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Comparative Studies of the Cross-Linked Regions of Elastin from Bovine Ligamentum Nuchae and Bovine, Porcine and Human Aorta

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1. The preparative Edman degradation of desmosine-containing peptides permitted the isolation of peptides *C*-terminal to the desmosine cross-links in bovine, porcine and human aortic elastin as well as bovine ligamentum nuchae elastin. This identifies the lysines in the tropoelastin which give rise to the desmosine cross-links. 2. The sequences from bovine aortic elastin were identical with those obtained from bovine ligamentum nuchae elastin but differed from those obtained from the other species. The most striking difference involves the occurrence of phenylalanine in bovine elastin and tyrosine in porcine and human elastin *C*-terminal to the desmosine cross-links. 3. The sequences of the *C*-terminal peptides were found to fall into two distinct classes, one starting with hydrophobic residues, the other starting with alanine. It is proposed that the hydrophobic residue prevents the enzymic oxidative deamination of the adjacent lysine ε-amino group and this then contributes the nitrogen to the pyridinium ring of the cross-links.

The classical methods of protein chemistry are not directly applicable to the study of mature elastin owing to the protein's high degree of cross-linking and its resulting insolubility in all non-hydrolytic solvents. The inhibition of cross-link formation results in the accumulation of a soluble elastin precursor called tropoelastin and this is being sequenced in order to determine the primary structure of elastin (Foster *et al.*, 1973*a*; Gray *et al.*, 1973).

The desmosine and isodesmosine cross-links of elastin are formed as the result of the deamination and subsequent condensation of four lysine side chains in the soluble precursor (Partridge *et al.*, 1966; Anwar & Oda, 1966). The study of the cross-linking regions of mature elastin was therefore required to identify those lysines that participate in the formation of the various cross-links and thus gain a more detailed understanding of the mechanism involved.

Although several groups have reported the isolation of cross-linked elastin peptides (Partridge, 1962; Keller *et al.*, 1969; Thomas, 1971; Shimada *et al.*, 1969; Foster *et al.*, 1973*b*; Davril & Han, 1974), virtually no sequence information was reported in these studies. The finding that elastase cleaves at and near the *N*-terminals of the desmosine cross-links permitted the release of single-chain peptides from the carboxyl groups of these cross-links by Edman degradation. The sequences of these single-chain peptides were then determined by classical protein-chemistry techniques (Gerber & Anwar, 1974).

Since four lysine side chains give rise to the desmosine cross-link, the sequences of some of the tryptic peptides of tropoelastin were expected to correspond to the *C*-terminal peptides obtained from mature elastin. More than one-half of the *C*-terminal peptides obtained from the cross-linked peptides of bovine ligamentum nuchae elastin were identical with *N*-terminal sequences of tryptic peptides of porcine aortic tropoelastin (Gerber & Anwar, 1974). However, significant differences were also observed. Since the sequences from bovine ligamentum nuchae elastin were compared with sequences from porcine aortic tropoelastin, these differences could be species-specific or tissue-specific, or both. A comparative study of bovine, porcine and human aortic elastins was therefore undertaken to distinguish between these possibilities and to gain further insight into the structural requirements for the desmosine cross-link formation. The results of this study are described in this paper.

General Methods

Detection of peptides

The elution profiles of peptides from the various columns were determined by measuring the E_{280} with a Hitachi spectrophotometer. The fractionation of uncross-linked peptides was monitored by assaying the column effluents with ninhydrin after alkaline hydrolysis by the method of Hirs (1967).

Amino acid analyses

Samples were dissolved in 1ml of 6M-HCl in evacuated sealed tubes and hydrolysed at 110°C for

20–72h. When peptides eluted from paper are hydrolysed under these conditions, some amino acids are destroyed; these samples were therefore hydrolysed in 200μl of 6M-HCl containing 0.2% phenol and 0.1% (v/v) mercaptoacetic acid in evacuated sealed tubes at 110°C for 24h (Moore & Stein, 1963). Amino acid analyses were carried out by a single-column modification of the procedure of Gerber & Kemp (1972). The basic amino acids were determined by extending the second buffer time to 4h followed by a third buffer (pH6.4, 1.0M-Na^+, as recommended in Beckman circular A-TB-059A) to elute arginine.

