Structural Studies on Cross-Linked Regions of Elastin*

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SUMMARY

A novel use of Edman degradation was made in the study of desmosine- and isodesmosine-containing elastolytic peptides of bovine ligamentum nuchae elastin. The elastolysis of elastin produced peptides with the cleavage at or near the NH$_2$-terminals of desmosine and isodesmosine cross-links. Therefore, it became possible to release single chain peptides from the carboxyl groups of these cross-links with the use of Edman degradation. This approach permitted the isolation and sequencing of seven unique peptides from bovine ligamentum nuchae elastin; the sequences of five such peptides from porcine aortic elastin were also determined. The comparison of the sequences of these peptides with the NH$_2$-terminal sequences of porcine tropoelastin tryptic peptides (Foster, J. A., Bruenger, E., Gray, W. R., and Sandberg, L. B. (1973) J. Biol. Chem. 248, 2876-2879) permitted the identification of 12 possible lysine sequences which may be involved in cross-link formation. On the basis of the available data on elastin and tropoelastin, it is proposed that the tropoelastin molecule contains six pairs of lysines in the sequence -Lys-Ala-Ala-Ala-Lys- and six additional pairs of lysines in the sequence -Lys-Ala-Ala-Ala-Lys- and that two such pairs meet to form desmosine or isodesmosine cross-links.

The elucidation of the primary structure of mature elastin is not readily amenable to study because of the protein's high degree of cross-linking and its insolubility. Lathyrisn is the result of the inhibition of cross-link formation, and this has enabled various groups (1-3) to isolate and partly characterize a soluble product called tropoelastin, which, on the basis of its NH$_2$-terminal sequence, is clearly required to identify the regions involved in cross-link formation. In order to determine the amino acid sequences, the cross-linked chains must be resolved into single chain peptides. The present paper describes an approach which permits the determination of sequences COOH-terminal to the desmosine and isodesmosine cross-links of elastin; seven such peptides were isolated and sequenced from bovine ligamentum nuchae elastin and five from porcine aortic elastin. It was found that some of these were identical with the sequences reported (1) for the NH$_2$-terminal regions of the tryptic peptides of tropoelastin. This evidence identifies some of the regions in tropoelastin which take part in the actual cross-linking. Furthermore, a model which takes into account all available data for elastin and tropoelastin is proposed.

EXPERIMENTAL PROCEDURE

Materials

Elastin—Elastin was prepared from fresh bovine ligamentum nuchae or fresh porcine aorta essentially according to the method of Partridge and Davis (5), as previously described (6).

Pure Elastase—Twice crystallized elastase prepared as described by Lewis et al. (7) from trypsin 1-300 was further purified to homogeneity by a chromatographic procedure (8), dialyzed, lyophilized, and stored as a dry powder at -20°C.

Fresh bovine ligamentum nuchae and porcine aorta were the gifts of Canada Packers, Toronto. Trypsin 1-300 was purchased from the Nutritional Biochemicals.

Cellex-P and Dowex 50-X2 were purchased from Bio-Rad Laboratories; Sephadex gels were from Pharmacia and AA-15 polystyrene resin from Beckman Instruments.

All reagents for sequencing were Sequenal grade purchased from Pierce Chemicals. Polymide sheets for thin layer chromatography were obtained from the Cheng-Chin Trading Co., Sec. 1, Hankow Street, Taipei, Taiwan. Sodium [H]$^3$borohydride at a specific activity of 180 mCi per mmole was purchased from Amersham-Searle and used undiluted. Potassium [H]$^3$cyanate with specific activity of 24.14 mCi per mmole was purchased from International Chemical and Nuclear Corporation.

Aquasil was purchased from New England Nuclear. All chemicals not listed were of ACS (American Chemical Society) grade and were obtained from Fisher Chemicals.

