ISOLATION OF LYSINONORLEUCINE FROM COLLAGEN

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Summary

A number of unique amino acid crosslinks have been isolated from collagen and elastin and, except in the case of the aldol formed from two residues of α-amino adipic semialdehyde, appear to be specific to each protein. In the course of isolating and identifying the crosslinks of collagen, we found considerable quantities of lysinonorleucine in insoluble calf skin collagen and smaller amounts in reconstituted calf skin collagen fibrils and in calf nasal cartilage. Lysinonorleucine was originally discovered in bovine elastin and has not been reported to occur in other connective tissue proteins. It may arise in collagen as a consequence of the incomplete hydroxylation of lysine residues.

The connective tissue proteins, collagen and elastin, undergo time dependent changes in primary structure, including hydroxylation and glycosylation of amino acids (1), conversion of peptide bound lysine to α-amino adipic semialdehyde (2) and condensation reactions of α-amino adipic semialdehyde to form several amino acid crosslinks (3). One of the latter compounds, lysinonorleucine, was discovered in elastin (4) but has not been described as occurring in collagen. Indeed, the only crosslink common to both proteins appears to be the aldol condensation product of two residues of α-amino adipic semialdehyde (5,6). In the present report we describe the isolation of lysinonorleucine from insoluble calf skin collagen and its presence in calf nasal cartilage and in reconstituted calf skin collagen fibrils.

Experimental

Insoluble skin collagen was prepared by exhaustively extracting finely

minced calf skin until no further soluble collagen could be detected. Calf nasal cartilage was prepared and finely divided by the method of Sajdera and Hascall (7). Reconstituted collagen fibrils and the collagenous tissues were treated with NaB^3H_4 as previously described (8). The samples were refluxed in 3N HCl for 48 hours (9) and then dried by flash evaporation. Chromatographic fractionation was carried out on an amino acid analyzer and radioactivity was monitored in a toluene based scintillation fluid employing Beckman Biosolve-3 according to the manufacturer's directions. Radioactive compounds were isolated from preparative columns and purified until homogeneous as determined by amino acid analysis and thin layer chromatography.

Satisfactory derivatives for mass spectrometry were obtained by reaction with acetic anhydride followed by permethylation using a modified Hakomori method (10)*. Permethylation was adopted to prevent the ring closure rearrangements of lysine derivatives in the mass spectrometer, thereby increasing the probability of detecting the higher mass fragments. Preliminary studies had shown that, without permethylation, only the lower mass fragments were detectable. Synthetic lysinonorleucine was isolated following reaction of brombutylhydantoin (Eastman Kodak) with α-N-CBZ lysine methyl ester. The method of synthesis closely paralleled that of Franzblau et al. (11) except that the methyl ester was synthesized from α-N-CBZ lysine (Cyclo) using thionyl chloride in methanol and the CBZ group was removed by HBr in glacial acetic acid just prior to alkaline hydrolysis. The hydrobromide salt was precipitated by cold ether and subjected to alkaline hydrolysis. Lysinonorleucine was isolated from the neutralized hydrolysate by ion exchange chromatography. Mass spectral analyses were performed on the medium resolution Hitachi-Perkin-Elmer RMU-6E machine by Mr. Edward Henson in Dr. Paul Gallop's laboratory.

