The Nature of the Intramolecular Cross-Links in Collagen. The Separation and Characterization of Peptides from the Cross-Link Region of Rat Skin Collagen*

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ABSTRACT: Isolated single-chain (α) and double-chain (β) components from rat skin collagen were cleaved at methionyl residues with cyanogen bromide and the resulting peptides were chromatographed on phosphocellulose. A lysyl-containing peptide of 15 amino acids was isolated from $\alpha 1$ ($\alpha 1$-CB-1) and another of 14 amino acids from $\alpha 2$ ($\alpha 2$-CB-1). These peptides are present as major components in digests of recently synthesized (salt extracted) collagen but are largely replaced in digests of less recently synthesized, more highly cross-linked collagen (acid extracted) by peptides ($\alpha 1$-CB-1a and $\alpha 2$-CB-1a) which react as aldehydes and differ in amino acid composition from the original peptides in that they lack lysine. Peptides $\alpha 1$-CB-1a and $\alpha 2$-CB-1a each contain instead a residue of lysyl-derived δ-semialdehyde of α-aminoadipic acid in peptide linkage. The aldehyde precursor lysyl residue is the fifth amino acid from the N-terminal end in both $\alpha 1$-CB-1 and $\alpha 2$-CB-1. Both the lysyl-containing and the lysyl-derived aldehyde-containing peptides from $\alpha 1$ and $\alpha 2$ are absent in cyanogen bromide digests of β_{12} while a new peptide, β_{12}-CB-1, is present. The amino acid composition, molecular weight, and other properties of β_{12}-CB-1 indicate that it is a dimer of $\alpha 1$-CB-1a and $\alpha 2$-CB-1a. Similar but less extensive studies demonstrate that an analogous peptide, β_{11}-CB-1, a dimer of $\alpha 1$-CB-1a, is present in digests of β_{11}. Both β_{12}-CB-1 and β_{11}-CB-1 react as aldehydes, but the spectra of derivatives of these peptides indicate that their aldehyde function differs from that in $\alpha 1$-CB-1a and $\alpha 2$-CB-1a and suggest the presence of an α,β-unsaturated aldehyde. It is tentatively proposed that the intramolecular interchain cross-link in collagen results from an aldol-type condensation of two lysyl-derived aldehydes on adjacent chains. The lysyl-containing peptides, $\alpha 1$-CB-1 and $\alpha 2$-CB-1, are present but their aldehyde-containing derivatives, $\alpha 1$-CB-1a and $\alpha 2$-CB-1a, are largely absent in cyanogen bromide digests of collagen from lathyritic rats. The data suggest that lathyrogens inhibit the process by which specific lysyl residues in peptide linkage are converted to their aldehyde derivatives.

Experimental support for the existence of interchain covalent bonds in collagen has come from molecular weight studies of gelatin fractions which reveal a spectrum of molecular weights ranging into the millions (see Veis, 1964) and from both biosynthetic and structural studies of soluble collagen in which single-chain (α) and double-chain (β) components were shown to exist in a precursor–product relationship (Martin *et al.*, 1961, 1963; Piez *et al.*, 1961, 1963). Since vertebrate collagens lack cystine, the nature of these cross-links has been the subject of considerable speculation and investigative effort. Recent reviews (Harding, 1965; Seifter and Gallop, 1966) examine the evidence for the presence of cross-links in collagen and the various structures which have been proposed. The structure, or structures, in question have not been investigated systematically partly because of the size and complexity of the collagen molecule and because of difficulty in complete purification of the protein.

In order to circumvent some of these problems we have employed as a starting material single- and double-chain components separated from denatured soluble collagen by chromatography on carboxymethyl- (CM-) cellulose (Piez *et al.*, 1961, 1963; Bornstein and Piez, 1964). It was reasoned that if the $\alpha 1$ and $\alpha 2$ chains of collagen were cleaved in such a way as to break only selected peptide bonds, a comparison of the products from $\alpha 1$ and $\alpha 2$ with those obtained by a similar treatment of β_{12}, the covalently linked dimer of $\alpha 1$ and $\alpha 2$, might be expected to reveal differences which could be ascribed to the presence of interchain cross-links in β_{12}. In view of the low methionine content of mammalian collagens (six to eight residues per α chain of about 95,000 mol wt) and the high specificity of cyanogen bromide (CNBr) for cleavage at methionyl bonds (Gross and Witkop, 1961), it appeared that CNBr might prove to be a useful reagent for this purpose.