Methods

Detection of Peptides—The elution profiles of peptides were determined by measuring the absorbance both at 280 and 330 nm with a Hitachi spectrophotometer as indicated in the legends. The fractions in 50% pyridine were assayed by reaction with ninhydrin after alkaline hydrolysis according to the method of Hirs (8).

Amino Acid Analyses—Samples were dissolved in 6 M HCl, in evacuated, sealed tubes and hydrolyzed at 110° for 20 to 72 hours. Amino acid analyses were carried out by a single column modification of the method described previously (10); basic amino acids were determined by extending the second buffer time to 4 hours followed by a third buffer (pH 6.4, 1.0 M Na$^+$ as recommended in Beckman circular A-TB-389A) to elute arginine.

* This investigation was supported by the Medical Research Council of Canada.
† Recipient of Medical Research Council of Canada Studentship.

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FIG. 1. Chromatographic separation of elastolytic peptides on Cellex P. The pH of the elastase digest was adjusted to 4.0 with glacial acetic acid. The digest was then applied to a column (2.0 × 30 cm) of Cellex P previously equilibrated with 0.01 N sodium acetate buffer, pH 4.5. The column was washed with 500 ml of the same buffer. The column was then eluted with 0.1 N NaCl in the same buffer and fractions (7.5 ml) collected and their absorbance measured after suitable dilution when necessary. The fractions were pooled as indicated.

FIG. 2. Chromatography of Fraction CPB (Fig. 1) on Sephadex G-50. Fraction CPB was dried, dissolved in 3 to 5 ml of buffer (0.01 M NH₄HCO₃, pH 8.8) and applied on a Sephadex G-50 (fine) column (4 × 80 cm) previously equilibrated with the same buffer. The column was then eluted with the above buffer at a flow rate of 100 ml per hour. Fractions (10 ml) were collected, their absorbance measured at 280 nm, and then pooled as indicated above.

Determination of Radioactivity—The radioactivity of fractions was measured with a Nuclear-Chicago Mark II refrigerated scintillation spectrophotometer. Aliquots to be counted (usually 100 μl) were added to 10 ml of Aquasol in glass scintillation vials and refrigerated for at least 24 hours before counting.

Elastase Digestion of Elastin—A suspension of 10 g of elastin powder in 1 liter of 0.01 M NH₄HCO₃ buffer at pH 8.8 was stirred overnight. All buffers were saturated with chloroform to minimize bacterial contamination. Two additions of elastase (20 mg each) were made, allowing 24 hours of incubation at room temperature after each addition; the pH was maintained between 8.7 to 8.9 with the addition of concentrated NH₄OH. The volume of the mixture was then reduced to 150 ml in a Diaflo apparatus by means of a UM 10 membrane under nitrogen pressure (50 to 80 p.s.i.). The retentate was diluted with 350 ml of the buffer and reconcentrated to 150 ml; this procedure was repeated two more times. The retentate was then diluted to 500 ml with the same buffer. Two further additions of elastase (20 mg each) were made, and the mixture allowed to incubate for 24 hours after each addition. The solution was then boiled for 5 min and filtered through Whatman No. 5 paper.

Purification of Cross-Linked Peptides—After numerous trials the following procedure was adopted. The elastolytic peptides produced by exhaustive digestion were chromatographed on a column of Cellex P as shown in Fig. 1. Fraction CPB (Fig. 1) was further purified by chromatography on a column of Sephadex G-50 (fine) as shown in Fig. 2.

Fraction G50-III (Fig. 1) was then chromatographed on a column of Sephadex G-25 (fine) and the peak divided into three fractions as shown in Fig. 3. Fraction G25.2 (Fig. 3) was separated on a column of Sephadex LH-20 by means of the conditions of Fig. 4. The excluded material (A) was dried on a rotary evaporator and used for the release
of single chain peptides from the COOH-terminals of the cross-links. Suitable aliquots from all pooled fractions were subjected to amino acid analysis (see Table I).

