ISOLATION AND STRUCTURAL IDENTIFICATION OF A LABILE INTERMOLECULAR CROSSLINK IN COLLAGEN

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Although considerable evidence is available on the chemistry of the intramolecular bond in soluble collagen (Piez 1968) the nature of the intermolecular bond in intact collagen is unknown. However, the absence of both intra and inter molecular bonds in lathyritic collagen (Martin et al 1963) suggests a basic similarity in their chemical constitution, and both may be mediated through condensations involving a 'lysine-derived aldehyde' formed during maturation of collagen (Piez et al 1966).

It has recently been shown that in addition to the stable intermolecular crosslinks present in collagen (Veis and Anesey 1965) young native collagen contains a proportion of labile intermolecular crosslinks, and it was proposed that they involved an aldimine bond (Bailey 1967: 1968).

Reduction of the fibres with tritiated sodium borohydride stabilized the bonds and other aldehydic components to acid hydrolysis, and afforded their location on the amino acid analyzer. Comparison of these analyses with those of fibres reduced after cleavage of the labile intermolecular bonds demonstrated the position of the stabilized bond on the amino acid analyzer (Bailey and Lister 1968). The present communication presents evidence that one of the labile intermolecular crosslinks is Δ7,8 dehydro N- ϵ -(5-amino 5-carboxypentanyl) hydroxylysine.

EXPERIMENTAL AND RESULTS

Isolation of Reducible Components. Rat tail tendons were reduced with tritiated sodium borohydride, hydrolysed, and analyzed with a Technicon amino

acid analyzer as described previously (Bailey 1963). Fractions of the radioactive peak eluting prior to hydroxylysine were obtained from several runs, bulked, and refractionated. The material finally eluted as poorly resolved twin peaks just before the hydroxylysine position. Analysis of the separate peaks by mass spectrometry showed them to be identical, indicating the presence of isomers.

Paper Electrophoresis. The material chromatographed as a single spot in ammonium carbonate buffer (0.1% w/v; pH 8.7) at 50 volts/cm. for 30 minutes. Comparison of its mobility, corrected for endosmotic flow, with lysine, lysinonorleucine and merodesmosine indicated that the material was very slightly less basic than lysinonorleucine.

Incorporation of C-14 Lysine. Rats (100 gm. body wt.) were sacrificed six days after intraperitoneal injection with C-14 lysine (0.025 mc.). The tail tendons were reduced with inactive sodium borohydride and after acid hydrolysis analysed as described above. The elution pattern of the C-14 labelled collagen displayed the same six major radioactive peaks observed for $NaBT_4$ reduced

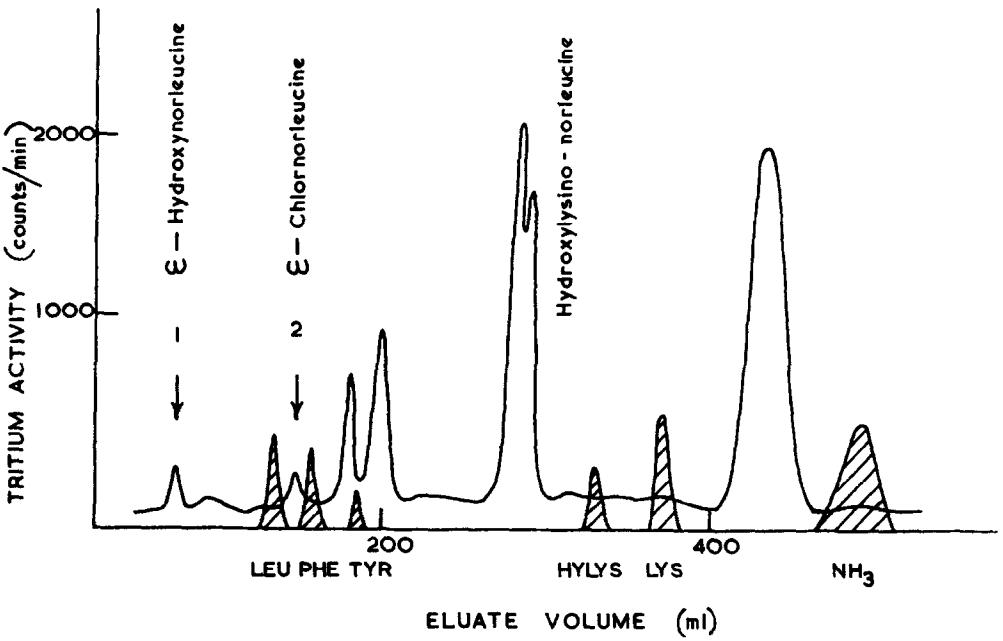


Fig. 1. Elution pattern of radio-active reducible components obtained from an acid hydrolysate of $NaBT_4$ reduced collagen.

collagen (Fig. 1). The reducible labile bonds must therefore be derived at least in part from lysine.

