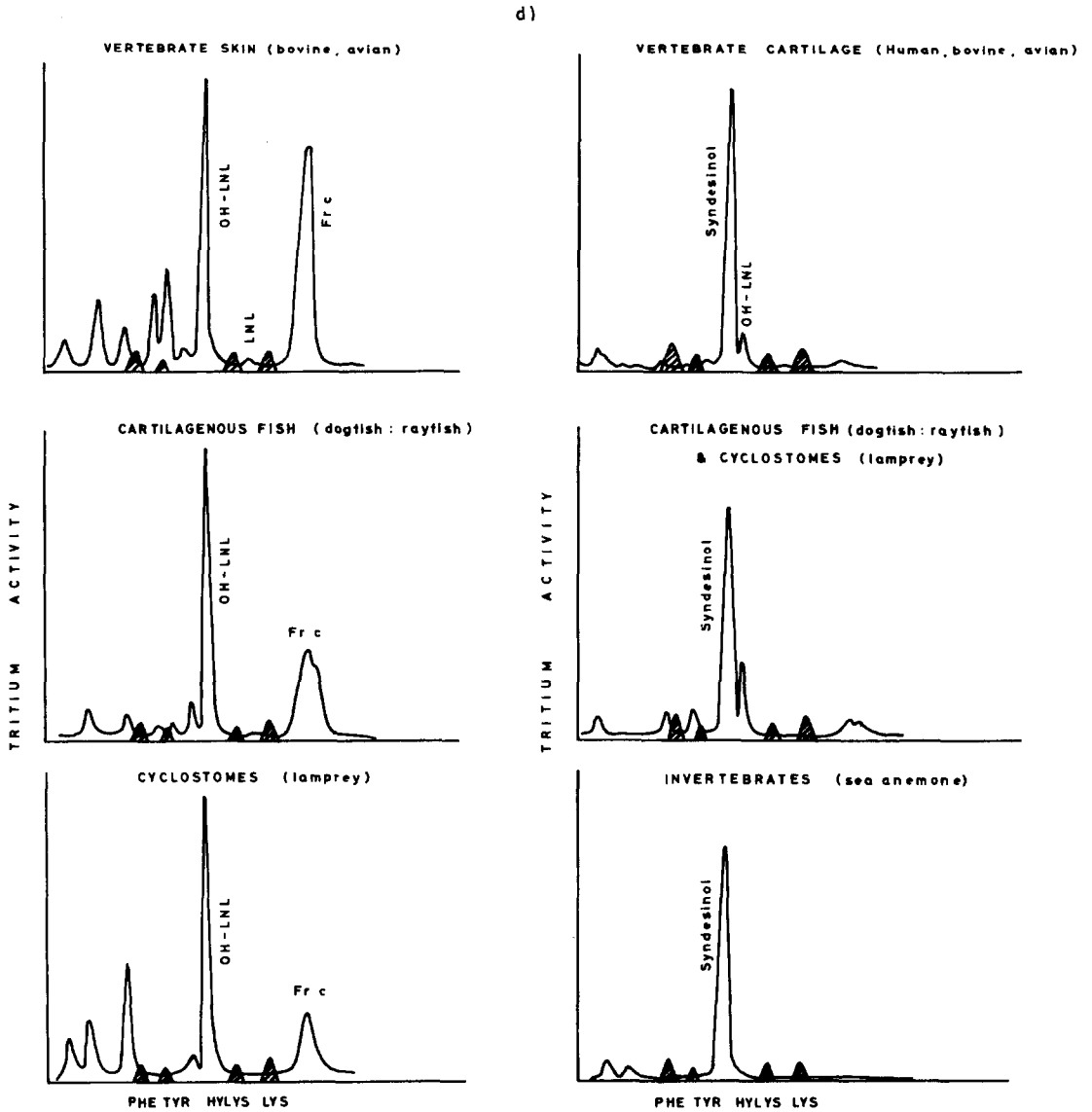

Fig. 2. Elution patterns of acid hydrolysates of reduced collagen from (a) skin of higher vertebrates, skin of elasmobranchs and skin from cyclostomes; (b) cartilage collagen from higher vertebrates, elasmobranchs and cyclostomes and from sea anemone.

gave similar elution patterns and are seen to contain hydroxylysinonorleucine and, the as yet unidentified, Fr. C. as the major inter-molecular cross-links (fig. 2a). The cartilagenous backbone of these fish again gave similar patterns, this time syndesine being the major reducible cross-link (fig. 2b).

3.2. *SDS-acrylamide electrophoresis*

Analysis of the electrophoretic patterns showed that all these collagens contain a single α component.