**Determination of Aald Condensation Product and Other Reducible Amino Acids**—The peptides (about 200 nmole) were reduced for 8 hours in 0.2 M NaBH₄ with at least a 200-fold molar excess over desmosines. The excess reagent was then destroyed by adjusting the pH to 3 with 3 M acetic acid. The sample was then applied on a Sephadex G-15 column (0.9 X 100 cm) previously equilibrated with 0.01 M NH₄HCO₃ buffer, pH 8.8. The column was eluted with the same buffer and the excluded fraction containing the carbamylated material was completely removed by chromatography on a Sephadex G-15 column (0.9 X 100 cm) previously equilibrated with 0.01 M NH₄HCO₃ buffer, pH 8.8. The excess reagents were then destroyed by adjusting the pH to 3 with 3 M acetic acid. The sample was then applied on a Sephadex G-15 column (0.9 X 100 cm) previously equilibrated with 0.01 M NH₄HCO₃ buffer, pH 8.8. The column was eluted with the same buffer and the excluded fraction containing the carbamylated material was collected. An aliquot was taken for the determination of the total radioactivity. Another aliquot of the purified carbamylated fraction was dried in a conical tube and incubated at 37°C for 12 hours in 6 N HCl under reduced pressure at 110°C and the desmosines determined on the amino acid analyzer.

The rest of the carbamylated material was subjected to the same procedure as described (11) except for the determination of hydantoin radioactivity. The Dowex 50-X2 fractions (A, B, and C) were collected separately. The radioactivity of an aliquot of each of these fractions was determined and the remainder then analyzed as described (11).

**Release of Single-chain Peptides from Desmosine and Isodesmosine-containing Cross-Linked Bovine Elastin by Preparative Edman Degeneration**—The COOH-terminal peptides were released from desmosine and isodesmosine cross-links by Edman degradation (for explanation and rationale see results and Fig. 7).

The fraction (Table I, A) containing the cross-linked peptides was dissolved in 4 ml of coupling buffer (50% pyridine, 2% triethylamine) and flushed with nitrogen. At least a 50-fold excess of phenylisothiocyanate was added, and the mixture incubated under nitrogen for 45 min at 50°C. The sample was dried at 60°C under reduced pressure, opened under nitrogen, and incubated with 1 ml of trifluoroacetic acid at 50°C for 15 min; it was then dried by rotary evaporation under reduced pressure.

The residue was chromatographed on a Sephadex LH-20 column as shown in Fig. 4. The excluded peak (Fraction A) was dried and the degradative cycle and LH-20 chromatography repeated two more times. The released single-chain peptides appeared in Fraction B (Fig. 4) and were then purified. The purified peptides were recovered from Fraction B (Table III) as described above for Fraction A. This fraction (Ap) was obtained from porcine aortic elastin in a manner identical with that described for the bovine Fraction A.

**Purification of Released Peptides**—Fraction B (Fig. 4) from each cycle was dried and chromatographed on the peptide analyzer as shown in Fig. 5. Suitable aliquots from all fractions were sub-

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**Table I**

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**Fig. 5.** Purification of released single chain peptides by ion exchange chromatography. A, B, and C represent elution profiles of the peptides obtained after the first, second, and third cycle of Edman degradation, respectively. The peptides are numbered in the order in which they are eluted. A column (0.9 X 55 cm) of Beckman AA-15 resin was equilibrated with Buffer I at 55°C at 34 ml per hour. The sample was dissolved in 2 ml of Buffer I and the pH adjusted to 2.2 with 1 N HCl; this sample was applied to the equilibrated column which was then developed with a gradient consisting of 200 ml of each of Buffers I, II, and III connected in sequence in the order of increasing pH and molarity. (I: 0.06 5 pyridine acetate, pH 2.6; II: 0.10 5 pyridine acetate, pH 3.15; III: 0.50 5 pyridine acetate, pH 3.7.) One-tenth of the total effluent was continuously monitored with ninhydrin after automatic alkaline hydrolysis according to Hill and Delaye (12). The remaining effluent was collected in 5-min fractions. The colorimeter chart was correlated with the tube number by a previously determined time constant; this was verified for each run by ninhydrin assay after alkaline hydrolysis of the last major peak in each chromatogram.
were removed by ultrafiltration. This was followed by further
larger peptides).

