IDENTIFICATION OF TWO INTERCHAIN CROSSLINKS OF BONE AND DENTINE COLLAGEN

A.J. Bailey, L.J. Fowler and Catherine M. Peach

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

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Bone and dentine collagens are virtually insoluble in the solvents employed to extract native tropocollagen from soft tissue collagens. The decrease in the ease of extraction of the tropocollagen is generally considered to be due to an increase in the presence of crosslinked components (see Piez, 1968; Bailey, 1968a for reviews). Bone and dentine collagen must therefore be extensively intermolecularly crosslinked, but the chemistry of the crosslink is unknown. Glimcher and his co-workers (1965a; b) suggested that non-covalent bonds are involved in the stabilization of bone. However a recent report by Miller et al. (1967) clearly demonstrated the presence of stable covalent inter-chain crosslinks in the denatured guanidine-HCl extract from bone.

Veis and Schlueter (1964) similarly concluded that dentine was extensively crosslinked by stable covalent bonds, and proposed phosphate-mediated ester bonds in addition to a system of periodate sensitive bonds.

Our recent studies on the crosslinks of soft tissue collagens have demonstrated the presence of both labile and stable intermolecular crosslinks (Bailey, 1968b; Bailey and Peach, 1968). The present communication describes the isolation and identification of two covalent inter-chain crosslinks stabilizing bone and dentine.

EXPERIMENTAL AND RESULTS

Preparation and reduction of collagen. One-day old chicks were killed and

the tibia removed and cleaned. The cartilaginous ends of the bone were removed and the thin cylinders of bone broken and washed in 0.9% saline (pH 7.4). The clean bones were decalcified in 0.2 M EDTA (pH 7.4) for 48 hrs. After dialysis to remove the EDTA the decalcified tibia were reduced as a suspension in 0.9% saline (pH 7.4) with tritiated sodium borohydride for 3 hrs., neutralized and extensively dialysed.

Lathyrism was induced by choreo-allantoic injection of varying doses of sterilized β amino propionitrile into 15 day-old fertile eggs. The embryonic chicks were killed after 48 hrs. further incubation, the bones extracted immediately and treated as for the one-day-old chick tibia. The decrease in magnitude of the reducible components (Fig. 1a) with increasing doses of the lathyrogen confirms the involvement of the components in the crosslinking mechanism.

Dentine was obtained from the roots of human and bovine teeth. The roots were sawn off the teeth, cleaned on an abrasive wheel, split with calipers and the soft central pulp removed. The small pieces of dentine were decalcified in 0.3 M EDTA for 7 days. After decalcification the dentine was reduced under the same conditions as the bone.

Isolation of the reduced components. Reduced bone and dentine were hydrolysed and analysed with a Technicon amino acid analyzer as described previously (Bailey, 1968). Elution patterns depicting the location of the radioactive components and the usual amino acid are shown in Fig. 1 and Fig. 2. Fractions of the radioactive peaks (Fr. 1 and Fr. 2) were obtained from several runs, bulked and refractionated.

Isolation of the radioactive components on a large scale was achieved by displacement chromatography employing the technique developed for amino acids by Partridge and Brimley (1952).

Incorporation of H^3-Lysine. 15 day old incubated fertile eggs were injected with H^3-Lysine (0.02 m Ci) and incubated a further five days. The tibia were reduced with inactive borohydride and after acid hydrolysis