Paper chromatography of peptides

The peptides were applied as a band 5cm from the edge of the paper (Whatman 3MM or no. 1) and the paper was developed by descending chromatography in butan-1-ol–*n*-butyl acetate–acetic acid–water (135:6:30:50, by vol.) as solvent (Bennett, 1967). The peptides were detected by staining guide strips with cadmium–ninhydrin reagent (Heilman *et al.*, 1957), and were eluted from the paper with water.

Experimental

Materials

Elastin was prepared from fresh bovine, porcine and human aorta and bovine ligamentum nuchae essentially by the method of Partridge & Davis (1955) as described previously (Anwar, 1966).

Twice-crystallized elastase prepared as described by Lewis *et al.* (1956) from crude trypsin 1-300 (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was further purified to homogeneity by a chromatographic procedure (Narayanan & Anwar, 1969), dialysed, freeze-dried and stored as a dry powder at −20°C.

The fresh bovine and porcine ligamentum nuchae and aorta were the gifts of Canada Packers, Toronto, Canada. The human aorta was kindly supplied by the Department of Pathology of the University of Toronto.

The Cellex-phosphate was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A. All Sephadex gels were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The AA-15 polystyrene resin was from Beckman Instruments Inc., Palo Alto, Calif., U.S.A.

All reagents for sequencing were Sequenal-grade and were purchased from the Pierce Chemical Co., Rockford, Ill., U.S.A. All other reagents were of ACS (American Chemical Society) grade and were obtained from Fisher Chemical Co., Silver Spring, Md., U.S.A. or BDH, Poole, Dorset, U.K.

Preparation of cross-linked peptides

Elastin (20g) was exhaustively (5 days) digested with chromatographically pure elastase (Narayanan & Anwar, 1969) and the elastolytic cross-linked peptides were purified by chromatography as described by Gerber & Anwar (1974). Briefly, the elastase digest of elastin was chromatographed on a column of Cellex-phosphate and a desmosine-rich peak was eluted with 0.01M-sodium acetate buffer (pH4.5)–0.1M-NaCl. This fraction was chromatographed on Sephadex G-50 and the small cross-linked fraction, eluted between 2.0 and 2.5 void volumes, was chromatographed on Sephadex G-25. The fraction, eluted between 1.0 and 1.5 void volumes (fraction G.25.2), was used for these studies. The amino acid compositions of fractions G.25.2 obtained from the various elastins are given in Table 1.

Release of C-terminal peptides

The peptides *C*-terminal to the desmosine cross-links were released from the cross-links by preparative Edman degradation as described by Gerber & Anwar (1974). Briefly, the peptides (20μmol of cross-link) were subjected to Edman degradation and the peptides separated by chromatography on Sephadex LH-20 after each cycle of the degradation. The cross-linked fraction was eluted between 1.0 and 1.35 void volumes and was subjected to further cycles of the degradation. The small released *C*-terminal peptides, eluted between 1.35 and 2.0 void volumes, were pooled from the first three cycles of the degradation and further purified.

Table 1. *Amino acid compositions of cross-linked peptides (fractions G.25.2) prepared from bovine ligamentum nuchae and bovine, porcine and human aortic elastins*

Values are expressed in residues per cross-link (i.e. desmosine plus isodesmosine).

Amino acid	Bovine ligament	Bovine aorta	Porcine aorta	Human aorta
Aspartic acid	0.2	0.3	0.9	0.9
Threonine	0.3	0.3	0.8	1.0
Serine	0.6	0.6	0.9	0.7
Glutamic acid	0.7	0.6	0.9	1.2
Proline	4.5	3.3	5.2	3.1
Glycine	10.1	9.1	11.2	8.0
Alanine	18.1	14.8	18.6	17.4
Valine	2.5	1.7	1.5	2.4
Isoleucine	0.7	0.4	0.6	0.5
Leucine	1.8	1.4	2.3	2.0
Tyrosine	0.8	0.7	1.7	2.1
Phenylalanine	1.8	1.5	0.8	0.6
Isodesmosine	0.41	0.32	0.42	0.37
Desmosine	0.59	0.68	0.58	0.63
Lysine	0.2	0.3	0.7	0.5
Arginine	0.3	0.3	0.5	0.6

