SYSTEMATIC IDENTIFICATION OF ALDEHYDES AND ALDEHYDE-DERIVED CROSSLINKS IN ELASTIN BY METHODS INCLUDING A MODIFIED STRECKER REACTION*

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SUMMARY: The crosslinks in elastin of bovine ligamentum nuchae and of the intimal-medial and adventitial segments of rat thoracic aorta were evaluated comparatively. Reaction with [^{14}C] -sodium cyanide and ammonia was used to stabilize and identify aldehydes and certain aldehyde-derived crosslinks. The derivatives obtained demonstrated the presence in the aortic elastins of α-amino adipic acid δ-semialdehyde, dehydrolysinonorleucine and the aldol condensate of α-amino adipic acid δ-semialdehyde. In addition to lysinonorleucine and the desmosines, other, but as yet uncharacterized compounds were found to be present.

We have previously shown (1,2) that reaction of elastin with [^{14}C] -NaCN and NH_3 leads to stabilization of its aldehyde precursors of crosslinks and of aldehyde-derived crosslinks. These can be isolated, after acid hydrolysis, as [^{14}C] -labelled amino acids. Three such amino acids were isolated and their structures established; they are α,ε -diaminopimelic acid (DAP)⊟, derived from α-amino adipic acid δ-semialdehyde; 1,9-diamino, 1,9-dicarboxy, glycino-non-4-ene (carboxyamino aldol (CAA)) derived from the aldol condensate of two residues of α-amino adipic acid δ-semialdehyde; and 2-amino, 6-lysinopimelic acid (carboxylysinonor-

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⊟Abbreviations used in this paper are: DAP = α,ε -diaminopimelic acid; CAA = carbox amino aldol (1,9-diamino, 1,9-dicarboxy, glycino-non-4-ene); CLNL = carboxylysinonorleucine (2-amino, 6-lysinopimelic acid); LNL = naturally reduced lysinonorleucine; I-DES = isodesmosine; DES = desmosine.

leucine (CLNL)) derived from dehydrolysinonorleucine (2). The structures of the isolated amino acids are in full agreement with structures previously deduced using procedures involving reduction with $NaBH_4$ (3-5). In this communication we suggest a scheme for the systematic detection of these and related compounds in elastins of various tissues. This scheme also provides for the detection of desmosines and lysinonorleucine.

Experimental Procedure

DAP was purchased from Cyclo Chemical Co.; $[^{14}C]$ -DAP, $[^{14}C]$ -CAA and $[^{14}C]$ -CLNL were isolated from elastin and their structures established (2). $[^{14}C]$ -NaCN samples of specific activity 1 mCi/mmole and 7 mCi/mmole respectively were purchased from New England Nuclear Corp., Boston, Mass. and stored at -20^{o}. The exact specific activity of a sample $[^{14}C]$ -NaCN was determined as $[^{14}C]$ alanine using a reaction with acetaldehyde (2). It could also be calculated from measurements of one peak of DAP isolated from modified elastin (see Table I). $[^{14}C]$ -NaCN with specific activity 7 mCi/mmole was obtained in vials containing approximately 1 mCi of NaCN (5-10 mg), and stored as such at -20^{o} C; immediately prior to use, 2 ml of 30% NH_4OH was added to a single vial. This solution was used for modification of rat aortic elastin; the ligamentum nuchae elastin was modified using NaCN of either specific activity. Elastin was prepared from bovine ligamentum nuchae by the method of Partridge et al. (6). Elastins of aortas of 6 month old male rats were prepared as follows: segments of thoracic aorta delimited by the left subclavian and celiac artery were dissected, and the intimal-medial and adventitial segments separated as described by Wolinsky and Daly (7). Corresponding segments from 5 rats were pooled, and the intimal-medial and adventitial segments treated identically. The segments were cut into small pieces, washed in cold distilled H_2O, cold 3:1 ethanol-ether, and then dried in a vacuum desiccator. In each case the dry residue (about 30 mg) was extracted with 7.5 ml of 0.5 M EDTA pH 8, for 24 hours and washed 3 times with cold distilled water. The residue was then extracted with 0.4 M acetic acid for 24 hours and again washed 3 times with water.

