DIRECT MEASUREMENT OF COLLAGEN CROSSLINKS WITH AUTOMATIC AMINO ACID ANALYZER – IDENTIFICATION OF PEAKS DUE TO CROSSLINKS

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1. Introduction

Crosslinks of collagen contribute to characteristic physico-chemical stability of connective tissues [1,2]. Measurement of crosslinks of collagen has a very important value especially in connective tissue disorders, inflammatory diseases and changes due to ageing [3,4]. In recent years many crosslinks of collagen have been identified and measured quantitatively by the methods using reduction of crosslinks with tritium-labelled $NaBH_4$ [5,6]. With these methods we cannot detect all of the crosslinks including non-reducible ones. So we devised new method of measuring crosslinks with automatic amino acid analyzer using the ninhydrin reaction. In this report we describe the system for analyzing crosslinks with automatic amino acid analyzer, identification of chromatographic peaks due to crosslinks and the existence of HMD** in larger quantity than previously reported.

2. Materials and methods

Purification of crosslinks: Diaphyses of long bones were obtained from male Wistar rats, about 250 g body weight. Periosteum, bone marrow and soft tissue were dissected away and removed completely. Bone cortices were decalcified in 0.1 M citrate buffer with 0.2 M EDTA pH 4.0 and acid-soluble collagen was removed in sufficient 0.5 M acetic acid. Defatting and dehydration were performed in mixture of ethanol and ether (1:1). The remaining insoluble collagen, frozen in dry ice–acetone, was pulverized, reduced with $NaBH_4$ [7] and hydrolyzed in 3 N HCl refluxing under flow of N_2 gas for 48 h. About 10 g of the hydrolysate of collagen was applied to the following column chromatographs for the purification of crosslinks.

The first chromatography was performed on a Dowex 50W-X8 column (2.6 × 50 cm), with eluent, 0.35 M Na^+ (citrate pH 5.29, third buffer of amino acid analyzer). With this chromatography we excluded acidic and neutral amino acids and other products of hydrolysis. Performing rechromatography under the same conditions we collected fractions between Phe and Hyl that amounted to nearly 200 mg. Next chromatography was carried out on a Sephadex G-10 column (2.6 × 60 cm) with eluent 0.05 N acetic acid. With this chromatography we obtained two peaks, the first composed of Hyl and crosslinks and the latter of Phe. Finally on the long column (0.8 × 50 cm) of automatic amino acid analyzer, JEOL-JLA-6AH, 25 mg of fractions containing crosslinks and a little amount of Hyl was applied and eluted according to our method for crosslinks described later. Fractions were collected directly from the outlet of the long column. On the each step of chromatography, fractions were desalted and the peaks of amino acids and crosslinks were detected with ninhydrin test,

**Abbreviations:* HMD, hydroxymerodesmosine; EDTA, ethylenediaminetetraacetic acid; DHLNL, 5,5'-dihydroxylysinonorleucine; HLNL, 5-hydroxylysinonorleucine.

identified qualitatively with thin layer chromatography of cellulose and with automatic amino acid analyzer. Peaks of crosslinks were named A,B,C and D in the order of elution with amino acid analyzer (see fig.1). Repeating above mentioned procedures five times, 10 to 100 μmol of crosslinks (leucine equivalent) were obtained.

Method for determination of crosslinks with automatic amino acid analyzer: Glucosamine and galactosamine emerged just at the position of peak