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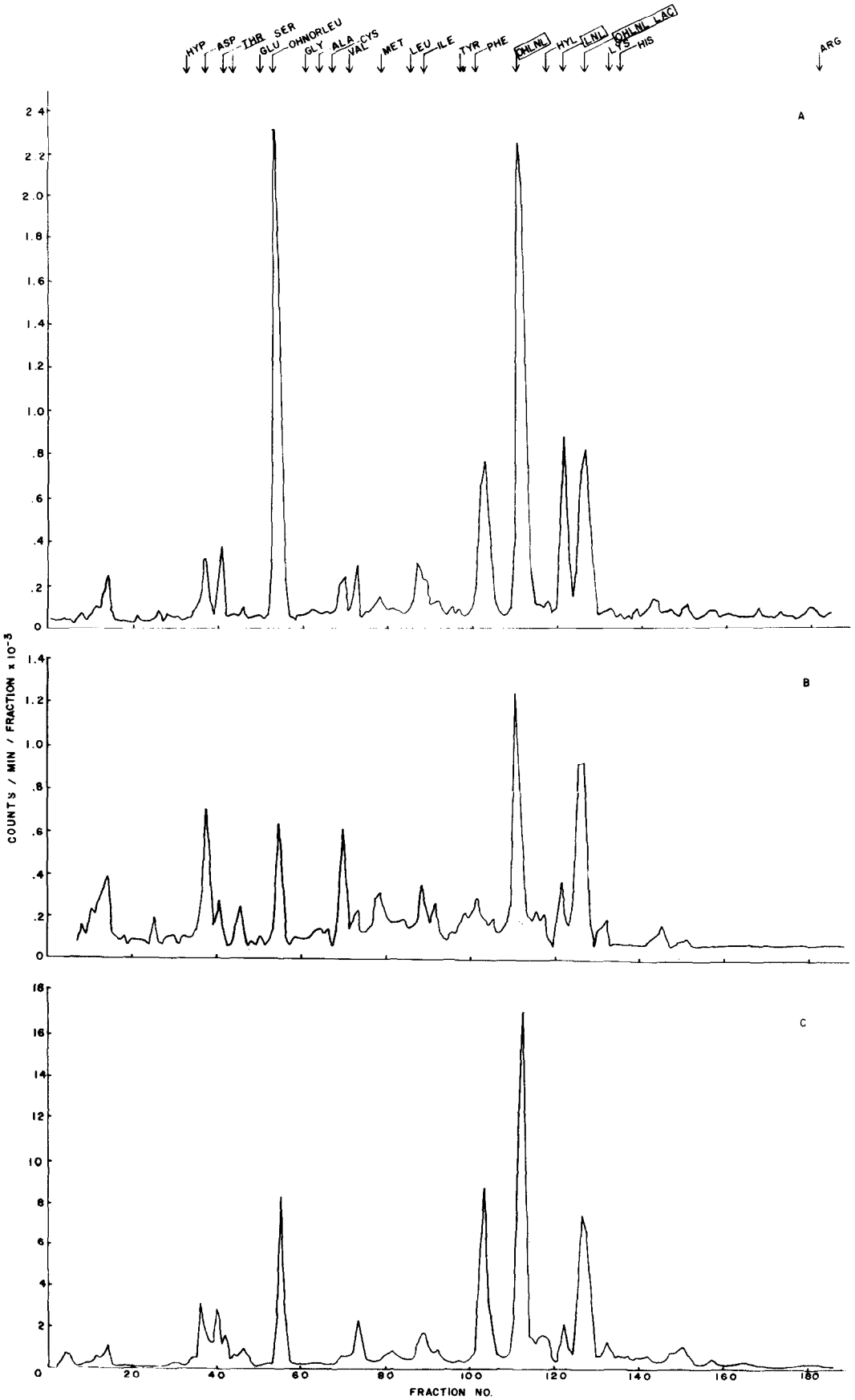
*Paz, M.A., Bernath, A., Henson, E., Blumenfeld, O.O., and Gallop, P.M., submitted for publication.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1. Chromatographic fractionation of acid hydrolysates of sodium borotritide-treated collagen preparations. (a) insoluble calf skin, (b) reconstituted calf skin fibrils, (c) calf nasal cartilage. The non-standard abbreviations are: OHNORLEU, hydroxynorleucine; OHLNL, hydroxylysinonorleucine; LNL, lysinonorleucine; OHLNL LAC, hydroxylysinonorleucine lactone.