The residue consisting predominantly of insoluble collagen and elastin, was digested with 0.3 mg of collagenase (Worthington Biochemical Corp.) in 5 ml of 0.004 M $NaHCO_3$ containing 0.0005 M $CaCl_2$ for 24 hours at 37° C. The final pH was 7.5. The solutions were centrifuged and the residues washed 3 times with distilled water and lyophilized. An amino acid analysis was performed on 0.5-1 mg of each residue to ascertain that no significant quantities of collagen remained and that the residue was predominantly elastin; the residue, however, was known to be associated with small amounts of "structural" glycoprotein (8,9). Rabbit aortic elastin was prepared in a similar fashion from rabbits weighing 3.5-4.5 kg.

The elastin of bovine ligaments was modified as follows: to 50 mg of elastin in 5 ml of water were added 10 mg of $[^{14}C]$ -NaCN of specific activity 1 or 7 mCi/mmole, and 5 ml of 30% NH_4OH. The pH value was 11.5. Two experimental conditions were used: the elastin was treated in suspension for 1 hour at pH 11.5 or over the pH range 11.5 to 7 (1 hour at pH 11.5, followed by a progressive decrease to pH 7, one unit at a time over a 5 hour period). After completion of the reaction the suspension was acidified (in the hood, with caution!) to pH 1, centrifuged and the precipitate washed 5 times with 0.1 N HCl, and hydrolyzed in 6 N HCl *in vacuo* for 36 hours at 105° C. The HCl was evaporated in a rotary evaporator; the hydrolysate was washed 3 times with distilled water and used for systematic analysis described here.

Modification of elastin of intimal-medial or adventitial segments of the aorta was carried out in vials of the kind used routinely for protein hydrolysis. Portions (5 mg) of the residues (predominantly elastin) of the intimal-medial or adventitial segments were introduced into a vial followed by 1 ml of water and 0.5 ml of $[^{14}C]$ -NaCN, NH_4OH solution (see above). Either or both experimental conditions described for the ligamentum nuchae elastin were used. After completion of the reaction, the contents of the vials were cooled and acidified to pH 1 (in the hood); 5 ml of cold 0.1 N HCl was added to each vial and the suspensions centrifuged in a Sorvall centrifuge at 206 x g for 10 min. The modified elastins were washed 3 times, and hydro-