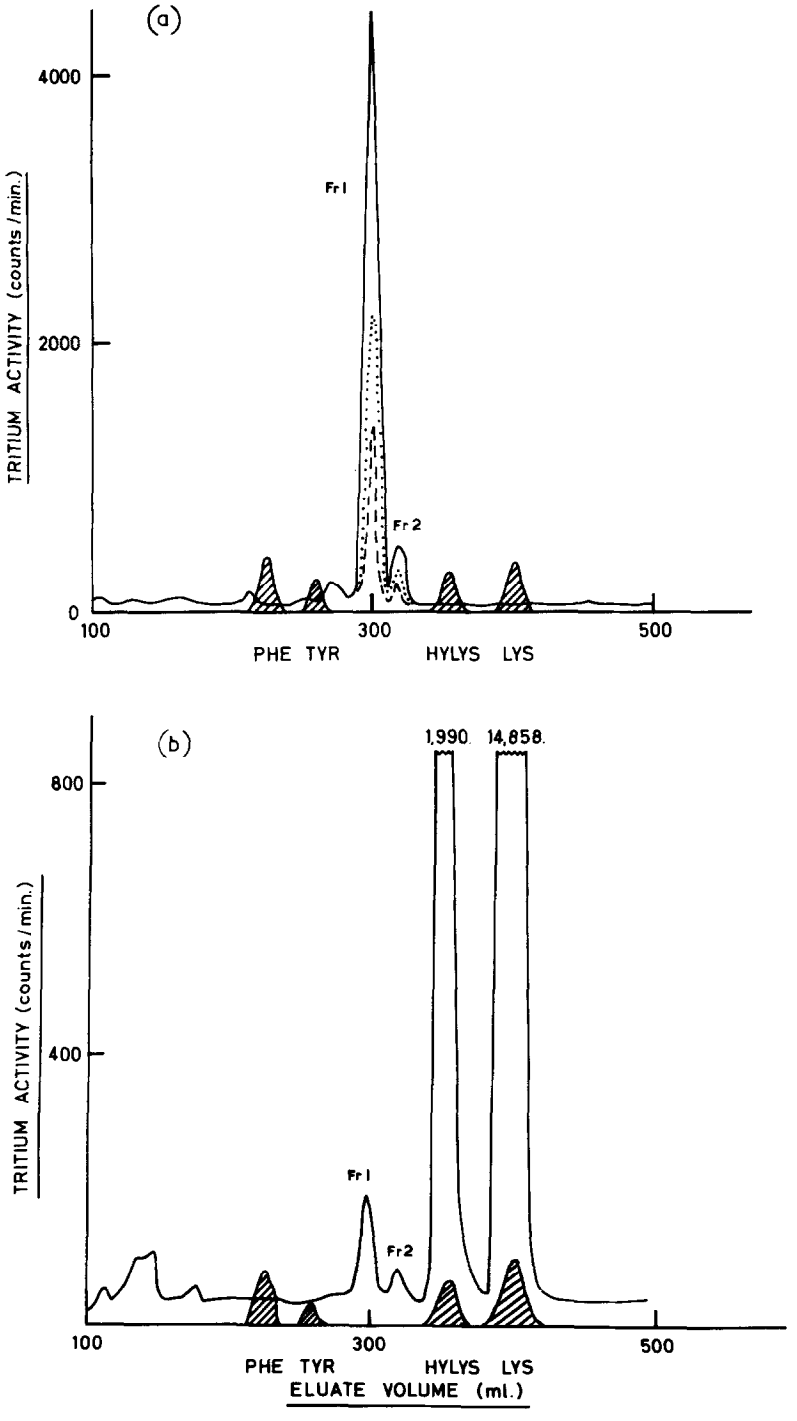


Fig. 1. Distribution of radioactivity (tritium) of acid hydrolysates of reduced decalcified bone collagen.
a) Lathyritic bone reduced with tritiated sodium borohydride; ——— control; .... 15 mgm β APN; — — 30 mgm β APN.
b) H^3-lysine bone collagen reduced with inactive sodium borohydride.
(hatched peaks denote positions of certain amino acids, peak areas not to scale)

analysed as described above. The elution pattern demonstrated that the two major reducible peaks, Fr. 1 and 2, were again radioactive indicating their derivation from lysine or hydroxylysine (Fig. 1b). From the tritium activities relative to lysine or hydroxylysine, the amount of Fr. 1 corresponded to one crosslinking amino acid per two tropocollagen molecules, and Fr. 2 to one crosslink per eight tropocollagen molecules.

Paper electrophoresis. The isolated reducible components were subjected to electrophoresis in ammonium carbonate buffer (0.1% w/v pH 8.7) at 50 volts/cm for 30 mins. Both components chromatographed as single ninhydrin-positive radioactive spots. Comparison of their mobilities with lysine, hydroxylysine, lysinonorleucine and hydroxylysinonorleucine confirmed the