Over 90% of the desmosines absorb on this ion exchanger under
the conditions used. The elution with salt produced two peaks.

Peptides released from Fraction Ap (porcine aortic elastin,
Table III) by the first cycle of Edman degradation were further
purified according to the system described in Fig. 5. The composi-
tions and elution volumes of the pure porcine peptides reported
in this paper are given in Table III.

Sequencing of Purified Released Peptides—Edman degradation
was carried out by a procedure adapted from one previously de-
scribed (14). The dried sample was dissolved in 300 μl of coupling
buffer (50% pyridine, 27% triethylamine), 7.5 μl of phenylisothio-
cyanate, 12.4 μl of dry trifluoroacetic acid were added,
reduced pressure, 200 μl of dry trifluoroacetic acid were added,
in this paper are given in Table III.

NH₂-terminal residues were identified by Hartley’s microdansyl
method (14) except that the hydralysis time in 6 M HCl was reduced to 4
hours as recommended by Gros and Labouesse (15). Dansyl
alanine and glycine were identified by chromatography on polyamide
sheets (5 × 5 cm) by means of Hartley’s system.

RESULTS

Isolation of Cross-Linked Peptides—The exhaustive digestion
of elastin with elastase produced limit peptides which could not
be further digested. Various small peptides (16) inhibit elastase.
Such peptides are released on estalysin of elastin (17, 18) and
were removed by ultrafiltration. This was followed by further
estalysin digestion (omission of this step resulted in significantly
larger peptides).

Chromatography on Cellex P was used as an initial step.
Over 90% of the desmosines absorb on this ion exchanger under
the conditions used. The elution with salt produced two peaks.
The second fraction (Fig. 1, CPB) which was the richest in
cross-links, was used for further purification.

Separation on the basis of size indicated a large range in ap-
parent molecular weight (Fig. 2). It was observed that the
elution profile did not change when repeated in the presence of
4 M urea. Thus the broad elution profile is not due to aggrega-
tion. This is further supported by the fact that various regions of
the broad peak when rechromatographed on the same column
eluted in their original position. Amino acid analysis showed a
range of 22 to 34 residues per cross-link (Table I).

The chromatography of Fraction G50-III on a column of
Sephadex G-25 showed that the bulk of the material (G52.2)
was slightly retarded (Fig. 3). Amino acid analysis indicated a
minimum molecular weight of approximately 2500. Therefore,
that G52.2 consists of cross-linked peptides containing only
1 cross-link per molecule. This fraction contains 25 to
30% of the total desmosine and isodesmosine present in elastin.

Determination of Aldol Condensation Product and Other Reduc-
ible Amino Acids—For the determination of aldol condensation
product and other reducible amino acids both large (G50-1) and
small (A) peptides (Table I) were reduced with sodium [³H]-borohydride. The products were chromatographed after
appropriate hydrolysis and the radioactivity determined as shown
in Fig. 6. The small peptides incorporated radioactivity only
in the region of the tetrahydridesmesonesines (Figues 6, C and D).
The reduction of the large peptides resulted in radioactivity in
several regions and these are identified on the chromatogram
(Fig. 6, A and B) (18, 19). The results show that lysinal, aldol
condensation products, dehydrinemerosines, and dehydroly-
sinonorleucines are essentially absent from the small peptides
(A) (<0.01 mole of reduced aldol condensation product, <0.01
mole of merodesmosine per mole of desmosines; no trace of ly-
sinonorleucine or hydroxynorleucine; values calculated from the
amino acid analyzer chart).