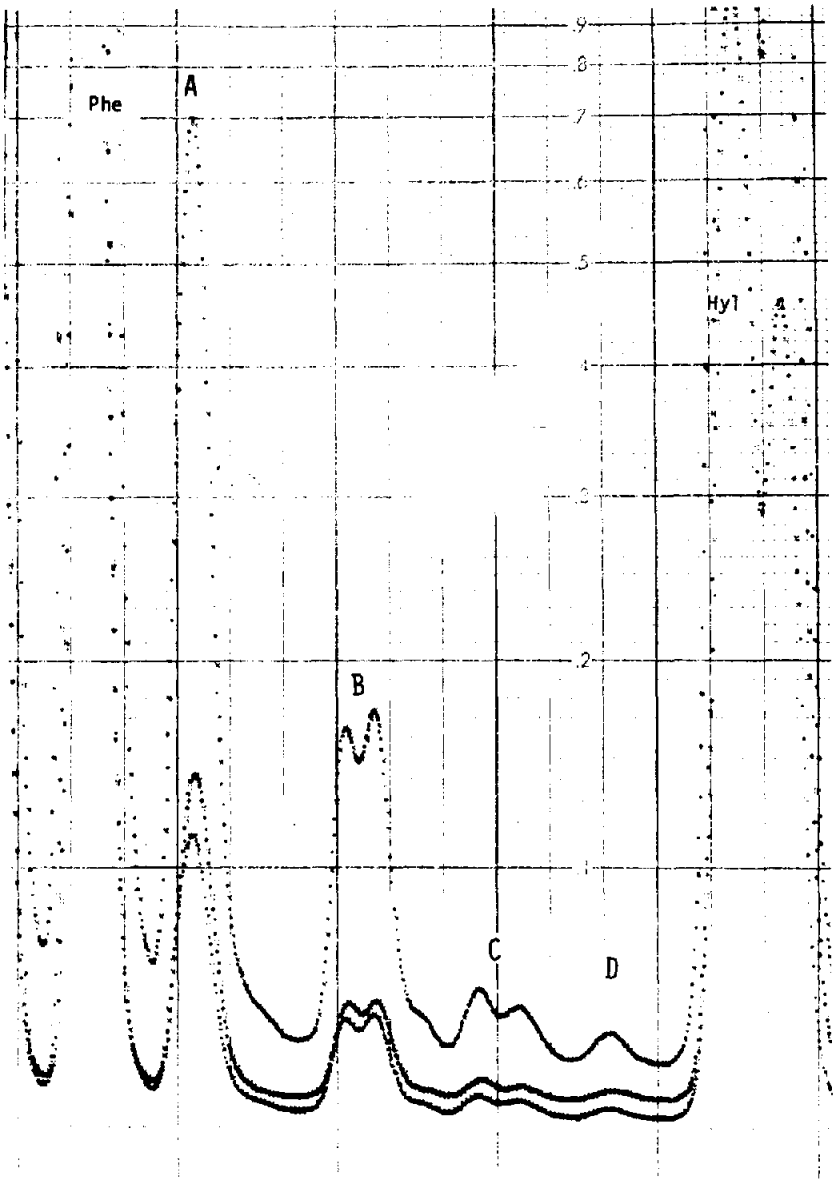


Fig.1. Elution of Phe, Hyl and crosslinks from a long column of automatic amino acid analyzer. This is obtained by application of 1 mg hydrolysate of reduced insoluble collagen of rat bone. Peak B and C consist of double peaks, respectively. Upper dotted line shows absorbance of 570 nm. Middle is absorbance of 440 nm. Lower is one third of the upper.

C and D according to the method of Davis [8]. Development of the long column with 0.2 M citrate buffer (pH 2.42) containing 0.15 M NaCl eluted hexosamine with acidic and some neutral amino acids. About 1 mg hydrolysate of collagen was applied. Elution of the column was performed with the buffer (pH 2.42) for 60 min and the third buffer for another 45 min at 0.98 ml min^{-1}. Amino acids eluting before 105 min were sent to drain, and switched from drain to coil just before emergence of Tyr. Then absorbance of ninhydrin reaction was recorded on a chart. Detection cell of 10 mm light path was used to increase the sensitivity.

N.m.r. method: 5 mg from peaks A and B were dissolved in 0.3 ml D_2O without standard material to avoid any contamination. P.m.r. was recorded on a Varian 100-XL NMR spectrometer after adjusting calibration with authentic amino acids containing 3-(trifluoromethylsilyl)-propane-sulfonic acid sodium salt. After measurement of sample the scale was rechecked and the true shift of each signal was calculated from the signal of H_2O.

Mass spectrography method: Purified materials from peak A, B and C were derivatized in two ways by permethylation according to Hakomori [9] and by trifluoroacetylmethyl esterification [10]. Derivatives were analyzed by direct inlet method in Shimazu-LKB 9000 GCMS or Jeol-JMS-01-SG-2 mass spectrometers.

3. Results and discussion

Fig.2 shows n.m.r. spectrum of purified peak A. Low solubility of material makes it difficult to interpret each signal in detail. If peak A is HMD, signal at 3.8 ppm is attributed to the proton on the hydroxylated carbon and protons of allylic methylene adjacent to imino group. Signal at 3.45 ppm although not forming clear triplet, is assigned to protons in α positions. Signal at 2.8 ppm is due to the protons of other allylic methylenes and protons on a carbon between the imino and the hydroxylated carbon. Signal at 1.7–1.55 ppm is due to ten protons of other methylenes. The signal of olefinic proton which usually appears in the region between 5.3 and 4.7 ppm does not appear and probably is overlapped by strong signal of H_2O (4.8–4.5 ppm). N.m.r. spectrum of peak B is not shown in this letter but it is also interpretable if B is DHLNL. In both spectra there are no peaks due to imidazol ring (7.8 and 7.0 ppm) or hexose (4.8 or 4.0 ppm) [11,12]. Both spectra cannot be interpreted by another known crosslinks. Fig.3

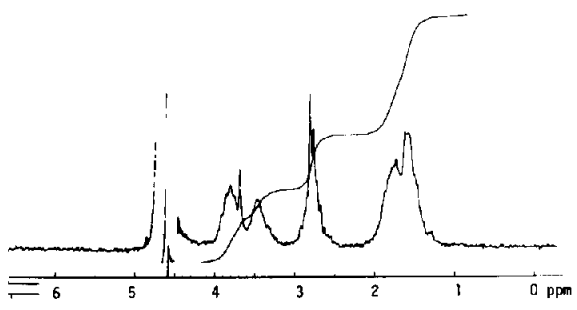


Fig.2. Proton magnetic resonance spectrum of purified peak A. 5 mg is dissolved in 0.3 ml D_2O.