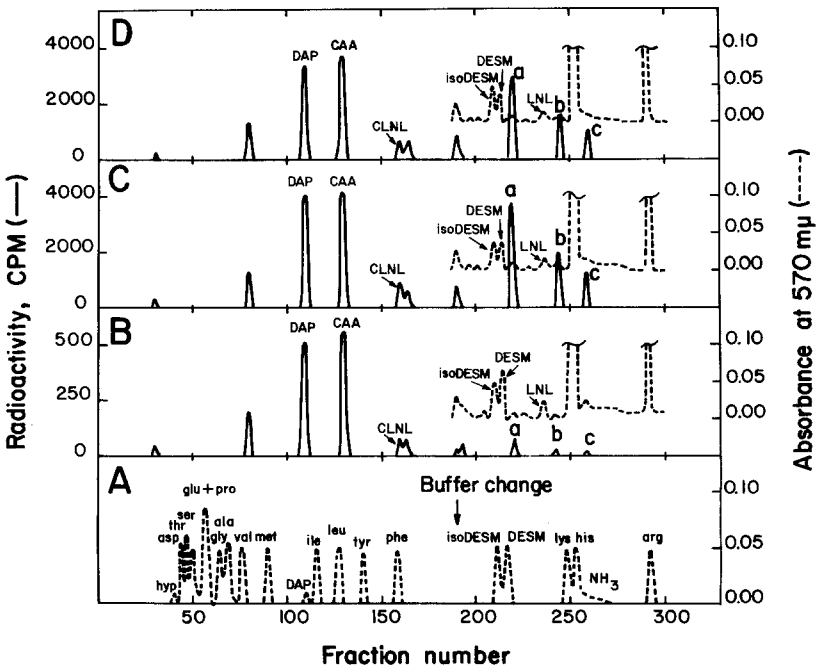

Figure 1.- Pattern of elution of modified crosslinks on a 60 cm column of a Jeol model JLC-6AH amino acid analyzer. The following gradients were used in a 9 chamber, 1 liter capacity Varigrad (Buchler Instruments): gradient I - 100 ml of 0.2 N sodium citrate buffer pH 3.25, 100 ml of 1:1 ratio of 0.2 N sodium citrate buffer pH 3.25 and 0.2 N sodium citrate buffer pH 4.25, and 100 ml of 0.2 N sodium citrate buffer pH 4.25; gradient II - 100 ml of 1:1 ratio of 0.2 N sodium citrate buffer pH 4.25 and 0.2 N sodium citrate buffer pH 5.25, and 100 ml of 1.2 N sodium citrate buffer pH 6.01; gradient II was initiated immediately after the elution of phenylalanine. Finally 1.2 N sodium citrate buffer pH 6.01 was used immediately after elution of lysinonorleucine. A) Standard amino acids including α,ε-diaminopimelic acid (DAP), isodesmosine (isoDESM) and desmosine (DESM); arrow indicates point at which the effect of change in buffer gradient occurs. B) An acid hydrolysate of modified elastin of bovine ligamentum nuchae. C) An acid hydrolysate of modified elastin of intimal-medial segment of rat thoracic aorta. D) An acid hydrolysate of modified elastin of adventitial segment of rat thoracic aorta. Conditions of reaction: pH 11.5, 1 hour. Specific activity of [^{14}C] -NaCN used: 0.86 mCi/mmole for ligamental elastin and 7.0 mCi/mmole for the aortic elastins.

lyzed in 6 N HCl as above. All subsequent steps were identical to those described for the elastin of ligamentum nuchae.

The crosslinking compounds or their derivatives were identified by chromatography using a Jeol model JLC-6AH amino acid analyzer. The buffer gradient described in the legend to Fig. 1 was used. In its development, the gradient was expanded in the region between methionine and lysine in a one column system; care

TABLE I

Comparative Contents of Crosslinks and Derivatives of Certain Crosslinks of Modified Elastins of Bovine Ligamentum Nuchae and of Intimal-Medial and Adventitial Segments of Rat Thoracic Aortas[a]

	Residues/1000 Total Amino Acid Residues					
	DAP[b]	CAA[b]	CLNL[b]	LNL/2[c]	I-DES/4[c]	DES/4[c]
Ligamental elastin	0.9[b,c]	1.1	0.2	1.1	4.1	6.9
Intimal-medial elastin	0.9	1.2	0.3	0.8	5.2	5.2
Adventitial elastin	0.8	1.0	0.2	0.5	7.6	4.8

a) Conditions of reaction: 1 hour at pH 11.5

b) Calculated from radioactivity: specific activity $[^{14}C]$ - NaCN = 0.86 mCi/mmole for ligamental elastin and 7.0 mCi/mmole for the aortic elastins.

c) Calculated from absorbance at 570 mμ (ninhydrin) using the color value of lysine; color for DAP relative to that of leucine was 1.35

was taken so that standard DAP emerged between the positions of valine and isoleucine. The positions of elution of samples of standard amino acids, including DAP, using such a gradient are shown in Fig. 1A. A hydrolysate prepared from approximately 2.5 mg of a given modified elastin was applied to the 60 cm column of the amino acid analyzer. Total radioactivity applied to the column was about 1.0×10^6 cpm for elastin modified with $[^{14}C]$-NaCN of specific activity 7 mCi/mmole. A split-stream device was used so that 50% of the sample was collected in 0.5 ml fractions at a rate of 48 ml/hr. Because the amount of hydrolysate applied to the column was so large, the ninhydrin used for analysis was introduced into the system only after emergence of valine from the column. The radioactivity of each fraction was determined on a 0.1 ml aliquot delivered into 10 ml of Bio-Solv scintillation mixture [2% Bio-Solv (Beckman) and 0.4% w/v Omnifluor (New England Nuclear Corp.) in toluene].