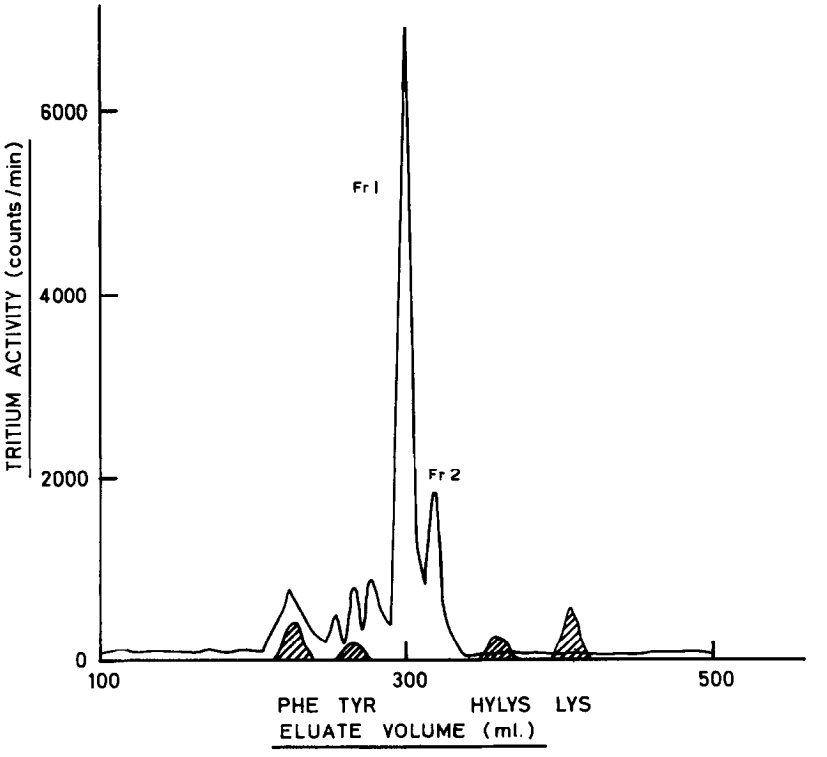


Fig. 2. Elution pattern of radioactive (tritium) reducible components from an acid hydrolysate of decalcified dentine reduced with tritiated sodium borohydride. (hatched peaks denote position of certain amino acids, peak areas not to scale)

identity of Fraction 2 with hydroxylysinonorleucine. Fraction 1 appeared to be less basic.

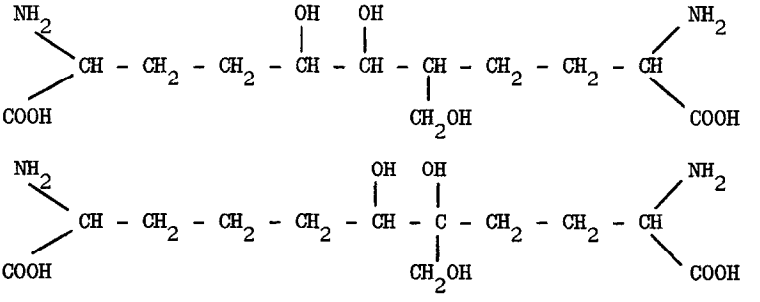
Characterisation of Fraction 1

Elemental analysis. Combustion analysis for the methyl ester; found N. 8.35, $C_{14}H_{28}N_2O_7$ requires N. 8.32%.

Mass spectrum of the trifluoroacetyl methyl esters. The volatile derivatives of the isolated Fractions 1 and 2 were prepared as described previously (Bailey and Peach, 1968). Direct injection of the sample into the ion source of the LKB 9000 GC-MS at 110° afforded a mass ion of m/e 816 (Fig. 3). Peak matching against p.f.k. gave an exact mass measurement of 816.10 ($C_{24}H_{23}N_2O_{12}F_{15}$ requires mol. wt. 816.10).

The ethyl ester was prepared from the methyl ester by reacting with anhydrous ethanol in the presence of HCl. Treatment with trifluoroacetic anhydride, as above, afforded the ethyl ester trifluoroacetate. The ethyl ester had a mass ion of 844 (Fig. 3b), the difference of 28 between this mass and the mass of the methyl ester confirmed the presence of two carboxy groups.