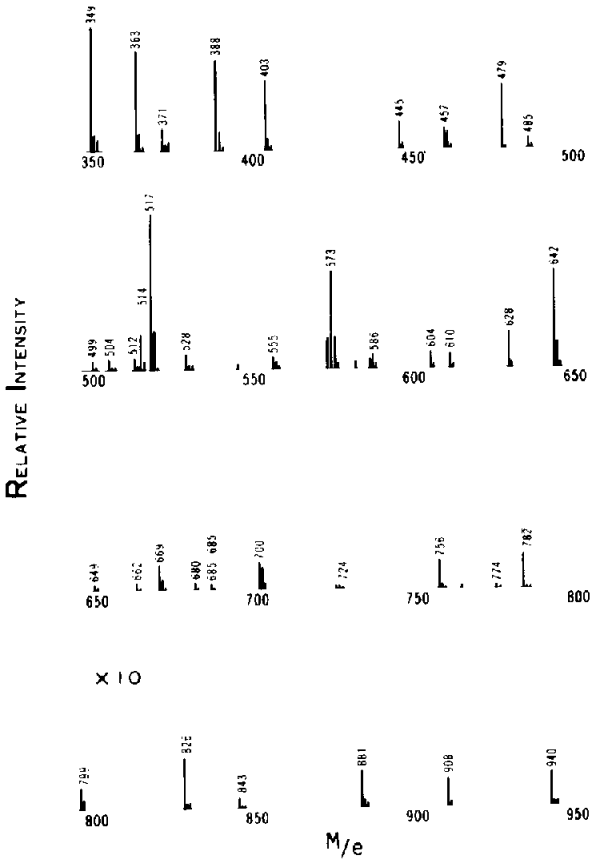
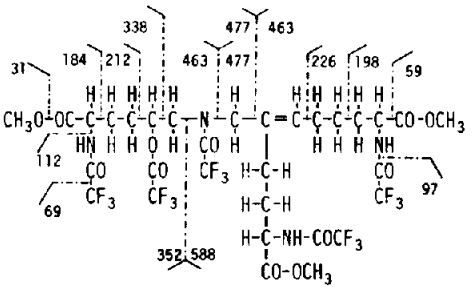


Fig.3. Mass spectrum of trifluoroacetylmethyl derivative of peak A.



M/e		M/e		M/e	
940	M	908	M - 32 (CH_3OH)	881	M - 59
843	M - 97 (CF_3CO)	826	M - 114 (CF_3COOH)	794	M - 114 - 32
782	M - 69 (CF_3)- 57 (Fx3)- 32			774	M - 97 - 69
756	M - 184	724	M - 184 - 32	700	M - 114 - 69 - 57 (Fx3) M - 112 - 69 - 59
685	M - 112x2 - 31 (CH_3O)	680	M - 114 - 32 - 113(CF_3COO)		
669	M - 212 - 59	662	M - 112 - 97 - 69	649	M - 184 - 69 - 38 (Fx2) M - 97x3
642	M - 184 - 114	628	M - 198 - 114 M - 184 - 69 - 59		
610	M - 184 - 114 - 32			604	M - 184 - 114 - 38
586	M - 114 - 112 - 69 - 59	573	M - 184 - 114 - 69	555	M - 212 - 114 - 59
528	M - 113 - 112 - 69 - 59x2			517	M - 212 - 114 - 97
514	M - 184 - 114 - 69 - 59	512	M - 212 - 114 - 32	504	M - 226 - 113 -97 M - 212 - 112x2
499	M - 198 - 184 - 32	485	M - 198x2 - 59 M - 212 - 184 - 59		
477	M'			459	M-113-112-69x2-59x2
457	M - 226 - 198 - 59	445	M - 212x2 - 113	403	M' - 69
388	M - 184x3	371	M'- 69 - 32	363	M' - 114

Fig.4. Derivative of HMD (pentatrifluoroacetyl-HMD-trimethyl ester), and interpretation of fragments higher than m/e 350 in the mass spectrum.

shows the mass spectrum of trifluoroacetylmethyl derivative of peak A. Parent peak ($M^+ = 940$) coincides with the derivative of HMD, pentatrifluoroacetyl-HMD-trimethyl ester. The peaks higher than m/e 350 are interpreted in fig.4. Mass spectrography of derivatives of peaks B and C was also performed and coincided with the derivatives of DHLNL and HLNL respectively. Peak D which can be obtained in a very small quantity from bone collagen of rat, was not analyzed with the n.m.r. spectrometer or with the mass spectrometer. The peak was determined coincidentally with the peak of lysinonorleucine which was purified from the hydrolysate of elastin. According to the method by Davis, HMD is partially included in the peak of Phe. When the citrate buffer, pH 2.42, is used as an eluent before the third buffer, the two peaks of Phe and HMD can be detected separately. We have proved HMD to exist in as large a quantity as that of DHLNL for the first time. This fact can be probably explained by the characteristic property of insoluble collagen of rat bone or by the difference in our procedure of sample preparation. In any way it is related to the fact that with our system for analyzing crosslinks we can hardly detect the remarkable peak corresponding to histidinohydroxy-merodesmosine whose existence is assumed to be an artificial product [13].

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