Quantitative Determination of NH₂-Terminals—The Stark and
Smyth procedure (11) is based on the separation of hydantoin-
s from amino acids and peptides. The hydantoines are hydrolyzed
to free amino acids which are then analyzed. The multifunc-
tional amino acids (desmosine and isodesmosine) present a
special problem since a large number of possible products can
be formed. This multiplicity of products is due to the fact that
carbamylation is possible at four different positions of the two
isomers and that peptides may remain attached to uncarbamyl-
ated (non NH₂-terminal) positions of the cross-links after cycli-
ization (see Fig. 7). To overcome this problem the total cyanate

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Yield 1.  19.2  27.6  30.0  51.5  7.2  b)  76.4  72.7  98.0

All values are expressed as residues per peptide. A blank indicates less than 0.2 residue per peptide. The peptides are numbered as in Figure 5.

a) The yields are expressed as a percentage of theoretical and are uncorrected for losses. The theoretical yield (6.3% of desmosine plus isodesmosine content) is based on the presence of 12 COOH-terminal peptides (see discussion).

b) See the yield for peptide 10. This yield is based on the assumption that 1 mole elastine plus alanylalanyl are released per mole crosslink due to elastase cleavage between lysine pairs (see discussion) in addition to Ala and Ala-Ala being released from the COOH-terminal positions.

The abbreviation used is: dansyl, 5-dimethylaminonaph-
thalene-1-sulfonyl.
bound (after carbamylination) was determined, and all of the cyanate recovered in Fraction C but not as hydantoins of known amino acids was taken to indicate cross-link NH$_2$-terminal.

This procedure was used for Fraction A (Table I), as described under “Methods.” The results indicate the presence of three amino-terminals per cross-link (1.7 moles of alanine, 0.2 mole of glycine, 1.0 eq of cross-link per mole of cross-link).

**Release and Purification of COOH-terminal Peptides from Cross-links**

The characterization of peptides released from the cross-links by means of chemical cleavage (21) indicated that elastase was cleaving at and very close to the NH$_2$-terminals of the cross-links. The NH$_2$-terminal analysis showed cross-links as one of the NH$_2$-terminals. It was therefore thought possible to release COOH-terminal peptides from the cross-links by Edman degradation, as shown in Fig. 7. To illustrate the rationale of the approach, the degradation of only one of the many possible structures present is shown. At those positions where the cross-link itself is the NH$_2$-terminal, Edman degradation will release the peptides attached to the α-carboxyl groups (e.g. H$_2$N-P$_1$). The separation of the released peptides from the cross-linked ones on the basis of size is then possible.

The peptide H$_2$N-P$_2$ will be released after a second cycle of Edman degradation. On the other hand, H$_2$N-P$_3$ will not be released until the cross-link NH$_2$-terminal immediately preceding P$_3$ becomes available (i.e. H$_2$N-P$_1$ will be released after four cycles of Edman degradation). This approach resulted in the release of the COOH-terminal peptides from the cross-links as expected.

Attempts to separate these released peptides from the cross-linked ones by means of chromatography on Sephadex G-15 were unsuccessful due to severe losses attributable to the production of insoluble peptides. This is apparently due to the presence of one or more phenylthiohydantoin moieties on the cross-links (see Fig. 7). This problem was solved by chromatography on Sephadex LH-20 in 50% pyridine and a typical elution profile is shown in Fig. 4.

The released peptides were recovered in Fraction B and further purified as shown in Fig. 5. This resulted in the isolation of eight pure peptides in addition to alanine from bovine elastin; the amino acid compositions of these peptides are shown in Table II. The amino acid compositions of the pure porcine peptides reported in this paper are shown in Table III.