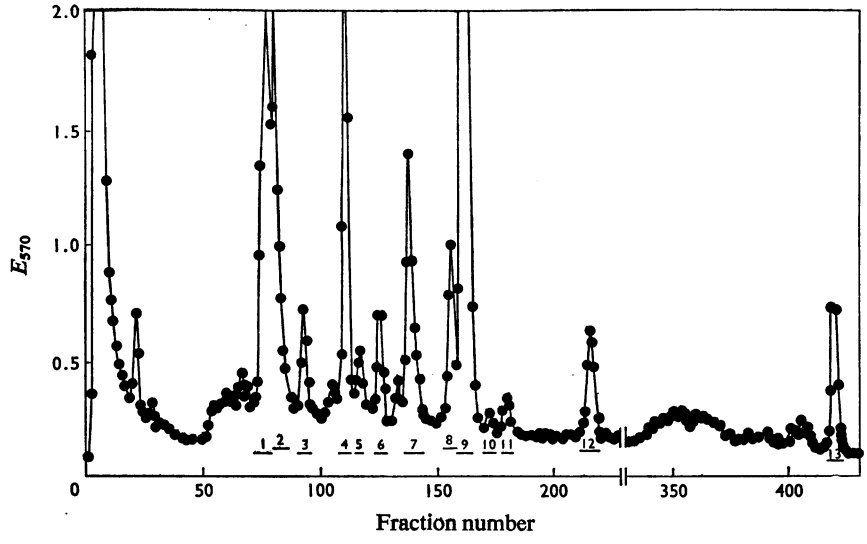


Fig. 1. *Chromatography of peptides released from the C-terminals of the desmosines of bovine ligamentum nuchae elastin*

The *C*-terminal peptides released from the desmosines by the first three cycles of Edman degradation were chromatographed on a column (0.9cm×55cm) of Beckman AA-15 resin. The column was equilibrated with buffer I (0.05M-pyridine acetate, pH2.6) at 55°C at 42ml/h. The sample was dissolved in 2ml of buffer I and the pH was adjusted to 2.2 with 1M-HCl; this sample was applied to the equilibrated column, which was then developed with a gradient consisting of 300ml of each of buffers I, II and III connected in sequence in the order of increasing pH and molarity (II: 0.10M-pyridine acetate, pH3.1; III: 0.50M-pyridine acetate, pH3.7). This was followed by a gradient consisting of 150ml of each of buffer III and buffer IV (2.0M-pyridine acetate, pH5.0). The effluent was collected in 4min fractions (2.8ml); these were then assayed with ninhydrin after alkaline hydrolysis as described in the General Methods section. Fractions were pooled and numbered as indicated.