Initial experiments in which the reaction products were fractionated on CM-cellulose demonstrated the

* From the National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland. *Received July 14, 1966.* A portion of this work has appeared in preliminary form (Bornstein *et al.*, 1966). The terminology in the present communication has been altered from that employed earlier to conform more closely to current designations for peptides derived from protein chains. The symbol CB is used to indicate derivation by cyanogen bromide cleavage. Hence, $\alpha 1$-CB-1 replaces $\alpha 1^{1}$, $\alpha 2$-CB-1a replaces $\alpha 2^{1a}$, β_{12}-CB-1 replaces β_{12}^{x}, etc.

feasibility of this approach (Bornstein and Piez, 1965). The larger peptides from β_{12}, ranging in molecular weight from about 3000 to 23,000, were identical in properties and amounts with those from $\alpha 1$ plus $\alpha 2$. However, the smaller peptides which travel with little or no retardation on CM-cellulose have now been separated by chromatography on phosphocellulose, and differences have been found which can be related to the cross-link. This report presents these results together with preliminary evidence regarding the nature of the intramolecular cross-link in collagen, its mechanism of formation, and the manner in which lathyrogenic agents inhibit cross-linking.

Materials and Methods

Preparation of Collagen. Rat skin collagen was obtained from the skins of 100–150-g male Sprague-Dawley rats. Salt- and acid-extracted collagens were prepared largely as described previously (Bornstein and Piez, 1964). However, instead of being subjected to repeated solution and salt precipitation at neutral pH, salt-extracted collagen was precipitated from a 1 M NaCl solution containing 0.05 M Tris, pH 7.5, by the addition of NaCl to 20% (w/v), washed three times with 20% NaCl, dissolved by the addition of distilled water, and then precipitated by the addition of acetic acid to pH 4. Acid-extracted collagen was obtained by extraction with 0.5 M acetic acid and was purified by precipitation at acidic pH with 5% NaCl and at neutral pH with 20% NaCl.

Lathyritic Collagen. Rats (15, 150 g) were placed on a diet containing 1 g of BAPN[1]/kg for 3 weeks. Each rat ingested an average of 15 g of diet daily and therefore received 15 mg of BAPN. After sacrifice, salt-extracted skin collagen was obtained as described above.

Lysine-^{14}C Collagen. L-Lysine-^{14}C (U.L.) was obtained from New England Nuclear Corp. A solution containing 0.5 mc (0.33 mg) in 5 ml of 0.01 N HCl was neutralized and diluted to 30 ml. Ten rats weighing 80–90 g received 1 ml (16.7 μc) of this solution intraperitoneally daily for 3 days. After the last injection of isotope (24 hr later), the rats were sacrificed and salt- and acid-extracted skin collagens were prepared.

Preparation of $\alpha 1$, $\alpha 2$, β_{11}, and β_{12}. Heat-denatured salt- and acid-extracted collagens were fractionated on columns of CM-cellulose (Piez *et al.*, 1961, 1963; Bornstein and Piez, 1964). A CM-cellulose which provides satisfactory separation is Whatman CM 32, 1.0 mequiv/g, available from Reeve Angel, N. J. This product, obtained as a dry microgranular preparation, was washed with 0.5 N NaOH, water, 0.5 N HCl, water, 0.5 M sodium acetate, pH 4.8, and finally with 0.06 M sodium acetate, pH 4.8. The washed cellulose, suspended in 0.06 M sodium acetate, was packed under gravity flow in 1.8 × 18 cm or 2.5 × 12 cm jacketed columns. Precision-bore glass columns with Teflon plunger-type fittings equipped with O rings at the top and bottom of the column were used. The plungers could be adjusted to eliminate air and liquid dead space within the column, thus permitting reverse-flow chromatography. Flow rates of 140 ml/hr, or 180 for the larger diameter columns, were tolerated without buildup of excessive pressure. Appropriate effluent fractions were desalted on columns of Sephadex G-25 using a pyridine acetate buffer (Piez *et al.*, 1963) and lyophilized.

