IN VITRO ENZYMIC BIOSYNTHESIS OF TWO INTER-CHAIN CROSSLINKS OF BONE COLLAGEN

A.J. Bailey and L.J. Fowler

Agricultural Research Council, Meat Research Institute, Langford, Bristol, England

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The presence of covalent crosslinks in bone and dentine presumably synthesised through the condensation of lysine and hydroxylysine-derived aldehydes has been recently demonstrated (Bailey, Fowler and Peach, 1969 - preceding paper). The formation of the lysine-derived aldehyde, α-amino adipic δ-semialdehyde, and its role as the crosslink precursor has been convincingly demonstrated for elastin by isolation of the actual crosslinks (Partridge, 1967) and inferred for collagen (Piez, 1968).

A number of workers have postulated the involvement of certain amine oxidases in the oxidative-deamination of lysine (Bird et al., 1966; Page and Benditt, 1967; Hill et al., 1967). However these enzymes are not inhibited by physiological levels of β amino propionitrile, and also act on free lysine. An enzyme system capable of converting lysine in peptide linkage to α-amino adipic δ-semialdehyde using elastin as the substrate has recently been detected in extracts from skin, bone and aorta (Pinnell and Martin, 1968).

We now report the biosynthesis of the aldol and aldimine crosslinks described in the preceding communication, by in vitro incubation of a similar enzyme system to that extracted by Pinnell and Martin, obtained from a crude extract of the cellular constituents of embryonic bone, with lathyritic chick bone.

EXPERIMENTAL AND RESULTS

Preparation of enzyme extract. Tibias and femurs were carefully dissected from 48 17-day embryonic chicks and the cartilagenous ends removed. The cleaned thin cylinders of bone in ice-cold 0.16 M saline, 0.1 M phosphate buffer pH 7.4 (50 ml) were macerated with a Silverson macerator, then centrifuged at 100,000 g for 2 hrs. The supernatant was lyophilized (1.1 g) and used as the enzyme source.

Preparation of lathyritic bone substrate. 24 embryonic chicks (15 day old) were made lathyritic by choreo-allantoic injection of 0.4 ml β-APN (100 mgm/ml). After 48 hrs further incubation the tibias and femurs were removed, cleaned, homogenized in a glass homogenizer, centrifuged at 11,000 g for 1 hr and the sediment resuspended in saline and re-centrifuged. This procedure was repeated three times and the final pellet obtained was used as the substrate.

Incubation of insoluble lathyritic bone collagen with enzyme extract. The lyophilized enzyme extract was reconstituted in water (0.5 g in 10 ml; pH 7.5) and mixed with the substrate suspension in water (2 ml) and incubated at 37° for 24 hrs with continuous shaking. Separate control incubations consisted of the enzyme extract and the lathyritic bone substrate.

Identification of the synthesised crosslinks. After incubation the test and control samples were reduced with tritiated sodium borohydride (1 m Ci/mg) for 3 hrs at pH 7.4 at a collagen to borohydride ratio of 30:1. The suspension was acidified with acetic acid, extensively dialysed, evaporated to dryness *in vacuo* and hydrolysed with 6N HCl. Amino acid analysis was then carried out with the Technicon amino acid analyzer as described previously (Bailey, 1968).

The distribution of tritium activity is shown in Fig. 1. The two radioactive peaks eluted in the identical position of the previously identified aldol and aldimine crosslinks. Despite the small amount of cross-linking amino acids produced, their high specific activity permitted

further confirmation of identity by their elution position on the Beckman amino acid analyzer and by electrophoresis. The location of the radioactive peaks was compared with the known positions of the previously isolated crosslinks.

DISCUSSION

The cell-free extract from embryonic bone has been clearly demonstrated to contain an enzyme system capable of synthesising the aldol (Fr. 1, Fig. 1) and aldimine (Fr. 2, Fig. 1) crosslinks previously shown to be present in normal bone (Bailey, Fowler and Peach, 1969).

Incubations were carried out with lathyritic bone since the lysine and

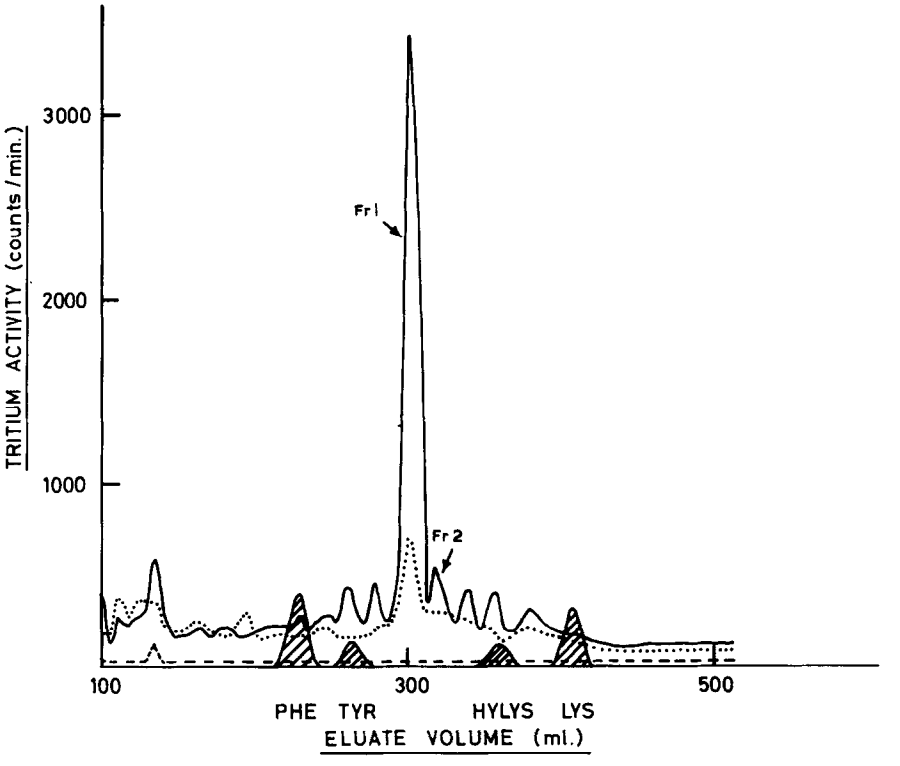

Fig. 1 Elution pattern of radioactive reducible components obtained from an acid hydrolysate of bone collagen reduced with tritiated sodium borohydride; ____ $NaBT_4$ reduced incubation mixture of lathyritic bone and enzyme extract; . . . . $NaBT_4$ reduced lathyritic bone; ---- $NaBT_4$ reduced enzyme extract. (Hatched peaks denote position of normal amino acids, areas of peaks not to scale.)

hydroxylysine residues normally involved in oxidation to aldehydes are still available. This procedure provided a low background of reducible pre-formed crosslinks thus permitting the detection of small amounts of new reducible crosslinks synthesised by the enzyme system. Similar studies have demonstrated that the system is capable of synthesising the various crosslinks in tendon collagen (Fowler and Bailey, 1969).

Further studies are being carried out to isolate both precursors, the lysine and hydroxylysine-derived aldehydes. Presumably, after the enzyme oxidation step the resulting aldehydes condense spontaneously to produce the crosslinks, but the precise details of the various modes of reaction of these aldehydes may require considerable effort to elucidate. A closer study of the crude enzyme system may demonstrate the involvement of more than one enzyme, or the role of other additional components, and lead to further information on the biosynthesis of the whole system of stabilizing cross-links in collagen.

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