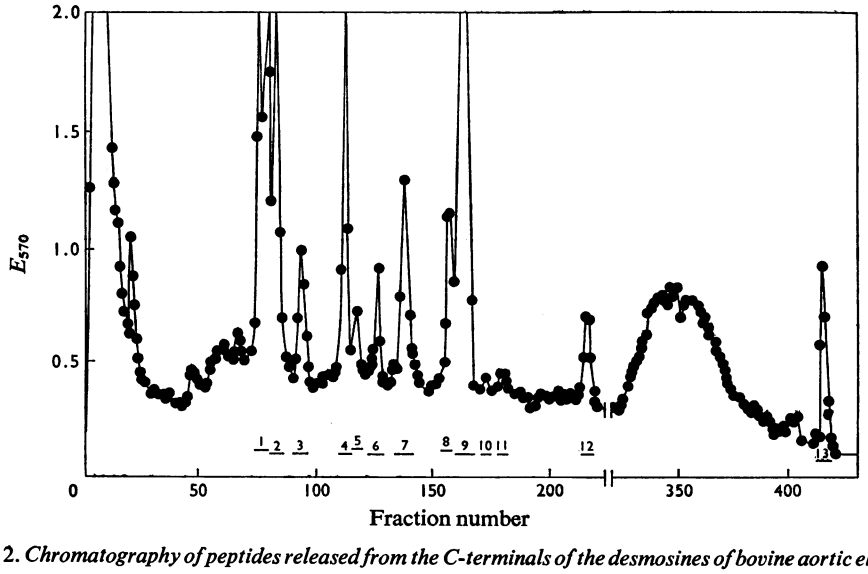


Fig. 2. *Chromatography of peptides released from the C-terminals of the desmosines of bovine aortic elastin*

The conditions of the chromatography were exactly as described in Fig. 1.

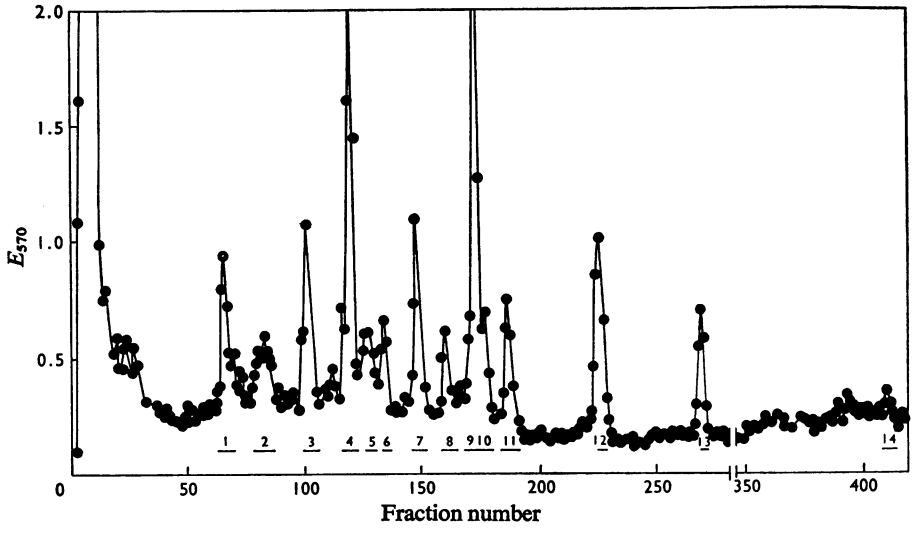


Fig. 3. *Chromatography of peptides released from the C-terminals of the desmosines of porcine aortic elastin*

The conditions of the chromatography were exactly as described in Fig. 1.

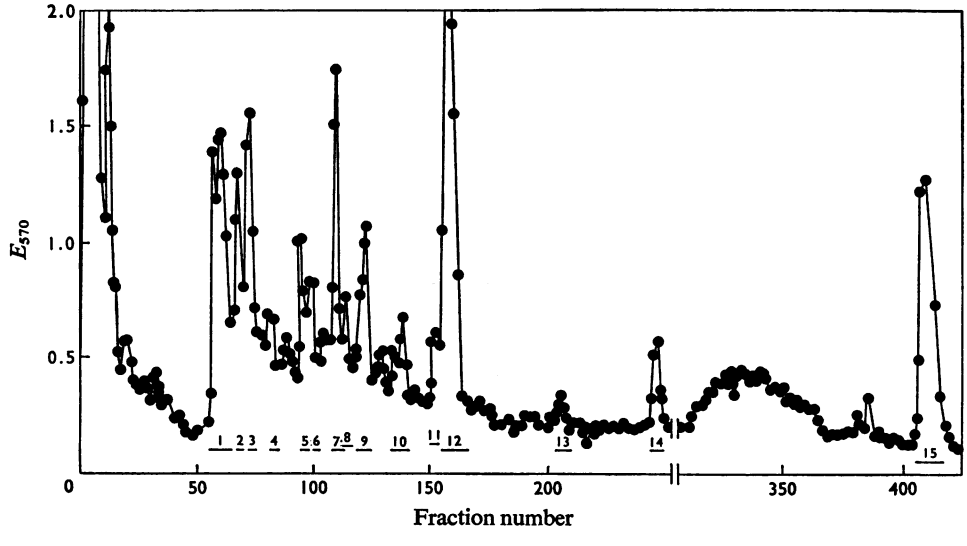


Fig. 4. *Chromatography of peptides released from the C-terminals of the desmosines of human aortic elastin*

The conditions of the chromatography were exactly as described in Fig. 1.