Although separation of β_{11} from $\alpha 1$ was never complete, the $\alpha 1$ chain could be obtained essentially free of β_{11} by pooling fractions representing the first half of the combined peak. Contamination with β_{11} was 5% or less as judged by polyacrylamide gel electrophoresis (Nagai *et al.*, 1964). The second half of the $\alpha 1$-β_{11} peak was taken as the β_{11} fraction and usually contained 50% or more of $\alpha 1$. The β_{12} component was separated completely from $\alpha 1$ and β_{11}, and almost completely from $\alpha 2$, by CM-cellulose chromatography. Acrylamide gel electrophoresis indicated that its purity exceeded 95%. The $\alpha 2$ chain, however, was usually contaminated with 5–10% of β_{12}.

Chromatography on Phosphocellulose. Whatman phosphocellulose, floc, capacity 7.4 mequiv/g, was obtained from Reeve Angel, N. J. The dry preparation was sieved through standard U. S. grade screens. The 140–230 mesh fraction was collected and washed with 0.5 N NaOH, 1 N HCl, distilled water, and then with 0.1 M sodium acetate buffer, pH 3.8. It was suspended in 0.001 M sodium acetate, pH 3.8, poured into a jacketed 1.8 × 18 cm column, and packed employing a finger pump at a flow rate of 140 ml/hr. Chromatography was performed at 40° in 0.001 M sodium acetate, pH 3.8, by superimposing a linear gradient of NaCl from 0 to 0.3 M over a total volume of 820 ml. The optical density of the effluent was monitored at 230 mμ in a Gilford Model 2000 multiple sample absorbance recorder attached to a Beckman DU monochromator. Fractions of 10 ml were collected and 0.5-ml aliquots of radioactive samples were plated on planchets and counted for 5 min in a low-background planchet counter. The phosphocellulose was regenerated and repoured after each run.

Molecular Sieve Chromatography. Sephadex G-25, fine beads, was a product of Pharmacia, Inc. Bio-Gel P-2, 100–200 mesh, was obtained from Bio-Rad Laboratories. Sephadex G-25 or Bio-Gel P-2 was suspended in ammonium propionate buffer, pH 4.5 (2.24 ml of propionic acid and 0.98 ml of concentrated NH_4OH diluted to 1 l.). Columns (2 × 40 cm) were packed and elution was carried out under gravity at a flow rate of 46 ml/hr for the Bio-Gel P-2 column and 85 ml/hr for the Sephadex G-25 column. The samples were applied in a volume of 1–3 ml of ammonium propionate buffer and 3.3-ml fractions were collected. The optical density of the effluent was monitored at 230 mμ.

Cleavage with CNBr. CNBr (Eastman Organic Chemicals) was used without further purification. The technique employed has been described (Bornstein and

[1] Abbreviations used: BAPN, β-aminoproprionitrilefumarate; MBTH, *N*-methylbenzothiazolone hydrazone.

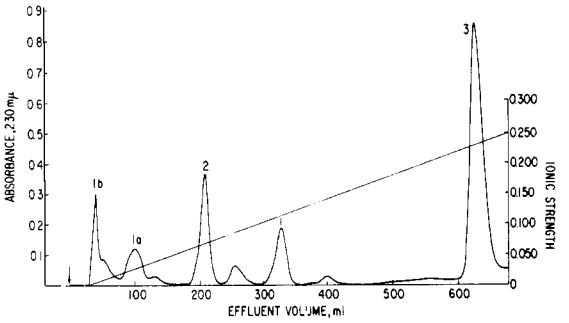

FIGURE 1: Elution pattern of peptides obtained by CNBr cleavage of the α1 fraction of salt-extracted collagen from normal rats. Chromatography was performed on phosphocellulose at pH 3.8, 40°. The arrow indicates placement of the sample (90 mg) dissolved in 10 ml of starting buffer, 0.001 M sodium acetate, pH 3.8. The straight-line plot indicates the ionic strength gradient. Peptides α1-CB-1b, α1-CB-1a, α1-CB-2, α1-CB-1, and α1-CB-3 are identified. The initial peak consists in part of nonpeptide material eluting at the gradient front.

Piez, 1965). Satisfactory conversion of methionine to homoserine (85–92%) in α chains and β components was achieved by incubation in 0.1 N HCl at 30° for 4 hr, employing a 100-fold molar excess (relative to methionine) of CNBr. After cleavage, all samples were diluted 10-fold with cold water and lyophilized.

Digestion with Trypsin and Chymotrypsin. A known quantity of peptide was dissolved in 2 ml of 0.1 M NH_4HCO_3, pH 7.8, containing 0.005 M $CaCl_2$. Trypsin (two times crystallized, Worthington) or α-chymotrypsin (three times crystallized, Worthington) was added as a 0.5% solution in 0.001 M HCl in a molar enzyme: substrate ratio of 1:50. After 4 hr at room temperature, the pH was lowered to 4 with a few drops of concentrated acetic acid and the solution was lyophilized.