Results and Discussion

The peak appearing between hydroxylysinonorleucine (12) and hydroxylysinonorleucine lactone+, shown in Fig. 1, was isolated and purified. The acetylated, permethylated derivative of this compound was analyzed by mass spectrometry. The results and their interpretation are shown in Fig. 2. This spectrum is notable for its intense fragments, prominent parent peak and characteristic half masses. Synthetic lysinonorleucine yielded the identical spectrum.

In light of the known incompleteness of lysine hydroxylation (13) and the fact that two of the major radioactive components in Fig. 1 are hydroxylysinonorleucine and its lactone, it seems likely that lysinonorleucine could arise as a consequence of the condensation of α-amino adipic semialdehyde and partially hydroxylated lysine residues. This would be less likely if hydroxylysinonorleucine arose from some other pathway. However, as shown in Fig. 3, periodate degradation of isolated hydroxylysinonorleucine yields almost stoichiometric amounts of radioactive lysine, as would be expected if the original Schiff base (prior to NaB^3H_4 exposure) arose from condensation of α-amino adipic semialdehyde and hydroxylysine. This result is entirely consistent with our previous finding that it is primarily α-amino adipic semialdehyde which contributes to the crosslinks in reconstituted collagen fibrils (9).

It is not apparent why lysinonorleucine is particularly prominent in insoluble calf skin collagen compared to fibrils derived from soluble collagen (Fig 1B) and compared to calf nasal cartilage (Fig. 1C). It might be argued that the lysinonorleucine arises from traces of elastic fibres