Purification of released peptides

The small peptides pooled from the first three cycles of preparative Edman degradation were dried and chromatographed on a column of Beckman AA-15 polystyrene resin as described in Fig. 1. The effluent was collected in 2.8ml fractions and these were assayed by reaction with ninhydrin after alkaline hydrolysis by the method of Hirs (1967). The fractions were pooled as indicated in Figs. 1–4.

Some of the peptides were pure as pooled and were sequenced directly whereas others required further purification as indicated in Tables 2–5.

Table 2. *Amino acid compositions of peptides released from the C-terminals of the desmosines of bovine ligamentum nuchae elastin*

Values are expressed as residues per peptide. A blank indicates less than 0.2 residue per peptide. For number see Fig. 1.

Amino acid	Residues/peptide																	
Peptides	... 1a	1b	1c	2	3a	4	5	6	7a	7b	8	9a	9b	9c	10	11	12	13
Threonine	0.9																	
Serine													1.0					
Glutamic acid								1.0		1.0								
Proline	1.0			1.0														
Glycine	1.0	3.1	3.1	3.3	2.2		1.0		1.1		1.0			1.0	1.2	1.0	1.2	1.2
Alanine	1.0	1.0	2.0		1.9	1.0	2.0	1.0	2.0	1.1	1.0	2.0	1.0	1.0	1.1	1.1	0.3	1.1
Valine		1.0		1.0														
Isoleucine		0.9													1.0			
Leucine			0.8											1.0				
Tyrosine	0.8																	
Phenylalanine				0.9					0.9							1.0	1.0	1.0
Arginine																		1.0
Comments	*	*	*		†R_F = 0.35			†R_F = 0.12	†R_F = 0.64	†R_F = 0.13		†R_F = 0.39	†R_F = 0.23	†R_F = 0.63				†R_F = 0.13

* Purified from fraction 1 by rechromatography on the same column (Fig. 1) by using a gradient of 400ml of each of 0.05M-pyridine acetate, pH2.6, and 400ml of 0.1M-pyridine acetate, pH3.1, and collecting 2min fractions.

† Purified by paper chromatography in solvent 2 (see the General Methods section).

Table 3. *Amino acid compositions of peptides released from the C-terminals of the desmosines of bovine aorta elastin*

Values are expressed as residues per peptide. A blank indicates less than 0.2 residue per peptide. For number see Fig. 2.

Amino acid	Residues/peptide																	
Peptides...	1a	1b	1c	2	3a	4	5	6	7a	7b	8	9a	9b	9c	10	11	12	13
Threonine	0.9																	
Serine													0.9					
Glutamic acid								1.0		1.0								
Proline	0.9			0.9														
Glycine	1.1	3.1	3.2	3.2	2.2		1.0		1.0		1.0			1.1	1.1	1.1	1.2	1.2
Alanine	1.0	1.1	2.0		1.9	1.0	2.0	1.1	2.0	1.1	1.0	2.0	1.0	1.0	1.0	1.1	0.2	1.1
Valine		1.0		1.0														
Isoleucine		1.0													1.0			
Leucine			0.8											1.0				
Tyrosine	0.8																	
Phenylalanine									0.9							1.0	1.0	1.0
Arginine																		1.0
Comments	*	*	*		†R_F = 0.33			†R_F = 0.10	†R_F = 0.65	†R_F = 0.11		†R_F = 0.40	†R_F = 0.25	†R_F = 0.66				†R_F = 0.12

* Purified from fraction 1 by rechromatography on the same column (Fig. 2) by using a gradient of 400ml of each of 0.05M-pyridine acetate, pH2.6, and 400ml of 0.1M-pyridine acetate, pH3.1, and collecting 2min fractions.