Mass spectrum of the trifluoroacetyl methyl ester. An aliquot of the isolated material (1 mg.) was added to anhydrous methanol (2 ml.) and dry hydrogen chloride bubbled through the solution for 35 minutes. After evaporation to dryness the residue was treated with trifluoroacetic anhydride (0.5 ml.) for 3 hours at room temperature. Evaporation of the solvent afforded the derivative for mass spectrographic analysis. The molecular ion was recorded at m/e 703. Exact mass measurement by peak matching against perfluorokerosene gave a mass ion of 703.44 ($C_{22}H_{25}F_{12}N_3O_9$; mol. wt. 703.44). From the exact mass ion, the fragmentation pattern, and the other evidence obtained about the molecule, it was deduced that the compound was O, N tetra trifluoroacetyl N ε- (5-amino 5-carboxypentanyl) hydroxylysine methyl ester.

Chemical synthesis of N- ε- (5-amino 5-carboxypentanyl) hydroxylysine.

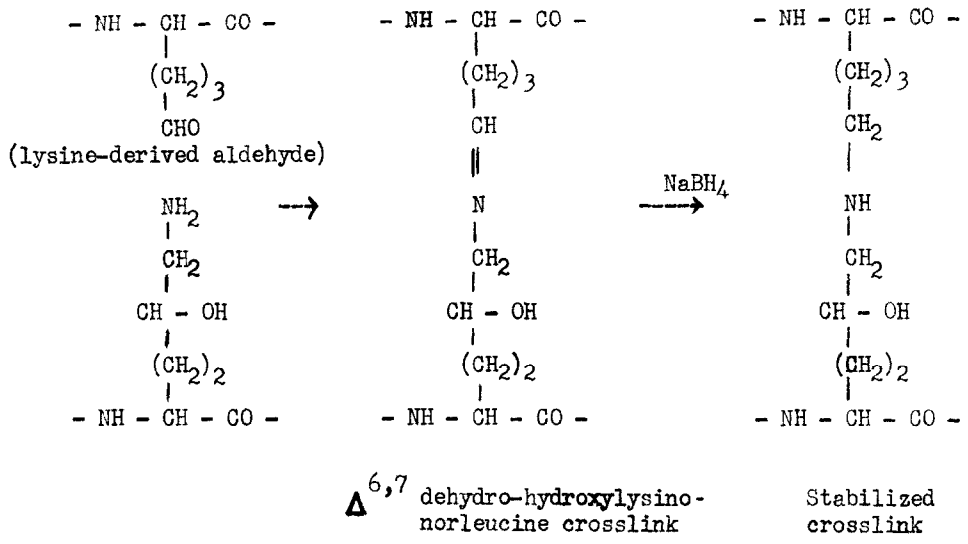
This compound was prepared by modification of the scheme presented by Franzblau (1968) for the synthesis of lysinonorleucine.

a) 5- δbromobutylhydantoin was prepared from 2;3 dihydropyran following the procedure detailed by Gaudry (1948).

b) Nα- trifluoroacetyl hydroxylysine methyl ester. Hydrogen chloride was bubbled into a methanolic solution (5 ml.) of DL - 5- hydroxylysine HCl (50 mg.) for 40 min. After evaporation in vacuo the residue was dissolved in trifluoroacetic acid (0.4 ml.), trifluoroacetic anhydride added (0.06 ml.) and the mixture stirred for 15 min. at 0°C. The solution was evaporated to dryness in vacuo to yield the Nα mono-trifluoroacetyl derivative.

c) The 5- δ- bromobutylhydantoin (44 mg.) and triethylamine (0.05 ml.) were added to the residue from b) dissolved in tetrahydrofuran (5 ml.) and the mixture refluxed 21 hr. The syrup obtained after evaporation of the solvent was hydrolysed with 2N NaOH (2 ml.) at 105° for 17 hr. The material was purified by fractionation using the amino acid analyser. The peak eluted in the same position as the compound isolated from collagen and mixture of the two showed no separation. Further analysis by electrophoresis and mass spectrum

a) Biosynthesis.



