In vitro Studies on the Enzymic Biosynthesis of the Collagen Crosslinks

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The *in vitro* incubation of a crude enzyme extract from bone with lathyritic porcine tendon resulted in the formation of all the reducible components previously demonstrated to be involved as the intermolecular crosslinks responsible for the stabilization of the collagen fibre. It is likely that the enzyme involved is a pyridoxal phosphate cupro-protein. Indirect support for the probable involvement of pyridoxal and copper in the enzyme system has been obtained by a series of model experiments demonstrating that the pyridoxal-copper system is capable of producing the crosslink precursors allysine and hydroxyallysine.

It is now generally accepted that the initial stage in the biosynthesis of the crosslinks of both elastin and collagen is the enzymic oxidative-deamination of the ε-amino groups of peptide bound lysine to give the δ-aldehyde (allysine). Pinnell and Martin (1968) demonstrated the presence of an enzyme in a crude extract of embryonic chick bone capable of producing the crosslink precursor allysine in lathyritic elastin. Incubation of a similar crude enzyme extract with lathyritic chick bone collagen has also been shown to result in the formation of a reducible component believed to be the major crosslink in the less soluble collagens (Bailey, Fowler and Peach, 1969).

In contrast to bone, the soft tissue collagens contain a high proportion of the labile intermolecular aldimine bonds. We now confirm that the enzyme system is capable of synthesising these additional crosslinks and their precursors in tendon collagen. From the nature of these crosslinks both allysine and the δ-aldehyde derived from hydroxylysine (hydroxyallysine)

would be required as precursors. Although we have isolated and characterised allysine from collagen (Bailey and Peach, 1968) the presence of hydroxyallysine has not yet been confirmed.

The enzyme system involved is probably copper and pyridoxal phosphate dependant since both copper deficiency (Chou et al, 1969; Miller et al, 1965) and pyridoxine deficiency (Hill and Kim, 1967) have been shown to impair the biosynthesis of the crosslinks in collagen and elastin. The amine oxidase involved may therefore be a pyridoxal phosphate-cupro protein. The action of these enzymes can often be 'mimicked' by the prosthetic group alone (Braunstein, 1960) and this action is markedly enhanced by the addition of copper ions (Ikawat and Snell, 1954). In support of the involvement of these co-factors we now report that the pyridoxal phosphate-copper complex is capable of producing both crosslink precursors, allysine and hydroxyallysine.

EXPERIMENTAL AND RESULTS

1. The Action of Crude Amine Oxidase on Lathyritic Achilles Tendon

Piglets were made lathyritic by the administration of 300 mg β APN per day from birth until 2 weeks old. The doses were given orally contained in gelatin capsules. The effects of dosing were noted by comparison with controls from the same litter and were entirely consistent with the view that the animals became lathyritic. This view was borne out by histological and X-ray examination of the tissues after death. The animals were killed by the captive bolt method and the tissues dissected out into ice cold physiological saline. The lathyritic achilles tendons were used as the substrate and prepared in the manner described previously (Bailey and Fowler, 1969).

The tibias and femora of normal 2 week old piglets were used to prepare an enzyme extract which was incubated with the substrate and assayed by reduction with tritiated sodium borohydride and analysis of the acid hydrolysates on ion exchange columns (Bailey & Fowler, 1969). The results are shown in Fig. 1. The peaks marked I and II are syndesine and hydroxylysinonor-

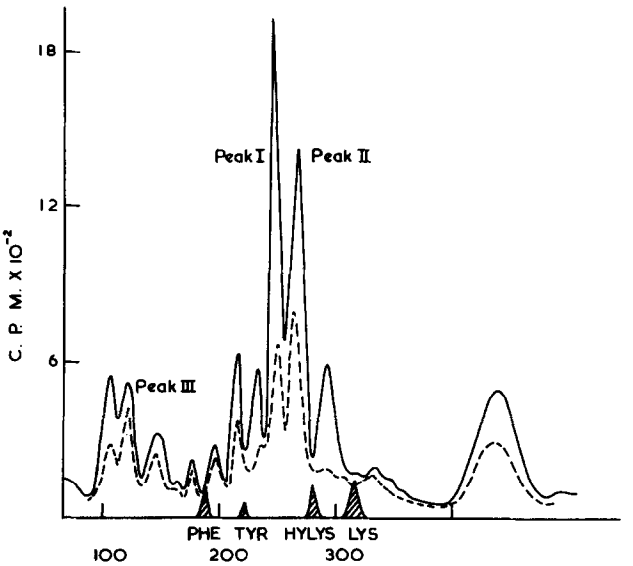

Fig. 1. Distribution of ^{3}H radioactivity of acid hydrolysates of reduced porcine achilles tendons a) lathyritic tendon reduced with $NaB^{3}H_4$ ..... ; b) lathyritic tendon reduced with $NaB^{3}H_4$ after incubation with the crude enzyme extract for 24 hr at 37°, pH 7.5; ——— .