**Sequences of Released Peptides**

The sequences of the purified peptides released from bovine elastin cross-links are shown in Table IV. Peptides 6 and 9 are redundant (see Peptides 10 and 5) and hence a total of seven unique sequences were found. Links—The characterization of peptides released from the cross-links by means of chemical cleavage (21) indicated that elastase was cleaving at and very close to the NH$_2$-terminals of the cross-links. The NH$_2$-terminal analysis showed cross-links as one of the NH$_2$-terminals. It was therefore thought possible to release COOH-terminal peptides from the cross-links by Edman degradation, as shown in Fig. 7. To illustrate the rationale of the approach, the degradation of only one of the many possible structures present is shown. At those positions where the cross-link itself is the NH$_2$-terminal, Edman degradation will release the peptides attached to the α-carboxyl groups (e.g. H$_2$N-P$_1$). The separation of the released peptides from the cross-linked ones on the basis of size is then possible.

The peptide H$_2$N-P$_2$ will be released after a second cycle of Edman degradation. On the other hand, H$_2$N-P$_3$ will not be released until the cross-link NH$_2$-terminal immediately preceding P$_3$ becomes available (i.e. H$_2$N-P$_1$ will be released after four cycles of Edman degradation). This approach resulted in the release of the COOH-terminal peptides from the cross-links as expected.

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tides obtained by chemical cleavage suggested that elastase single chain COOH-terminal peptides from the cross-linked ones This evidence led us to use Edman degradation for the release of on a Sephadex LH-20 column and the tail end of the peak dis-

The starting material, the peptides (G25.2) were chromatographed the starting material, the peptides (G25.2) were chromatographed cleaves bonds at or near the NH₂-terminals of the cross-links. Full 
mole of glycine, 1.0 eq of cross-link per mole of desmosine or Total NHP-terminal determination (1.7 moles of alanine, 0.2

unidentified. The low yield of this peptide might be due to major elastolytic cleavages at the alanyl bonds. The un-

seven unique sequences, Table IV) than could be expected from

This point will become clearer as the discussion proceeds. A homoge-

eous species (one or two COOH-terminal peptides). All three cycles of Edman degradation. (Some peptides were released on

Analysis of the peptide in Peak 7 indicated the sequence Ala-

The peptide fraction used for the release of COOH-terminal peptides from the cross-links was apparently not homogeneous. This is clear from the number of peptides released and the manner in which they were released. (Some peptides were released on all three cycles of Edman degradation.) However, it is also apparent that this nonhomogeneous nature of the peptides in no way detracts from the validity of the results or conclusions. In fact, the use of nonhomogeneous peptides provided more information about the structure of elastin (identification of seven unique sequences, Table IV) than could be expected from a homogeneous species (one or two COOH-terminal peptides). This point will become clearer as the discussion proceeds.

As mentioned under "Results," the characterization of pep-
tides obtained by chemical cleavage suggested that elastase cleaves bonds at or near the NH₂-terminals of the cross-links. Total NH₂-terminal determination (1.7 moles of alanine, 0.2 eq of glycine, 1.0 eq of cross-link per mole of desmosine or isodesmosine) provided additional confirmation of this view. This evidence led us to use Edman degradation for the release of single chain COOH-terminal peptides from the cross-linked ones (Fig. 7). To make sure that no small peptides were present in the starting material, the peptides (G25.2) were chromatographed on a Sephadex LH-20 column and the tail end of the peak discarded. The chromatography of the Edman degradation products on the Sephadex LH-20 column resulted in the clear separa-

Table IV
Amino acid sequences COOH-terminal to desmosines in bovine ligamentum nuchae elastin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Des-Ala-Gly-Tyr-Pro-Thr</td>
</tr>
<tr>
<td>2</td>
<td>Des-Ile-Gly-Ala-Gly-Gly-Val</td>
</tr>
<tr>
<td>3</td>
<td>Des-Phen-Gly-Pro-Gly-Gly-Val</td>
</tr>
<tr>
<td>4</td>
<td>Des-Leu-Gly-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>5</td>
<td>Des-Ala-Gly</td>
</tr>
<tr>
<td>6</td>
<td>Des-Ala</td>
</tr>
<tr>
<td>7</td>
<td>Des-Phe-Gly-Ala-Ala</td>
</tr>
<tr>
<td>8</td>
<td>Des-Ala-Gly</td>
</tr>
<tr>
<td>9</td>
<td>Des-Ala</td>
</tr>
<tr>
<td>10</td>
<td>Des-Ala-Ala</td>
</tr>
</tbody>
</table>

