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Note

Determination of ε-hydroxynorleucine and "reduced aldol condensate" by standard amino acid chromatography

Collagen and elastin contain several types of aldehydes as intermediates in the cross-linking process, and it is convenient to identify these reducible compounds by the use of NaB^3H_4. When these two proteins are reduced with tritiated $NaBH_4$, hydrolyzed in alkali and resolved in an amino acid analyzer, the α-amino-δ-semi-aldehydes (AL), derived from oxidative deamination of lysine, are converted into residues of tritiated ε-hydroxynorleucine (HNL) and the "aldol condensate" formed by aldol condensation of two AL residues is recovered as tritiated "reduced aldol" (RA).

The amounts of HNL and RA are determined by counting on the radioactive peaks and relating the number of counts to the specific activity of the standardized $NaBH_4$–NaB^3H_4 mixture used for the reduction. The amount of "reduced aldol" can also be determined by the ninhydrin reaction in a Technicon AutoAnalyzer using the buffer system of BURNS *et al.*[1], which spreads out the amino acid chromatogram between isoleucine and ammonia.

However, neither method is easy to use on a routine basis. While the first method requires the use of radioactive material and of an analyzer equipped with a stream-splitting device, the second requires unusual chromatographic conditions.

Using a multi-sample, fully automated JLC-5AH analyzer, we have found that both HNL and RA are very easily detected and determined with a standard amino acid procedure and buffer system used for the resolution of collagen and elastin amino acids.

Methods

Bovine *ligamentum nuchae* elastin was prepared essentially according to the procedure of PARTRIDGE *et al.*[2]. The reduction of elastin was carried out as described by LENT *et al.*[3]. About 40 mg of $NaBH_4$ was added to 200 mg of bovine elastin suspended in 20 ml of 0.001 *M* EDTA solution (pH 9.0). Reduction was followed by automatic titration (Radiometer), keeping the pH constant by the addition of small amounts of HCl as required. The reaction was then stopped and the remaining $NaBH_4$ destroyed by adjusting the reaction mixture to pH 3.0 with HCl.

A sample of bovine *ligamentum nuchae* elastin reduced in the same way in a standardized mixture of $NaBH_4$ and NaB^3H_4 was obtained from Dr. C. FRANZBLAU.

The radioactivity of fractions from the analyzer was determined in a Nuclear Chicago liquid scintillation counter using Bray's solution as the scintillation fluid. Hydrolyses were performed in constant-boiling HCl at 110° for 22 h or in sealed alkali-resistant vials in NaOH for 22 h at 110°. When required, a stream-splitting device was used. A JLC-5AH analyzer was used for the experiments. The long

column (70 × 0.8 cm) and the short column (15 × 0.8 cm) were both packed with JLC-R-2 resin.

The basic amino acids were resolved on the short column by using 0.35 *N* sodium citrate buffer of pH 5.28. The acidic and neutral amino acids were separated on the long column by using a stepwise buffer gradient starting with 0.2 *N* sodium citrate of pH 3.30 and changing to 0.2 *N* sodium citrate of pH 4.25 after about 220 min.

Results and discussion

Aliquots of $NaBH_4$-reduced elastin were digested in 2 *N* NaOH, neutralized with concentrated HCl and diluted with water and an aliquot was placed on the amino acid analyzer. Figs. 1 and 2 represent portions of the analysis showing the presence of two unknown peaks, which are not present either in an acid hydrolyzate of reduced elastin or in an alkaline hydrolyzate of unreduced elastin, indicating that these peaks are derived from reduced compounds that are stable only on alkaline hydrolysis.

To show that these two peaks correspond to the two compounds HNL and RA, we repeated the experiment of LENT *et al.*[3], examining the radioactivity elution profile of a sample of NaB^3H_4-reduced elastin hydrolyzed under alkaline conditions. Two radioactive peaks appeared that corresponded exactly with the two unknown peaks. LENT *et al.*[3], in their Fig. 5, showed that the two peaks were HNL and RA, respectively.