† Purified by paper chromatography in solvent 2 (see the General Methods section).

Table 4. *Amino acid compositions of peptides released from the C-terminals of the desmosines of porcine aorta elastin*

Values are expressed as residues per peptide. A blank indicates less than 0.2 residue per peptide. For number see Fig. 3.

Amino acid Peptides	Residues/peptide																
	... 1	2	3	4	5	6	7	8	9	10	11	12a	12b	12c	13	14	15
Threonine			0.7														
Serine														0.9			
Glutamic acid									1.0	1.0							
Proline	0.9	0.8	0.9			0.9											
Glycine	4.1	3.0	1.2	2.2	1.0	2.0		1.0			1.0	1.0			1.1	1.0	1.1
Alanine	2.0	2.0	1.0	2.2	2.2	1.4	1.0	2.1	1.0	1.0	1.0	2.0	2.0	1.0	1.0		1.1
Valine																	
Isoleucine																	
Leucine				1.0	1.0												
Tyrosine		0.7	0.8			0.9						0.9			0.9	0.8	0.8
Phenylalanine																	
Arginine																	1.0
Comments	$*R_F =$ 0.44	$*R_F =$ 0.43			$*R_F =$ 0.64	$*R_F =$ 0.43			$*R_F =$ 0.11	$*R_F =$ 0.12		$*R_F =$ 0.47	$*R_F =$ 0.38	$*R_F =$ 0.23			$*R_F =$ 0.15

* Purified by paper chromatography in solvent 2 (see the General Methods section).

Table 5. *Amino acid compositions of peptides released from the C-terminals of the desmosines of human aorta elastin*

Values are expressed as residues per peptide. A blank indicates less than 0.2 residue per peptide. For number see Fig. 4.

Amino acid Peptides ...	Residues/peptide									
	2a	4	6a	8	9	10a	10b	10c	12	13
Threonine	0.8									
Serine								0.9		
Glutamic acid			1.0							
Proline	0.9									
Glycine	1.0			2.0	1.0	1.0			1.2	1.0
Alanine	1.0	1.0	1.0	1.2	1.0	2.0	2.0	1.0	1.0	
Tyrosine	0.8					0.9			0.6	0.8
Phenylalanine				0.8					0.3	
Comments	†		*R_F=0.14			*R_F=0.49	*R_F=0.39	*R_F=0.25		

* Purified by paper chromatography in solvent 2 (see the General Methods section).

† Purified from fraction 2 by rechromatography on the same column (Fig. 4) by using a gradient of 400ml of 0.05M-pyridine acetate, pH2.6, and 400ml of 0.1M-pyridine acetate, pH3.1, and collecting 2min fractions.

Sequencing of purified released peptides

The peptides were subjected to Edman degradation as described by Gerber & Anwar (1974). The *N*-terminals were identified by Hartley's (1970) microdansyl method except that the hydrolysis time in 6M-HCl was decreased to 4h as recommended by Gros & Labouesse (1969). The dansyl amino acids were identified by chromatography on polyamide sheets (5cm × 5cm) by Hartley's (1970) system.

Results and Discussion

The amino acid compositions of the comparable cross-linked peptide fraction from the various sources used for the release of the *C*-terminal peptides are shown in Table 1. The detailed methods of isolation have been reported by Gerber & Anwar (1974) and are summarized in the present paper in the General Methods section. The peptides released from the desmosine cross-links on each of the first three cycles of Edman degradation were pooled and chromatographed as shown in Figs. 1–4; this allowed us to sequence three peptides (B6, B9b and B13) which had previously escaped detection because the released peptides after each cycle of Edman degradation were chromatographed separately. This procedure resulted in the isolation of all the other peptides in higher yields and was therefore adopted for all further studies.

The elution profile of the peptides released from the cross-linked bovine ligamentum nuchae peptides is shown in Fig. 1, and was identical with that obtained from bovine aortic elastin as shown in Fig. 2. The elution profile for the peptides obtained from porcine elastin is shown in Fig. 3, and that for human aortic elastin is shown in Fig. 4.