From the exact mass ion, the presence of two amino acid residues, the electrophoretic mobility, the nitrogen content and its derivation from lysine it was concluded that the compound was a reduced aldol derived from the condensation of the δ-semi aldehydes of lysine and hydroxylysine. The configuration of two of the possible isomers may be written:



In view of the number of possible isomers resulting from the aldol condensation of the δ-semi aldehydes derived from hydroxylysine and lysine we suggest the non-systematic name 'syndesine' (Greek σύνδεω meaning bind together) and

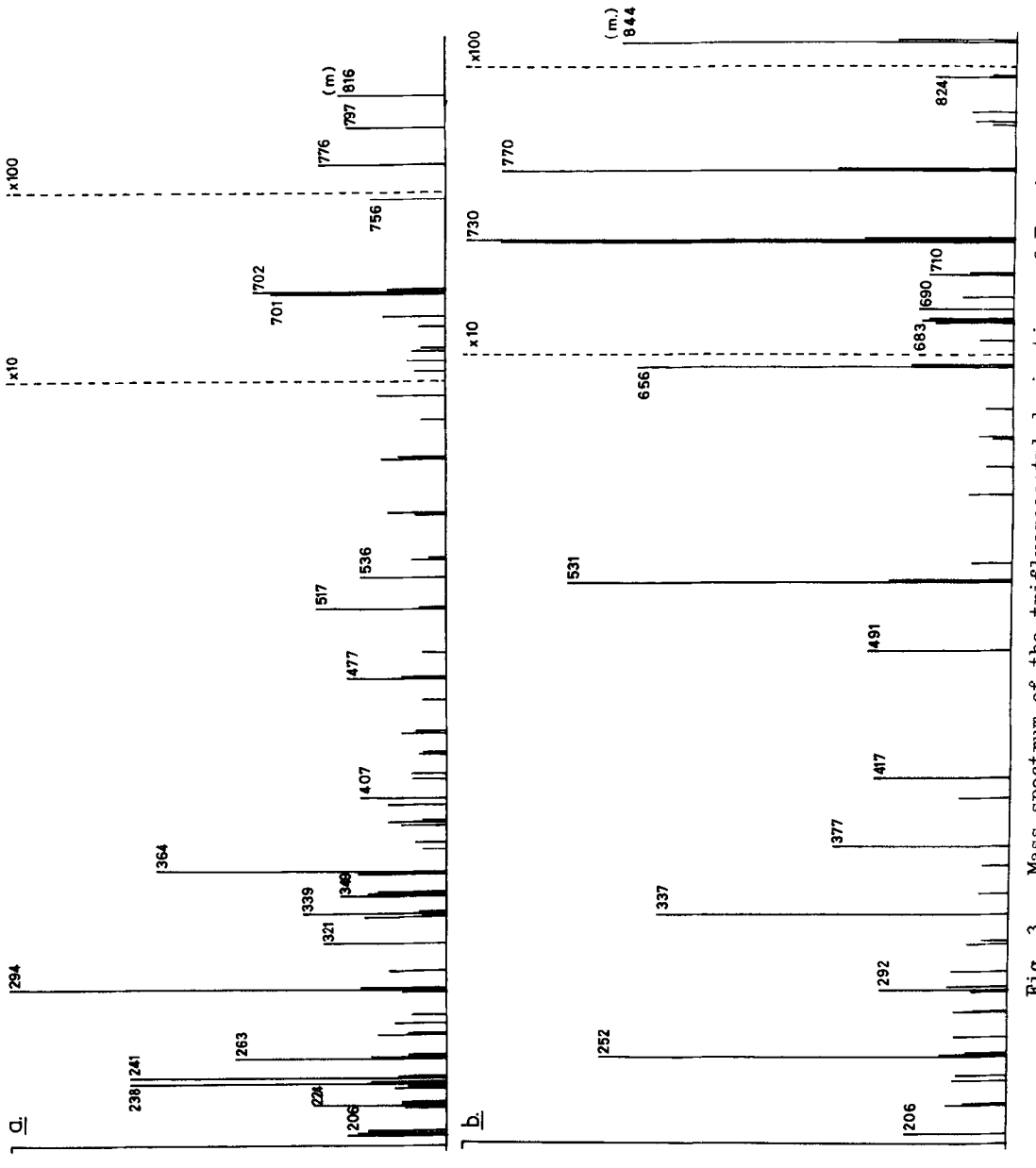


Fig. 3. Mass spectrum of the trifluoroacetyl derivatives of Fr. 1.
a) methyl ester, b) ethyl ester.