The sea anemone, partially solubilized in SDS, revealed a single α component of lower mobility than mammalian α 1. After solubilization by pepsin

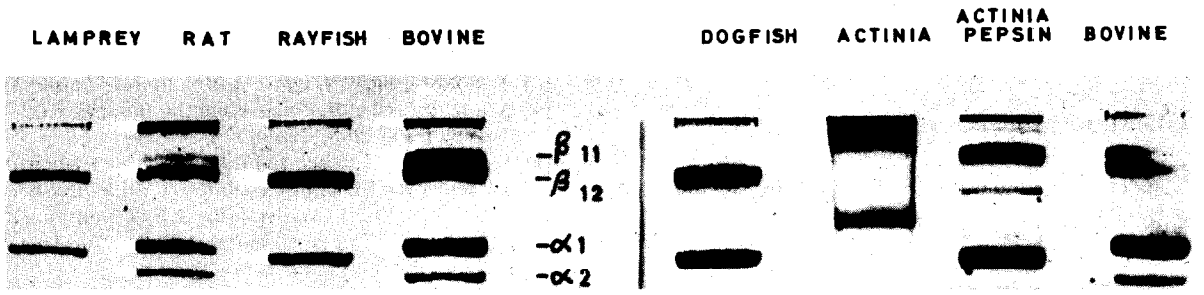

Fig. 3. SDS-acrylamide electrophoretic patterns of denatured collagen derived from a number of different phyla: lamprey skin, rat skin, ray fish skin, bovine tendon, dogfish skin, actinia equina, actinia pepsin treated, bovine tendon.

treatment a single α component with a mobility similar to that of mammalian α 1 (fig. 3) was observed.

The elasmobranch and cyclostome both gave a single α component but in this case it had a mobility intermediate between those of mammalian $\alpha 1$ and $\alpha 2$. The β component similarly had a higher mobility than the mammalian β components (fig. 3).

4. Discussion

The stabilizing inter-molecular cross-links of collagen fibres from even the most primitive animals have been clearly shown to be based on the same biochemical system as those previously shown to exist in higher vertebrates [4–8]. Comparison of the elution patterns of the reduced collagen from the body wall of the sea anemone, the backbone of the cyclostomes and the elasmobranchs, with those of cartilage and bone of higher vertebrates shows them to be basically similar. All the tissues contained syndesine as the only significant reducible cross-link and are known to be virtually insoluble in acid buffers [2]. Although it is not possible to confirm a direct correlation with solubility, all the tissues found to possess syndesine as the major cross-link have a very low solubility. The predominance of syndesine in the invertebrate collagens suggests that the lysine residues normally involved in cross-linking are fully hydroxylated.

Similarly the elution patterns of the skin of the lamprey and dogfish were basically identical to those of the higher vertebrates, all containing the same two Schiff base cross-links, hydroxylysinonorleucine and, the as yet unidentified, Fr. C. as the major reducible cross-links. The labile character of these bonds readily accounts for the higher solubility of these tissues.

It would appear that there is a basic pattern to the type of cross-links present in skin, at least in all the skins analysed from lamprey up to the higher vertebrates. Cartilage possesses the cross-link syndesine and this is the only significant reducible cross-link in these tissues from sea anemone up to man. Although considerable differences occur in the types of cross-link present in different tissues, a particular tissue has preserved the same type of cross-links thoughout the phylogenetic scale.

In contrast to the sea anemone which has a high hydroxylysine content this residue has been reported to be absent from the earthworm cuticle [11]. However, analysis of the reduced collagen revealed the cross-link syndesine, indicating the presence of at least one residue prior to conversion to hydroxylysine aldehyde. *Ascaris lumbricoides* cuticle is stabilized by disulphide bonds [12] and as might be expected did not contain any of the lysine-derived aldehyde bonds as additional cross-links.

SDS-acrylamide electrophoresis produces an excellent resolution of the various subunits of mammalian collagen and the different mobilities of the $\alpha 1$ and $\alpha 2$ have suggested a slight difference in molecular weight of these chains [10]. The solubilized primitive collagens showed a single α component as originally demonstrated by Kulonen and his colleagues [2]. An interesting observation was that the mobility of the sea anemone collagen was increased by pretreatment with pepsin as a means of solubilizing the fibre, suggesting an initial molecular weight higher than mammalian $\alpha 1$.

The absence of the $\alpha 2$ chain in these primitive collagens does not appear to affect the type of cross-links present in the different tissues, suggesting that

it has little relationship to the type of cross-linking present.

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