TABLE II

Effect of Conditions of Reaction on the Contents of Crosslinks

Source of Elastin	Conditions	Residues/1000 Total Amino Acid Residues					
		DAP[a]	CAA[a]	CLNL[a]	LNL/2[b]	I-DES/4[b]	DES/4[b]
Ligamentum Nuchae	pH 11.5 (1 hour)	0.8	0.9	0.2	1.5	4.5	6.3
	pH 11.5-7.0 (5 hours)	1.2	2.3	0.7	1.3	4.8	7.0
Rat aorta (intimal-medial segment)	pH 11.5 (1 hour)	0.9	1.2	0.3	0.8	5.2	5.2
	pH 11.5-7.0 (5 hours)	1.8	2.9	0.9	1.0	3.8	4.5
Rabbit aorta (intimal-medial segment)	pH 11.5 (1 hour)	0.9	2.0	0.3	3.1	3.8	6.1
	pH 11.5-7.0 (5 hours)	1.3	2.3	0.9	2.3	4.0	6.1

a) Calculated from radioactivity: specific activity $\left[^{14}C\right]$-NaCN = 7.0 mCi/mmole.

b) Calculated from absorbance at 570 mμ (ninhydrin) using the color value of lysine.

Results and Discussion:

The elution pattern of hydrolyzed modified elastin of ligamentum nuchae is shown in Fig. 1B. Three of the radioactive peaks obtained corresponded respectively to DAP, CAA and CLNL. Their identities were demonstrated by co-chromatography respectively with $\left[^{14}C\right]$-labelled standard compounds that had been isolated from modified elastin and whose identity had been established (2). The nature of compounds present in the other radioactive peaks has not as yet been determined. However, ligamental elastin yielded DAP and CAA as dominant derivatives. The positions of elution of desmosine, isodesmosine and lysino-

norleucine (LNL) are seen from their ninhydrin absorbance at 570 mμ. These crosslinks did not incorporate the label in the overall reaction with cyanide and ammonia, and their quantity could be determined from usual amino acid analysis. Other small ninhydrin-positive peaks were present, and some could in fact have arisen from other aldehydes or aldehyde-derived crosslinks in the native elastin. The contents of the radioactive modified crosslinking compounds can also be estimated when the specific activity of the $[^{14}C]$ -NaCN is known.

The elution pattern of hydrolyzed modified elastin of the intimal-medial segment of the aorta is shown in Fig. 1C, and that of the adventitial segment in Fig. 1D. Clearly, DAP, CAA and CLNL are present in addition to the desmosines and LNL. Three additional peaks corresponding to other compounds (as yet unidentified) are also present in relatively larger amounts than in the ligamental elastin. One may note that the same radioactive compounds are present in the elastins of intimal-medial and adventitial segments. Also, some differences in contents of the various compounds are apparent (Table I and II). The significance of these differences are presently under study. One should consider the possibility that the conditions used in these experiments may not have been optimum for modification of each kind of aldehyde or crosslink in a particular elastin. The conditions used here were considered optimum on basis of experimentation with a "model" collagen bound aldehyde (2), α-aminoglutaric acid γ-semialdehyde. For precise quantitative estimation, optimum conditions should be established for each particular aldehyde or crosslink. For example, when the reaction was carried out over the pH range 11.5 to 7, the pattern of $[^{14}C]$ -labelled amino acids derived from the crosslinks was similar but more radioactivity was incorporated into DAP, CAA and CLNL (see Table II). Nevertheless, the modifications under the conditions described here allow one to obtain comparative profiles of aldehydes, aldimines and aldehyde-derived crosslinks of elastin of different tissues in both normal and pathological conditions.

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