The relevant portions of the NH₂-terminal sequences of tryp-
tic peptides of porcine aortic tropoelastin (1) are compared with the sequences of the peptides released from the COOH-terminals of the elastin cross-links (Table V). The sequence of Peptide 5 is identical with the NH₂-terminal sequence of Peptide T6. Similarly, Peptide 1 is identical with the NH₂-terminal sequence of Peptide T7b and Peptide 10 is identical with the NH₂-terminal sequences of Peptides T2 and T9b. The only difference between Peptide 8 and the NH₂-terminal sequences of Peptides T1 and T10 is the replacement of tyrosine by phenylalanine, a highly common mutation (25). Since the sequences from bovine ligamentum nuchae elastin are being compared with porcine aortic tropoelastin sequences (two different tissues and species), such differences would be expected. A single base mutation of proline to alanine, again a highly common mutation (25), makes the

Table V
Comparison of sequences of peptides COOH-terminal to elastin cross-links with NH₂-terminal sequences of tryptic peptides of tropoelastin

<table>
<thead>
<tr>
<th>Source</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine ligament</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>porcine</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>aorta</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>T9c</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>tropoelastin</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>aorta</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>T10</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>ligament</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
<tr>
<td>T9c</td>
<td>Tyr-Gly-Ala-Ala-Gly-Gly-Ala</td>
</tr>
</tbody>
</table>

a) Data for tropoelastin taken from reference 1.
Our preliminary results with porcine aortic elastin show the release of Tyr-Gly-Ala-Ala (P12) and Tyr-Gly-Ala-Pro-Gly (P4), but not of Phe-Gly-Ala-Ala. Thus Peptide 8 corresponds to the NH2-terminal sequences of Peptides T1, T9c, and possibly T4. We have also isolated the peptide with the sequence Ala-Pro-Gly-Gly-Gly-Ala from porcine aortic elastin (Peptide P11, Table V). This peptide is identical with the NH2-terminal sequence of Peptide T14b. The fact that this peptide could not be isolated from bovine ligamentum elastin may be due to a single base mutation of proline to alanine, resulting in the sequence Ala-Ala-Gly- which is readily susceptible to elastase cleavage (8). The yield of the Ala-Ala peptide (Table II) was sufficiently high to account for this sequence being repeated three times (Peptides T2, T9b, and T14b). Peptides 2, 3, and 4 are not readily identifiable with tryptic peptides of tropoelastin. As over one-half of the tropoelastin sequences are not yet reported, it is very likely that the sequences corresponding to these peptides will be found, especially in view of the fact that porcine aortic elastin yields a similar peptide (see Table V, Peptide P3). From these results it can be concluded that the lysines preceding the tryptic peptides T1, T2, T4, T6, T7b, T9b, T9c, and T14b may be involved in cross-links and that at least three more tryptic peptides, corresponding to Peptides 2, 3, and 4, will be discovered. This identifies a total of 11 lysine sequences which may take part in cross-linking. If one of these peptide sequences is repeated, as is the case with the NH2-terminal sequences of Peptides T1 and T9c and also T2 and T9b, this would bring the number of cross-linked lysine sequences to 12. A possible candidate for this repeated sequence is Peptide 4, which was obtained in twice the yield of Peptides 2 or 3.