The amino acid compositions of the purified released peptides from bovine ligamentum nuchae and bovine, porcine and human aortic elastin are shown in Tables 2–5 respectively. The amino acid sequences of the pure released *C*-terminal peptides from the various sources are shown in Table 6. These sequences should correspond to and are thus compared with the *N*-terminal sequences of tryptic peptides of porcine aortic tropoelastin. Where overlapping sequences were found, only the longest peptide obtained is shown in Table 6. Ten unique peptides were sequenced from porcine aortic, bovine ligamentum nuchae and bovine aortic elastin and seven such peptides from human aortic elastin (Table 6).

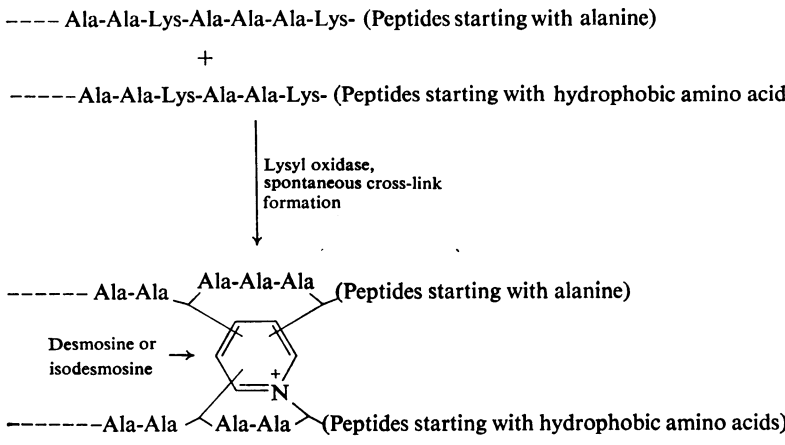
The peptides obtained from bovine ligament elastin were identical with those obtained from bovine aortic elastin (Tables 2 and 3; Figs. 1 and 2). The sequences of these peptides are given in Table 6, column 3. Thus the differences between these sequences and the *N*-terminal sequences of tryptic peptides of porcine aortic tropoelastin or the sequences of *C*-terminal peptides from porcine aortic elastin (Table 6) must be species- rather than tissue-specific. It is therefore likely that the morphological differences of elastin from different tissues are determined by something other than the tropoelastin. The microfibrils are likely candidates for this function and a comparative study of microfibril structure and function in different tissues would therefore be important to our understanding of the morphogenesis of elastic fibres.

Although the bovine aortic and bovine ligament peptides are identical, there are obvious differences between the porcine, bovine and human elastin peptides. The most striking difference involves the occurrence of phenylalanine in bovine elastin, and

Table 6. *Comparison of the N-terminal sequences of tryptic peptides of porcine tropoelastin with the sequences of peptides C-terminal to the desmosines in elastins from porcine aorta, bovine aorta and ligament and human aorta*

Porcine tropoelastin*		Porcine aorta elastin		Bovine aorta and ligament elastin		Human aorta elastin	
T1	Tyr-Gly-Ala-Ala-Gly-Gly-Leu-						
		PA12b	Tyr-Gly-Ala-Ala	B7b	Phe-Gly-Ala-Ala	HA10a	Tyr-Gly-Ala-Ala
T9c	Tyr-Gly-Ala-Ala-Gly-Ala-Leu-						
T4	Tyr-Gly-Ala-Pro-Gly-Ala-Gly-	PA2	Tyr-Gly-Ala-Pro-Gly-Ala-Gly				
T2	Ala-Ala-Gln-Phe-Gly-Leu-						
		PA9	Ala-Gln	B6	Ala-Gln	HA6a	Ala-Gln
T9b	Ala-Ala-Glu-Phe-Gly-Val-						
T14b	Ala-Pro-Gly-Gly-Gly-Gly-Ala-	PA1	Ala-Pro-Gly-Gly-Gly-Gly-Ala				
T7b	Ala-Gly-Tyr-Pro-Thr-Gly-	PA3	Ala-Gly-Tyr-Pro-Thr	B1a	Ala-Gly-Tyr-Pro-Thr	HA2a	Ala-Gly-Tyr-Pro-Thr
	Tyr-Gly-Ala-Arg	PA15	Tyr-Gly-Ala-Arg	B13	Phe-Gly-Ala-Arg		
	Ser-Ala-Lys	PA12c	Ser-Ala	B9b	Ser-Ala	HA10c	Ser-Ala
	Ala-Ala-Lys	PA12a	Ala-Ala	B9a	Ala-Ala	HA10b	Ala-Ala
T6	Ala-Gly-Ala-Gly-Leu-Gly-	PA8	Ala-Gly-Ala	B3a	Ala-Gly-Ala-Gly	HA9	Ala-Gly
				B1c	Leu-Gly-Ala-Gly-Gly-Ala		
		PA5	Leu-Gly-Ala-Ala	B1b	Ile-Gly-Ala-Gly-Gly-Val	HA8	Phe-Gly-Ala-Gly
				B2	Phe-Gly-Pro-Gly-Gly-Val		