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leucine respectively (Bailey, Fowler and Peach, 1969). The level of tritiated peaks seen in the lathyritic tendon control was still relatively high since it was not possible to make the piglets completely lathyritic. The increase in peak size seen after incubation with the enzymic extract restores the level of crosslinks almost completely to that seen in normal tissue.

2. In vitro Nonenzymic Production of Crosslink Precursors.

I. Reaction of L-Lysine with Pyridoxal 5'-Phosphate and $CuSO_4$. The reactants (α-N acetyl L-Lysine, Pyridoxal 5'-Phosphate and $CuSO_4$) were all present at 1 μm/ml in 0.01 M sodium acetate solution pH 7.0 and were incubated at 38° for 24 hr with continuous shaking. The reaction mixture was then reduced with tritiated sodium borohydride at a lysine:borohydride ratio of 30:1. After 2 hr. the excess borohydride was removed by acidification with acetic acid.

The α-N acetyl group was removed by HCl hydrolysis, the solution evaporated to dryness in vacuo and assayed on the Technicon amino acid analyzer using volatile buffers (Bailey, 1968). The tritium pattern obtained is shown in Fig. 2 (a). Employing the techniques previously described in detail (Bailey and Peach, 1968), peak I was identified as ε-hydroxynorleucine, the reduced form of the crosslink precursor. The larger peaks (IV and V) eluting in the basic region were identified as pyridoxal derivatives by ultraviolet spectrometry and were present in non-incubated control mixtures. Peak II was identified as pipecolic acid by comparison with authentic material on paper chromatography (Butanol:Acetic acid:water 4:1:1), high voltage paper electrophoresis (pyridine:acetic acid:water 1:10;89, pH 3.50) and on the Beckman amino acid autoanalyzer. Pipecolic acid could only have been formed from the lysine-derived aldehyde by ring closure to give dehydropipecolic acid which was subsequently reduced by the borohydride. Incubation of L-Lysine without the protecting acetyl group and the pyridoxal-copper system resulted in a high yield of pipecolic acid, no hydroxynorleucine being detected.

II Reaction of DL-Hydroxylysine with pyridoxal 5'-phosphate and $CuSO_4$

Pyridoxal 5'-phosphate and $CuSO_4$ were incubated as described above with α-N-trifluoracetyl hydroxylysine. After incubation and reduction the trifluoracetyl group was removed by reaction in 0.2N NaOH for 10 mins. The tritium pattern of the reaction mixture when assayed on the analyzer is shown in Fig. 2(b). Ultraviolet spectrometry indicated that the peaks eluting after lysine were all derivatives of pyridoxal phosphate and were also present in reaction mixtures which had not been incubated. The peak eluting at 100 ml. was isolated and purified further using the Beckman amino acid analyzer and then characterized by mass spectroscopy. The mass spectrum of the di O, N-trifluoracetylated methyl ester is shown in Fig. 3. The mass ion at 465 and the fragmentation pattern are entirely consistent with the structure of the original material being dihydroxynorleucine which is the reduction product of hydroxylysine aldehyde (hydroxyallysine).