Reaction with 2,4-Dinitrophenylhydrazine. The procedure used is largely that described by Rojkind *et al.* (1964). Acid-extracted collagen was suspended in cold water and dissolved by stirring for 12 hr at 4°. The solution was mixed with an equal volume of 2 N HCl containing a 50-fold molar excess of 2,4-DNP-hydrazine (relative to the equivalents of α chain in the sample) and stirred for 5 min at 25°. Excess 2,4-DNP-hydrazine was removed by exhaustive dialysis in the cold and the preparations were lyophilized.

Azine Formation with MBTH. The spectrophotometric assay described by Paz *et al.* (1965) was used without modification. MBTH was a product of Eastman Organic Chemicals. The reaction was followed in a Cary Model 11 recording spectrophotometer with the cell compartment jacketed at 40°.

Reduction with Borohydride. The peptide was dissolved in 2.0 ml of cold water. Sufficient solid $NaBH_4$ was added to a cold 0.1 M, pH 8.5, sodium phosphate buffer to make a 0.1 M borohydride solution, and 2.0 ml of this was added to the peptide solution. The mixture was allowed to stand in ice for 30 min, then at room temperature for 1 hr, and at 37° for an additional hour. The reaction was stopped by addition of sufficient acetic acid to lower the pH to 4 and salts were separated by passage through a P-2 column equilibrated with the ammonium propionate buffer. The solution was then lyophilized.

Oxidation with Performic Acid. Oxidation was performed by a modification of the method of Moore (1963). The peptide was dissolved in 2 ml of the performic acid reagent and oxidation was carried out at room temperature for 5 hr. The solution was then cooled to 4°, 0.3 ml of 48% HBr was added, and the solution was taken to dryness in a rotary evaporator under reduced pressure at 40°. The oxidized peptide was then dissolved in several milliliters of water and lyophilized.

Amino Acid Analysis. Analyses were performed on a single-column automatic amino acid analyzer modified for high-speed analysis (Miller and Piez, 1966). Peptides were hydrolyzed under nitrogen in 6 N HCl at 108° for 24 hr. Corrections for loss of serine, threonine, and tyrosine and incomplete release of valine were made using previously determined correction factors (Piez *et al.*, 1960). Radioactivity in the effluent from the analyzer was measured by continuous scintillation counting (Piez, 1962). The number of micromoles in solution of a small peptide of known composition was determined by performing an amino acid analysis on an aliquot.

Molecular Weight Determinations. The molecular weights of peptides β_{12}-CB-1 and α1-CB-2 were determined by high-speed sedimentation equilibrium in a Spinco Model E ultracentrifuge as described by Yphantis (1964) using a titanium rotor at 67,770 rpm. The peak fraction containing the peptide in the effluent from a Bio-Gel P-2 column equilibrated with pH 4.5 ammonium propionate buffer was used directly as the peptide solution. The buffer contained in the fractions immediately preceding the emergence of the peptide peak served as a solvent reference. Liquid columns 6 mm in height were used in a cell with a double-sector Kel-F-coated aluminum centerpiece. Initial protein concentrations were approximated from the fringes at equilibrium and the known dilutions. Measurements of interference patterns and calculations were performed as described by Yphantis (1964) utilizing a computer program written by Drs. Parker Small and Jerome Resnick (see Piez, 1965). The partial specific volumes employed for α1-CB-2 and β_{12}-CB-1, calculated from their amino acid compositions, were 0.69 and 0.70, respectively.

Results

The Chromatographic Separation on Phosphocellulose of Peptides Derived from α1, α2, β_{11}, and β_{12}. When α chains from neutral salt- or acid-extracted normal collagen or from lathyritic collagen were cleaved with CNBr and the resulting peptides compared, limited

TABLE I: Amino Acid Composition of Peptides Eluted from Phosphocellulose after Cleavage of Collagen Chains with CNBr.[a]