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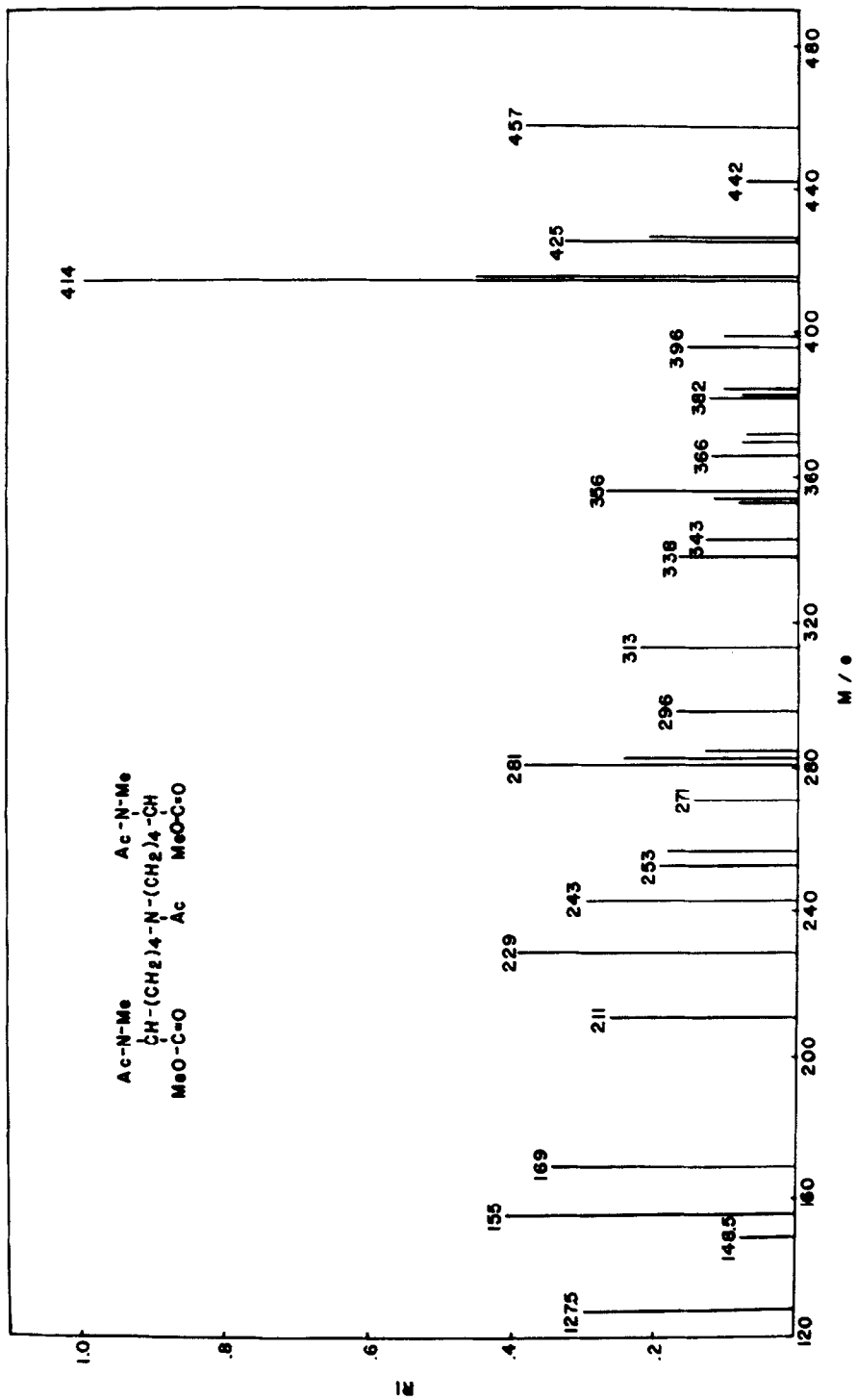
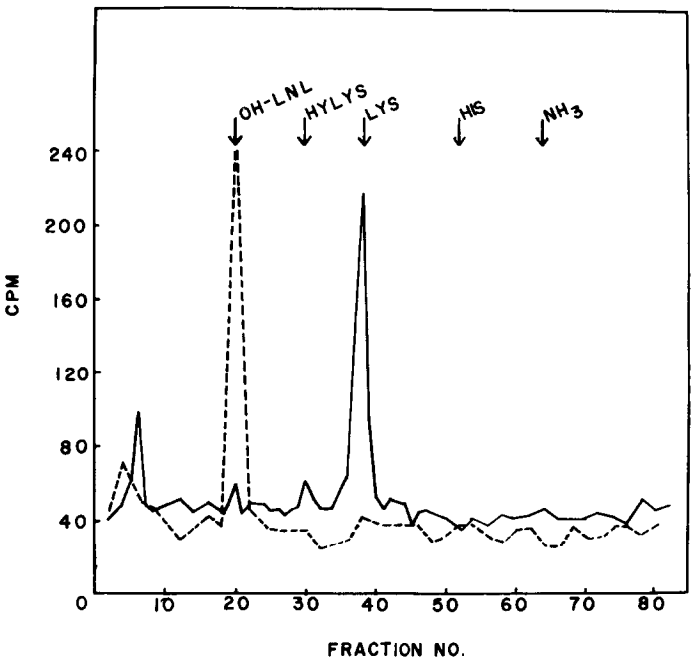


2. Mass spectrum of acetylated, permethylated lysinonorleucine.



3. Chromatography of hydroxylysinonorleucine and of a reaction mixture containing sodium metaperiodate and hydroxylysinonorleucine. The periodate oxidation was according to Bohak (15). (----), hydroxylysinonorleucine; (———), reaction mixture.

present in the insoluble calf skin. However, the reduction products of the desmosines (14), which are characteristic of elastin, do not seem to be present in the chromatogram. Furthermore, since lysinonorleucine is present in reconstituted fibrils prepared from highly purified collagen, and is also in cartilage, which has no elastic fibres, a portion of it must arise from collagen. It is apparent from Fig. 1 that quite a number of radioactive components are present; these may be more prominent in other tissues or animal species and perhaps also occur in elastin.

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