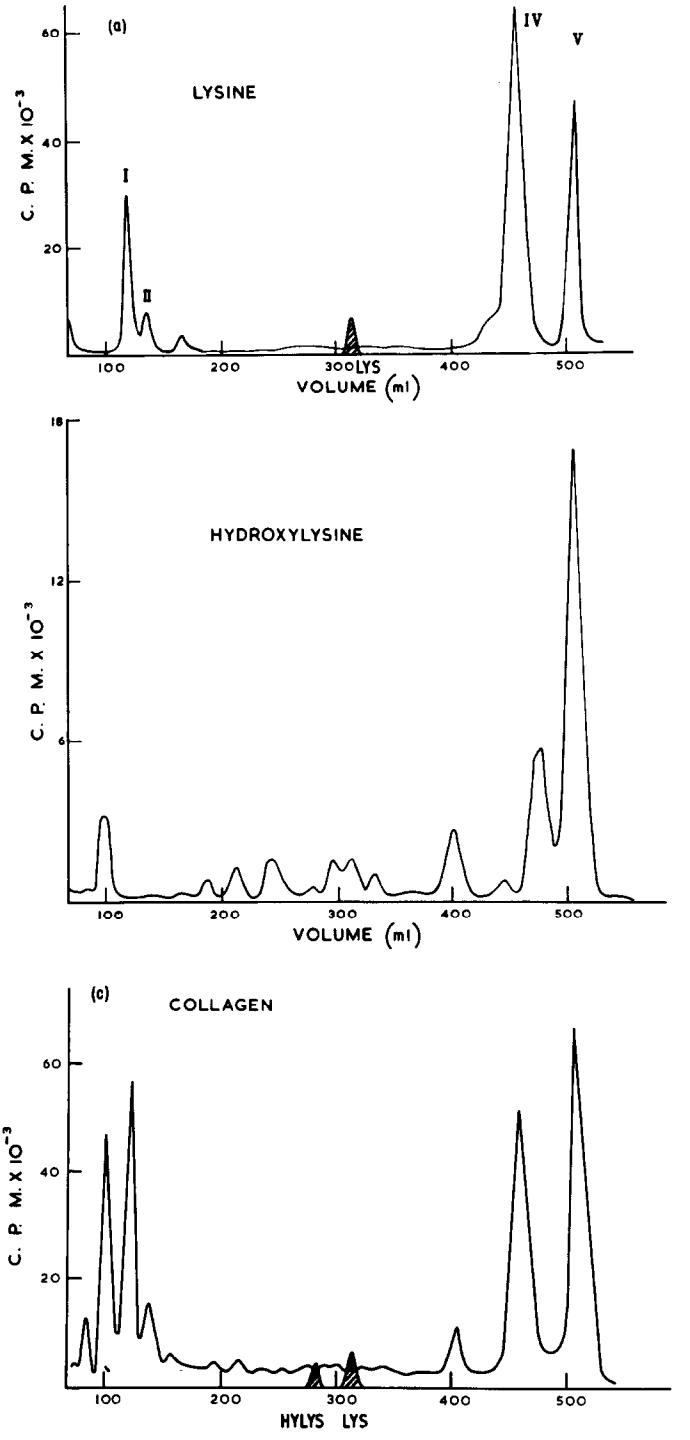

Fig. 2. Elution patterns of ^{3}H-labelled reducible components obtained on $NaB^{3}H_4$ reduction of the reaction products formed after incubation with pyridoxal 5' phosphate and copper a) α-N acetyl lysine b) α-N-trifluoroacetyl-hydroxylysine c) Neutral salt soluble collagen fibres.

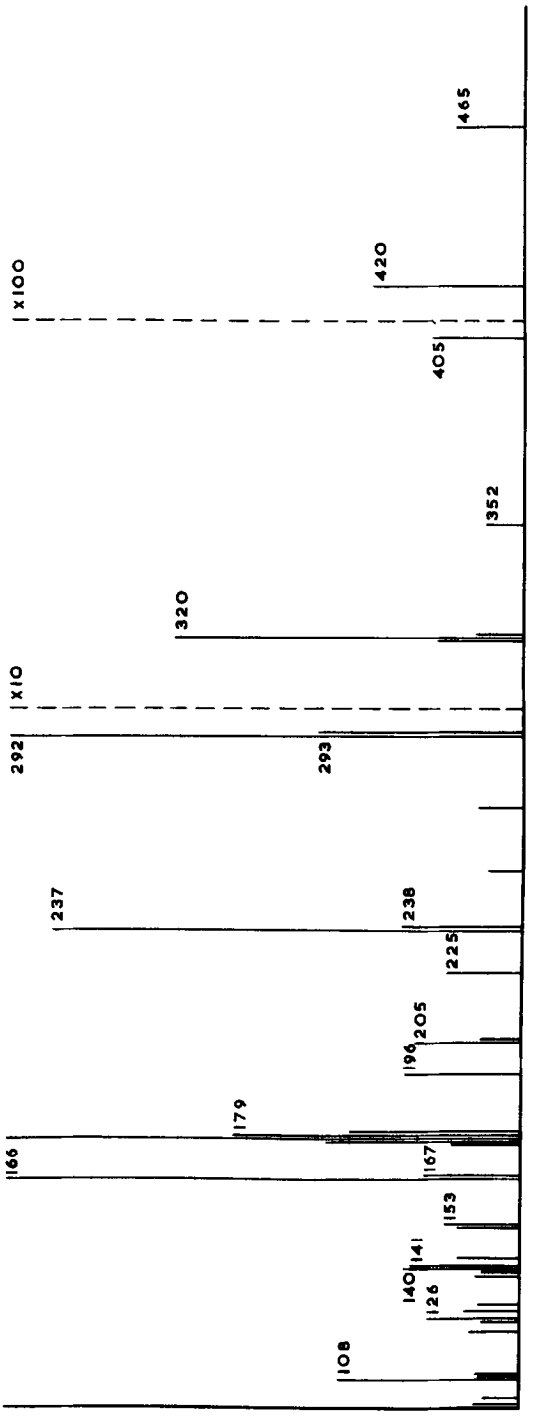

Fig. 3. Mass spectrum of the trifluoroacetyl methyl ester of 5:6 dihydroxynorleucine.