'syndesinol' for the reduced product $C_{12}H_{25}N_2O_7$ isolated in these experiments.

Characterisation of Fraction 2

The molecular ion of Fr. 2 was recorded at 703. The value of the mass ion, electrophoretic mobility, elution position of the component on the amino acid analyzer confirmed that this component was identical with hydroxylysinonorleucine, previously isolated from tendon collagen (Bailey and Peach, 1968).

DISCUSSION

The isolation and identification of the reduced form of two interchain crosslinks of bone and dentine offers direct confirmation that the stability of these collagens is dependent on a system of covalent bonds. The cross-linking mechanism has a basic similarity to that found in soft tissue collagen (Bailey and Peach, 1969). The aldol bond is stable during the thermal denaturation of collagen and resists dissociation by weak acids. In chick bone collagen it constitutes one crosslink for every two collagen molecules and may therefore contribute the major stabilizing bond. However, further studies should establish whether these bonds remain as aldols and aldimines, or are intermediate bonds that undergo further reaction with other active groups. The nature of the crosslink accounts for the previously observed ability of periodate to solubilize dentine (Veis and Schlueter, 1964) and possibly also for the decrease in hydroxylysine content of bone with increase in age of the tissue (Miller et al., 1968).

The crosslinks would arise biosynthetically through the condensation of the aldehydes derived from lysine and hydroxylysine by enzymic oxidation. Hydroxylysine is involved in both the crosslinks thus establishing a further role for this unique amino acid residue. It is significant that conditions inhibiting the hydroxylation of the lysine e.g. in vitamin C deficiency, lead to an impairment of the fibril stability (Gould, 1968).

A lysine-derived aldehyde (allysine) has been demonstrated to reside in the telopeptide region of tropocollagen from rat skin (Bornstein and

Piez, 1966). A very recent report by Miller et al. (1969) shows that in lathyritic bone collagen the telopeptide lysines may be hydroxylated to the extent of about 50%. In normal bone collagen a proportion of these hydroxylysines may undergo oxidative-deamination to give a hydroxylysine-derived aldehyde. Based on the present identification of the bone and dentine collagen crosslinks we propose that the telopeptides of these collagens possess an aldehyde derived from hydroxylysine.

We suggest that the role of the hydroxylysine in the telopeptides may be to control the formation of the crosslink. The greater the proportion of lysine hydroxylated the more hydroxylysine available for enzymic conversion to the aldehyde and subsequent crosslink formation. Future investigations should therefore reveal a smaller proportion of telopeptide lysine converted to hydroxylysine in calf tendon collagen. This would be consistent with the smaller proportion of the aldol crosslink in calf tendon and the virtual absence in rat tendon collagen (Bailey and Peach, 1969).

Further studies are being carried out to isolate the hydroxylysine-derived aldehyde and to establish the location of the aldol crosslink.

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REFERENCES

Bailey, A.J. (1968a) Comprehensive Biochemistry, Vol. 26B. (M. Florkin and E.H. Stotz eds.) p. 297. Elsevier Publishing Co., Amsterdam.
Bailey, A.J. (1968b) Biochim. Biophys. Acta, 160, 44.
Bailey, A.J. and Peach, C.M.(1968) Biochem. Biophys. Res. Communs. 33, 812.
Bailey, A.J. and Peach, C.M. (1969) In press.
Bornstein, P. and Piez, K.A. (1966) Biochemistry, 5, 3803.
Glimcher, M.J. and Katz, E.P. (1965) J. Ultrastructure Res., 12, 705.

Glimcher, M.J., Katz, E.P. and Travis, D.F. (1965) J. Ultrastructure Res., 13, 163.
Gould, B.S. (1968) In: Treatise on Collagen (G.N. Ramachandran ed.), Vol. 2, Part A., p. 323. Academic Press, N.Y.
Miller, E.J., Martin, G.R., Piez, K.A. and Powers, M.J. (1967) J. Biol. Chem., 242, 5481
Miller, E.J., Lane, J.M. and Piez, K.A. (1969) Biochemistry, 8, 30.
Partridge, S.M. and Brimley, R.C. (1952) Biochem. J., 51, 628.
Piez, K.A. (1968) Ann. Rev. Biochem., 37, 547.
Veis, A. and Schlueter, R.J. (1964) Biochemistry, 3, 1650