The position in which HNL appears is the same as that found by LENT *et al.*[3]. In our system, the RA appears between methionine and *allo*-isoleucine, while it elutes between tyrosine and phenylalanine in the system of LENT *et al.*

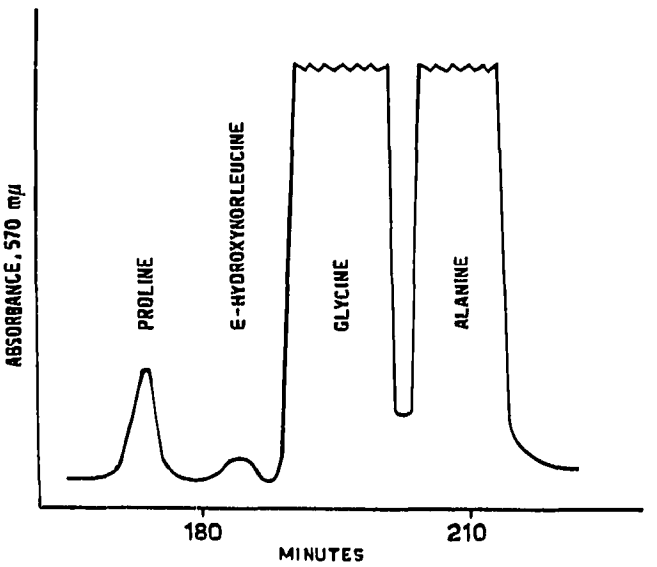

Fig. 1. Part of an amino acid chromatogram showing the separation of ε-hydroxynorleucine from a sample (0.8 mg) of adult bovine ligament elastin reduced with $NaBH_4$ and hydrolyzed in 2 *N* NaOH.

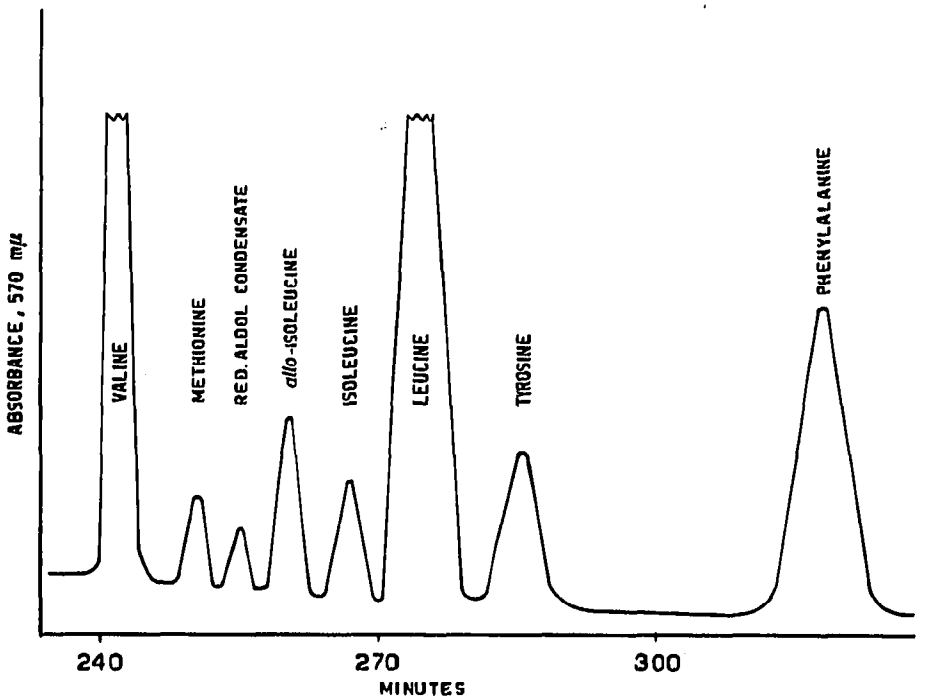

Fig. 2. Part of an amino acid chromatogram showing the separation of "reduced aldol condensate" from a sample (0.3 mg) of adult bovine ligament elastin reduced with $NaBH_4$ and hydrolyzed in 2 *N* NaOH.

Without modifying the buffer conditions for a standard multisample fully automated analyzer, we have achieved the separation, from all other amino acids, of ε-hydroxynorleucine and "reduced aldol condensate", two amino acids present in small amounts after reduction of elastin. We have therefore avoided the introduction of unusual chromatographic conditions and the determination of these compounds can be carried out in a standard amino acid chromatographic system on a routine basis.

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Institute of Histology,
University of Padova,
Padova (Italy)

D. VOLPIN
M. G. GIRO
I. CASTELLANI

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