The elution position of this compound on both the Technicon volatile buffer system and the Beckman amino acid analyzer corresponds to the previously unidentified peak in reduced collagen eluting just prior to the established position of hydroxynorleucine (Fig. 1, peak III). Since hydroxyallysine is the proposed precursor of the syndesine crosslink identified in collagen (Bailey et al., 1970) this peak may correspond to its reduced form. However insufficient material could be isolated from the reduced collagen to confirm the identification.

An alternative chemical synthesis of 5:6 dihydroxynorleucine was achieved by oxidation employing N-bromo succinimide. Hydroxylysine was reacted with phenyl acetate to give Nε-acetyl hydroxylysine (Leclerc and Benoiton, 1968). The product was then treated with benzyl chloroformate and MgO to give Nα-Cbz-Nε acetyl hydroxylysine (Baer and Maurukus, 1955). The acetyl groups were then removed by acid hydrolysis to give Nα-Cbz-hydroxylysine which was then oxidized with N-Bromosuccinimide (Franzblau et al., 1969). The resulting aldehyde was reduced with tritiated potassium borohydride and the α-Cbz group removed by hydrogenation using Pd catalyst. The compound was isolated from the Beckman amino acid analyzer and gave a mass spectrum with a mass ion of $^{m}/e$ 465 and a fragmentation pattern consistent with the product being 5:6 dihydroxynorleucine.

III Reaction of Collagen fibres with Pyridoxal 5'-phosphate and $CuSO_4$

10 mg. Neutral salt soluble collagen (1.5 mgm/ml) was heat gelled at 38° and the resulting fibres mixed with pyridoxal 5'-phosphate (5 mgm) and 0.05 M $CuSO_4$ (1 ml) and incubated for 72 hr at 38°. After reduction with tritiated potassium borohydride and hydrolysis (6N HCl) the products were analysed on the Technicon autoanalyzer.

The reduced fibres produced a radioactive elution pattern in which the two radioactive peaks I and II eluting in the acidic region (Fig. 2.(c)). possessed elution positions and paper chromatographic mobilities corresponding to the synthesised dihydroxynorleucine and hydroxynorleucine respectively.

Although readily detectable by their radioactivity, insufficient material was available to confirm their identity by isolation and mass spectrometry.

DISCUSSION

The crude enzyme extract obtained from normal piglet bone when incubated with lathyritic piglet Achilles tendon has been shown to catalyze the formation of all the reducible inter-chain crosslinks detectable in normal tendon. Two of these crosslinks have previously been identified, one as dehydro-hydroxylysinonorleucine and the second tentatively as the aldol reaction product of lysine aldehyde and hydroxylysine aldehyde (syndesine) (Bailey, Fowler and Peach, 1969). This is essentially similar to the previously reported *in vitro* formation of these two crosslinks in bone collagen. However, dihydro-hydroxylysinonorleucine and the other reducible components in tendon collagen are barely detectable in bone collagen where syndesine predominates (Bailey, Peach and Fowler, 1970).

The production of the crosslink precursors, allysine and hydroxyallysine from neutral salt soluble collagen by reaction with the pyridoxal copper complex is indirect evidence for the participation of these co-factors in the *in vivo* enzymic process. The complex may be present at the active site of the enzyme. Insufficient material was produced to confirm their identity as would be expected since it is known that only a few specific lysine and hydroxylysine residues in collagen react to form an aldimine bond with pyridoxal phosphate (Page *et al.*, 1968). It would be of considerable interest to locate the position of these highly specific residues as the same residues must be involved in the formation of the precursors.

It is presumed that the only enzymically controlled step in the biosynthesis of the collagen crosslinks is the initial oxidative-deamination and that the aldehydes formed react spontaneously with one another or with ε-amino groups. The chemical reactivity of these groups is probably increased simply by their close proximity in the highly ordered collagen structure. In order to investigate this problem it would be an advantage

to be able to form these aldehydes in model systems using relatively large amounts of material. This has proved possible using pyridoxal 5'-phosphate which may be involved in the *in vivo* process and further studies should demonstrate whether it is possible to form the crosslinks *in vitro* and thus to give further information on the process involved *in vivo*.

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