	α1-CB-							α2-CB-			β_{11}-	β_{12}-	(β_{12}-CB-1) +
	1b	1a	1	2	3	(1 + 2)	(1a + 2)	1a	1	2	CB-1	CB-1	(α1-CB-2)
4-Hydroxyproline	—	—	—	4.9	14.0	5.2(5)	5.6(5)	—	—	2.8	—	—	5.4(5)
Aspartic acid	0.9	0.9	1.0	—	6.6	1.2(1)	1.0(1)	1.0	1.0	2.8	1.7	1.9	2.0(2)
Threonine	—	—	—	—	2.2	—	—	—	—	1.0	—	—	—
Serine	2.1	1.9	2.0	2.2	3.3	3.8(4)	4.1(4)	2.0	2.0	1.1	4.2	4.2	6.1(6)
Homoserine	0.9	0.9	0.9	1.0	0.8	1.1(1)	1.3(1)	0.9	1.0	0.9	1.9	2.0	2.3(2)
Glutamic acid	1.1	1.2	1.0	4.0	14.4	4.7(5)	4.9(5)	1.0	1.0	1.1	2.2	2.0	5.9(6)
Proline	1.9	1.9	1.8	6.9	13.4	8.2(9)	8.4(9)	1.9	1.9	3.1	3.8	3.8	9.9(11)
Glycine	3.1	3.3	3.2	12.1	48.3	14.2(15)	14.5(15)	3.2	3.1	9.6	6.2	6.0	17.2(18)
Alanine	1.0	1.1	1.1	2.1	19.0	3.2(3)	3.0(3)	1.0	1.0	2.1	2.1	2.0	4.0(4)
Valine	2.0	1.9	1.8	—	2.9	1.9(2)	2.0(2)	1.0	1.0	0.8	4.1	3.0	3.1(3)
Methionine	—	—	—	—	—	0.6(1)	0.7(1)	—	—	—	—	—	0.8(1)
Isoleucine	—	—	—	—	—	—	—	—	—	—	—	—	—
Leucine	—	—	—	1.0	2.8	1.1(1)	0.9(1)	—	—	1.0	—	—	1.2(1)
Tyrosine	1.1	1.0	1.2	—	—	0.9(1)	1.1(1)	1.0	1.1	—	1.4	2.0	2.1(2)
Phenylalanine	—	—	—	0.9	2.6	0.9(1)	1.0(1)	—	—	—	—	—	0.9(1)
Hydroxylysine	—	—	—	—	—	—	—	—	—	—	—	—	—
Lysine	—	—	1.0	—	4.5	0.6(1)	—	—	0.9	—	—	—	—
Histidine	—	—	—	—	—	—	—	—	—	—	—	—	—
Arginine	—	—	—	1.0	5.8	1.9(1)	0.9(1)	—	—	2.6	—	—	0.9(1)
Total residues	14	14	15	36	141	50(51)	50(50)	13	14	30	28	27	62(63)

[a] The values, given as residues per peptide, are the averages of two or more determinations performed on different preparations of each peptide. In almost every instance duplicate determinations agreed within 10%. The number of residues in peptides derived from β components was calculated assuming two residues of homoserine per peptide. Homoserine includes homoserine lactone. The numbers in parentheses indicate the number of residues predicted for the proposed structure of peptides α1-CB-(1 + 2), α1-CB-(1a + 2), and (β_{12}-CB-1) + (α1-CB-2). A dash indicates that the amino acid was either entirely absent or present as less than 0.1 residue. The amino acid compositions of α1-CB-2 and α1-CB-3 have been reported previously (Bornstein and Piez, 1965) as peptides C and B, respectively.

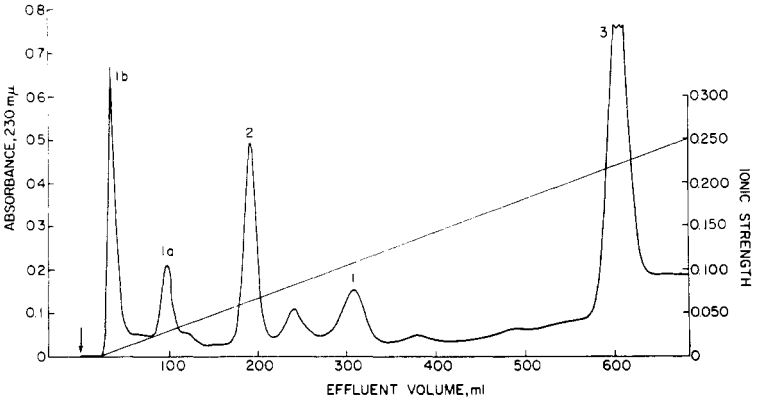

FIGURE 2: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the α1 fraction (120 mg) of acid-extracted collagen from normal rats. See legend to Figure 1 for further details.

but reproducible differences were noted. The phosphocellulose elution pattern of the peptides obtained by methionyl bond cleavage of α1 derived from salt-extracted normal collagen is illustrated in Figure 1. The peptides eluted with the gradient accounted for 15–20% of the protein sample, the larger peptides remaining on the column.