The possibility of 12 lysine sequences being involved in cross-linking raises an interesting point. Sandberg et al. (18) reported the isolation of 6 moles of Ala-Ala-Lys and 6 moles of Ala-Ala-Ala-Lys peptides per mole of tropoelastin after tryptic digestion. On the basis of these data they proposed the presence of 6 repeated sequences of Lys-Ala-Ala-Lys-Ala-Ala-Lys per tropoelastin molecule. However, these data could also be interpreted to indicate the presence of 6 pairs of lysines in the sequence Lys-Ala-Ala-Lys and 6 pairs of lysines in the sequence Lys-Ala-Ala-Lys (a total of 12 such pairs of lysines) dispersed in the molecule. This would then provide for 12 potential cross-link regions in the molecule. If this interpretation is correct, the formation of desmosine and isodesmosine would involve two such pairs of lysines, as shown in Fig. 8. This may take place in some random fashion (26). All the pairs do not necessarily meet another pair due to steric and other factors and thus some will remain as aldol condensation products, dehydrolysinonorleucines, lysinonorleucine, etc. Some pairs may form cross-links with lone lysines to form dehydromerodesmosine and three allysine condensation products. The formation of additional desmosine and isodesmosine after oxalic acid solubilization of elastin (27) suggests that such pairs do exist and apparently react upon the removal of these steric restraints. Thus it is possible that only about one-half of these pairs actually form desmosine and isodesmosine (about 3 residues per molecule). This would be consistent with the observed values in mature elastin of 1.8 moles of desmosine plus 1.4 moles of isodesmosine (6) per 850 to 880 residues (the total number of residues in the proposed monomer) (1, 2). If six clusters of 3 lysines are present as suggested by Sandberg et al., then six other regions, probably of single lysines, must be involved to account for the 12 COOH-terminal peptides. Although several other explanations are possible, in our opinion, the presence of 12 pairs of lysines, with two such pairs forming desmosine or isodesmosine is the simplest explanation of all the available data on elastin and tropoelastin and is consistent with the isolation of an alanine-containing aldol condensation cross-link by Foster et al. (28).

As the cross-linked peptides showed a total of three NH2-terminals (1.7 moles of alanine, 0.2 mole of glycine, 1.0 eq of cross-link per mole of cross-link), one will have to conclude that two of the four cross-link amino groups were in continuous peptide chain. If one assumes that the cross-link forms between two pairs of lysines, as discussed above, then the third NH2-terminal must be produced by elastolytic cleavage of alanyl bonds between the pair of lysines (elastase readily cleaves such bonds (8)). This raises the possibility that elastolytic cleavages were occurring between both pairs of lysines, as indicated in Fig. 8, and that the observed value of NH2-terminals is the average of these cleavages. The fact that COOH-terminal peptides are released after the first, second, and third cycle of Edman degradation supports this conclusion. No peptides will be released from those portions of the peptide chains which were "not nicked" by elastase until after the fourth cycle of Edman degradation (Fig. 7). The fact that peptides were released even after the fourth, fifth, and sixth cycle of degradation, although in reduced yields, indicates that "un-nicked" peptide chains were present. Therefore, the theoretical yields of the released peptides in the first three cycles (Table II) were calculated.
assuming 50% "nicks" distributed evenly between 12 pairs of lysines forming the cross-links.

The novel use of the Edman degradation described in this paper permits more meaningful study of the cross-link regions of elastin. It identifies the lysines in tropoelastin which are involved in desmosine and isodesmosine cross-link formation. We are now surveying different enzymes which will solubilize elastin without cleaving the alanyl bonds, thus enabling us to provide evidence for or against the proposed model.

Acknowledgments—We are grateful to June Radley for technical assistance and to Dr. T. Hofmann for his critical review of the manuscript.

Note Added—After the submission of our paper, a paper by Gray et al. appeared (29) which reported the occurrence of the sequence -Ala-Ala-Ala-Ala-Lys-Ala-Ala-Lys-Tyr-Gly-Ala-Ala- in tropoelastin (soluble elastin). This is consistent with our model (Fig. 8) and in fact this sequence was suggested (P12, Tyr-Gly-Ala-Ala, Table V, preceded by Ala-Ala-Lys-Ala-Ala-Lys).

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Structural Studies on Cross-Linked Regions of Elastin
Gerhard E. Gerber and Rashid A. Anwar


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