* Taken from Gray *et al.* (1973).



Scheme 1. *A proposed scheme for the formation of the desmosine cross-links*

tyrosine in porcine and human elastin *C*-terminal to the desmosine cross-links. The replacement of tyrosine by phenylalanine is a highly common mutation (Dayhoff *et al.*, 1972). Even large-scale preparations from bovine sources failed to show any peptides containing tyrosine other than Ala-Gly-Tyr-Pro-Thr. All the other differences are also consistent with single point mutations as shown in Table 6.

It should be pointed out that a sequence Ala-Ala-Glx- corresponding to peptides T2 and T9b (Table 6) from porcine aortic tropoelastin was not detected in the elastins studied. This is probably due to rapid elastolytic cleavage of this sequence at alanine. However, a sequence Ala-Gln was detected from all four sources studied suggesting that the sequence Lys-Ala-Gln- also occurs in tropoelastin and takes part in cross-link formation.

Foster *et al.* (1974) have suggested a unique role for tyrosine in the formation of the desmosine and isodesmosine cross-links. It was suggested that the tyrosine inhibits the deamination of the neighbouring lysine. In the light of our results, no such unique role can be assigned to tyrosine as the cross-linked peptides studied from bovine elastin, both aortic and ligament, do not contain tyrosine *C*-terminal to the desmosines. However, it is clear from the sequences of the *C*-terminal peptides that they fall into two distinct classes, about one-half having all alanine adjacent to the *C*-terminals of the cross-links, the others starting with hydrophobic residues such as tyrosine, phenylalanine, leucine or isoleucine. Mainly alanine is *N*-terminal to the desmosines. Further, Gray *et al.* (1973) have demonstrated the occurrence of pairs of lysines separated by two or three amino acid residues in porcine aortic tropoelastin (Lys-Ala-Ala-Lys, Lys-Ala-Ala-Ala-Lys, Lys-Ser-Ala-Lys etc.). The release of Ser-Ala from the cross-linked peptides from all four sources suggests that the sequence Lys-Ser-Ala-Lys also takes part in the desmosine cross-link formation. These facts indicate that three of the four lysines (in two pairs) are preceded and followed by alanine or similar small amino acids whereas the fourth lysine is followed by a hydrophobic residue. As shown in Scheme 1, out of the four lysines that give rise to the desmosine cross-links, three are to be deaminated whereas one retains its ε-amino group. It is therefore proposed that all lysines preceded and followed by alanine or similar small amino acids are oxidized whereas those followed by hydrophobic amino acids retain their ε-amino groups and donate their nitrogen to the desmosines, dehydrolysinonorleucine and lysinonorleucine.

Since most of the lysines of tropoelastin occur in pairs separated by either two or three residues of alanine, this proposal could account for the number of lysines that retain their ε-amino groups. On the basis of steric considerations, it is likely that the ring nitrogen is derived from the pairs of lysines which are separated by only two alanine residues and that the peptides starting with hydrophobic residues are *C*-terminal to these pairs as shown in Scheme 1.

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