A comparison of the elution pattern in Figure 1 with

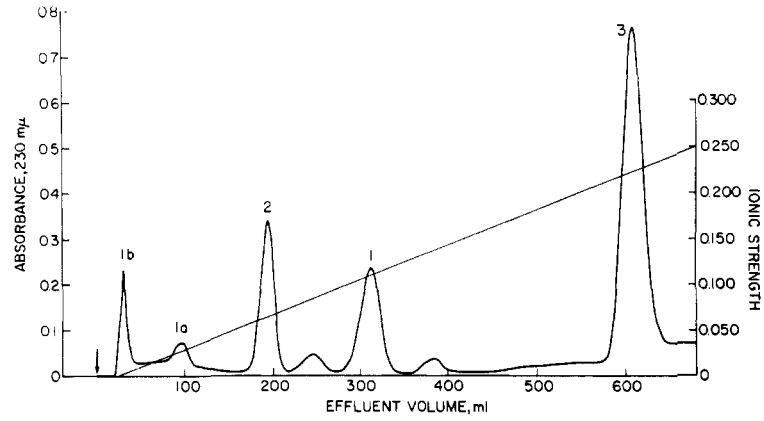

FIGURE 3: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the $\alpha 1$ fraction (80 mg) of acid-extracted collagen from lathyritic rats. See legend to Figure 1 for further details.

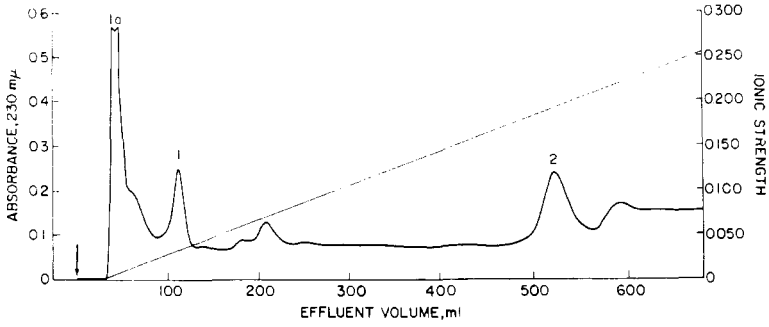

FIGURE 4: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the $\alpha 2$ fraction (75 mg) of acid-extracted collagen from normal rats. Peptides $\alpha 2$-CB-1a, $\alpha 2$-CB-1, and $\alpha 2$-CB-2 are identified. See legend to Figure 1 for further details.

those of the peptides derived in an identical fashion from normal acid-extracted collagen (Figure 2) and from lathyritic salt-extracted collagen (Figure 3) revealed that whereas peptides $\alpha 1$-CB-2 and $\alpha 1$-CB-3 were always present in the same proportion to each other and to the sample size, the amount of peptide $\alpha 1$-CB-1 relative to $\alpha 1$-CB-1a (as indicated by their absorbance at 230 mμ) varied with the source of the α chain. Peptide $\alpha 1$-CB-1 predominated in digests of $\alpha 1$ obtained from lathyritic collagen whereas it was present in smaller amounts in preparations derived from the $\alpha 1$ chain of acid-extracted normal collagen. Digests of $\alpha 1$ from salt-extracted collagen were intermediate in their content of $\alpha 1$-CB-1.

The peak labeled $\alpha 1$-CB-1b in Figures 1–3 was composed largely of nonpeptide ultraviolet-absorbing material which eluted as an unretarded peak at the start of the salt gradient. Although a peptide ($\alpha 1$-CB-1b) could be separated from this mixture by molecular sieve chromatography on Bio-Gel P-2, its composition was indistinguishable from that of $\alpha 1$-CB-1a (Table I). The reason for its chromatographic separation from the latter peptide is not understood. Since the ionic strength at which peptide $\alpha 1$-CB-1a elutes during phosphocellulose chromatography is low, the presence of a small amount of salt in the sample may cause the elution of a fraction of $\alpha 1$-CB-1a prior to the establishment of the gradient. Alternatively $\alpha 1$-CB-1b may represent a small fraction of $\alpha 1$-CB-1a which has lost one or more amide groups or in which the homoserine is present in the open rather than the lactone form, causing an increase in acidity of the peptide and thus accounting for its earlier elution from phosphocellulose. In any event, $\alpha 1$-CB-1b represented less than 15% of the total of $\alpha 1$-CB-1a and $\alpha 1$-CB-1b and will not be further considered in this communication. The remaining small peaks in Figures 1–3 were consistently present as minor components. Two of them, one eluting between $\alpha 1$-CB-2 and $\alpha 1$-CB-1 and the other following $\alpha 1$-CB-1a, are of considerable significance and will be discussed below.