b) Chemical Synthesis

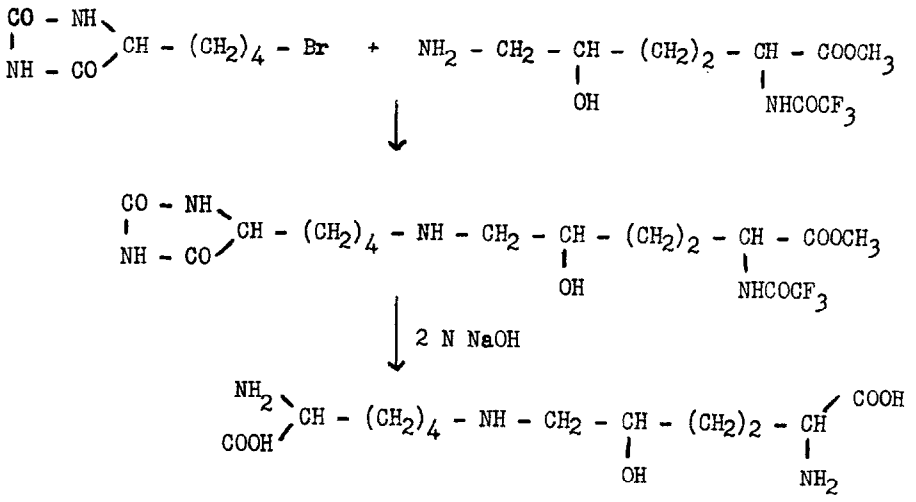


Fig. 2. Synthesis of hydroxylysino-norleucine.

confirmed that the two compounds were identical.

Volatile derivatives of ε-hydroxynorleucine and ε-chlornorleucine.

The peaks designated 1. and 2. (Fig. 1) were identified as ε-hydroxynorleucine and ε-chlornorleucine respectively. The latter compound is an artifact

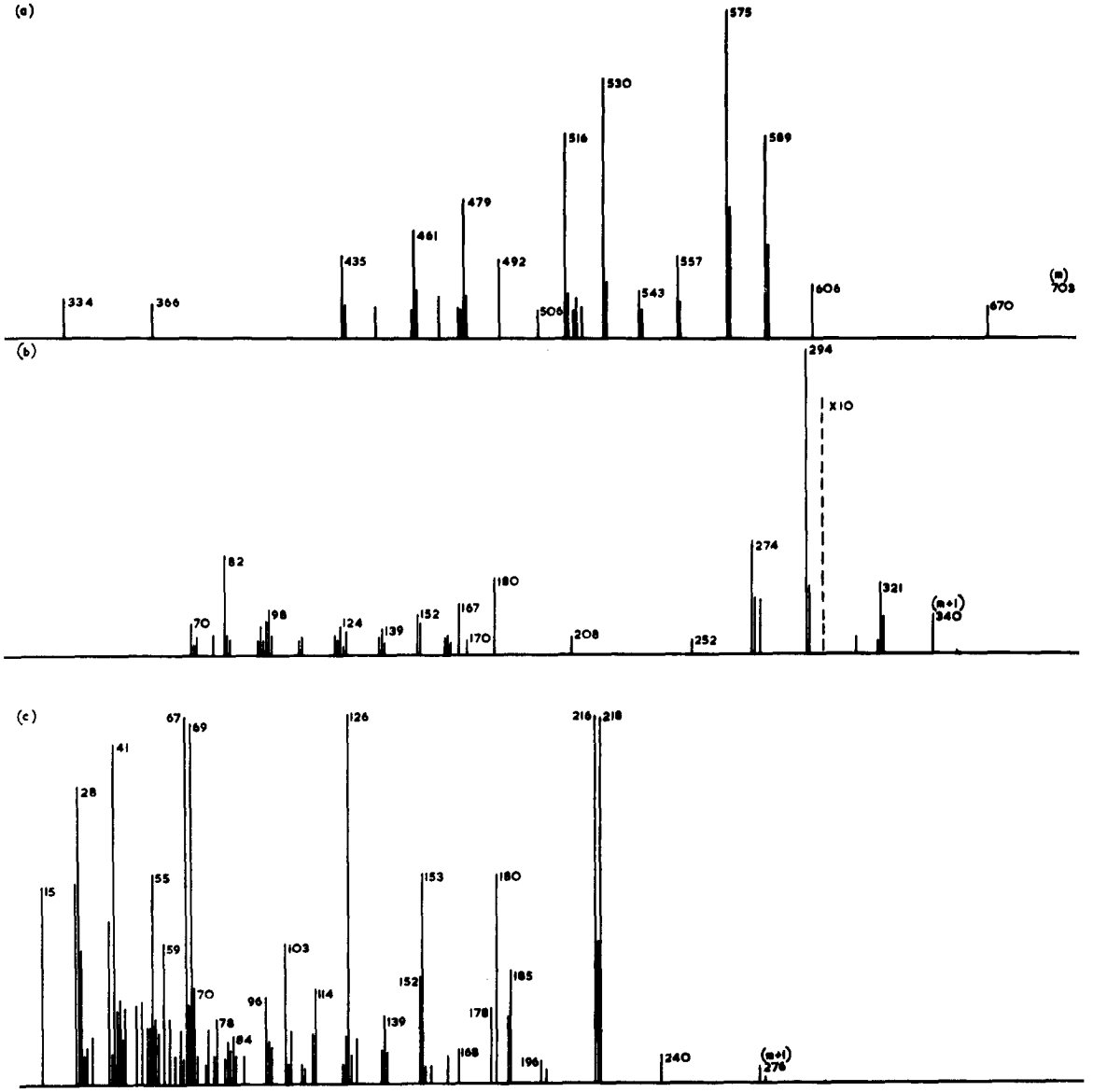


Fig. 3. Mass spectrum of a) O,N tetra trifluoroacetyl N-ε-(5-amino 5-carboxypentanyl) hydroxylysine methyl ester.
b) O,N di-trifluoroacetyl ε-hydroxynorleucine.
c) N-trifluoroacetyl ε-chlornorleucine methyl ester.

formed from ε-hydroxynorleucine during acid hydrolysis. Identification was confirmed by comparison with authentic material synthesised from 2,3 dihydropyran (Gaudry 1948). Both these compounds were also isolated from the hydrolysate of the dialyzable telopeptide fragments cleaved from $NaBT_4$ reduced collagen by peptic digestion.

Hydroxynorleucine. O,N di-trifluoroacetyl ε-hydroxynorleucine ($C_{10}H_{11}F_6NO_5$; mol. wt. 339.05) gave an exact mass ion of 340.06 using $C_{23}H_{48}$ as the mass standard. This corresponds exactly to the molecular mass ion + 1.

Clornorleucine. N-trifluoroacetyl ε-chlornorleucine methyl ester ($C_9H_{13}F_3ClNO_3$; mol. wt. 275.05) gave a mass ion +1 of 276.06 using $C_{19}H_{40}$ as the mass standard.

DISCUSSION

Our results clearly indicate that the new amino acid isolated from reduced native collagen is N ε-(5-amino 5-carboxypentanyl) hydroxylysine, or hydroxylysino-norleucine adopting the nomenclature used for lysinonorleucine (Franzblau et al 1965), and its structure has been confirmed by synthesis. Both the synthetic and the isolated compounds were a mixture of isomers. The presence of isomers is to be expected since the molecule is derived from hydroxylysine which exhibits diasterioisomerism. Epimerisation at C-2 occurs during the acid hydrolysis of hydroxylysine (Hamilton and Anderson 1955).

Since the compound appears in the hydrolysis products only after reduction it is assumed to exist in the collagen fibre in the labile Schiff base form, Δ6,7 dehydro-hydroxylysino-norleucine. Evidence that this component constitutes one of the labile intermolecular bonds in native fibres has been presented previously (Bailey 1968, Bailey and Lister 1968). The crosslink could be formed biosynthetically through the condensation of the carbonyl function of the lysine-derived aldehyde, α-amino adipic δ-semi aldehyde, with the ε- amino group of hydroxylysine. The reduced form of this aldehyde, ε-hydroxynorleucine, together with its acid hydrolysis product ε-chlornorleucine, were isolated from the telopeptide region of reduced collagen. Both ε-hydroxynorleucine and ε-chlornorleucine have very recently been isolated from reduced gelatin (Gallop et at 1968).

The lysine-derived aldehyde which appears to be the fundamental precursor for the crosslinking process in collagen has been shown to reside solely in the telopeptide region. This region does not contain hydroxylysine (Kang et al 1967) indicating that the new amino acid must exist as an inter molecular bond from

the telopeptide region of one molecule to the body of the second molecule. This would be consistent with the generally accepted "quarter stagger" alignment of the molecules in the native fibril. Further support is afforded by the evidence presented by Tanzer (1968) that in contract to reconstituted native-type fibrils the SLS form was not rendered insoluble on reduction.

Analysis of reconstituted fibrils, despite a more complex pattern, due to fragmentation products of the native labile bonds produced during dissolution, also revealed the presence of hydroxylysino-norleucine. This supports our belief that it is a primary intermolecular bond. It is presumably one of the unknown components recently reported by Tanzer and Mechanic (1968). Since the labile bonds are ruptured during gelatin formation no relationship to the components isolated from reduced gelatin by Blumenfeld and Gallop (1966) would be expected.

The proportion of this labile crosslink decreased with increase in age of the tissue and the route by which it is stabilized remains to be elucidated. It is significant that the most insoluble collagens, bone and dentine, contain the highest proportion of hydroxylysine. The second labile crosslink eluting prior to ammonia appears much more complex and probably crosslinks several molecules. Analysis of this component and the route by which it is stabilized is currently in progress.

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