Figure 4 represents the phosphocellulose elution pattern of peptides derived by methionyl bond cleavage of the $\alpha 2$ chain of acid-extracted normal collagen and Figure 5 depicts the peptides similarly derived from the $\alpha 2$ chain of lathyritic salt-extracted collagen. The quantity of peptide $\alpha 2$-CB-2 in these digests was always proportional to the amount of protein placed on the

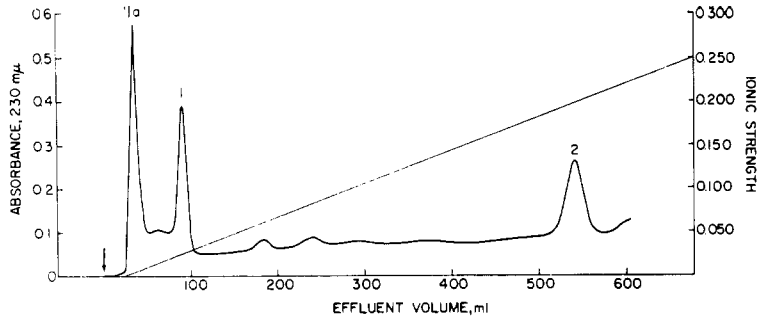

FIGURE 5: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the $\alpha 2$ fraction (70 mg) of salt-extracted collagen from lathyritic rats. Peptides $\alpha 2$-CB-1a, $\alpha 2$-CB-1, and $\alpha 2$-CB-2 are identified. See legend to Figure 1 for further details.

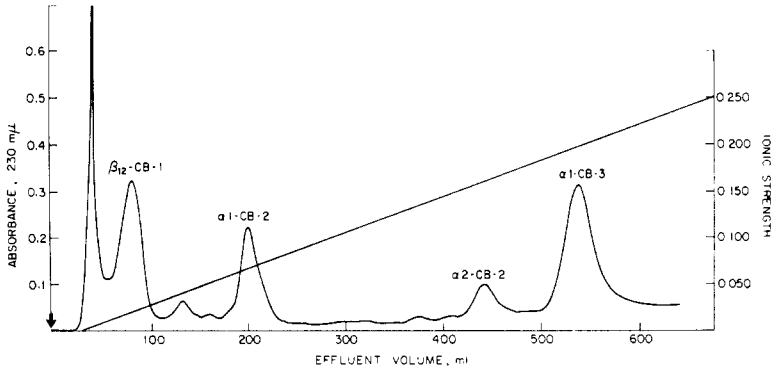

FIGURE 6: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the β_{12} fraction (95 mg) of collagen from normal rats. Minor differences in the position of elution of peptides $\alpha 1$-CB-2, $\alpha 2$-CB-2, and $\alpha 1$-CB-3 result from small variations in the elution gradient or flow rate. See legend to Figure 1 for further details.

column. Peptides $\alpha 2$-CB-1 and $\alpha 2$-CB-1a however varied in relative amounts with the source of $\alpha 2$ in the same fashion described for peptides $\alpha 1$-CB-1 and $\alpha 1$-CB-1a. Since $\alpha 2$-CB-1a eluted at the start of the ionic strength gradient and was contaminated with varying amounts of nonpeptide material, its quantitation was more difficult to establish than that of $\alpha 1$-CB-1a. However, purification of $\alpha 2$-CB-1a on columns of Bio-Gel P-2 confirmed the impression, gained from the phosphocellulose chromatograms, that acid-extracted collagen contained more $\alpha 2$-CB-1a than did lathyritic collagen. Salt-extracted normal collagen again contained an intermediate amount of this peptide. In the case of $\alpha 2$ the presence or absence of a peptide analogous to $\alpha 1$-CB-1b could not be evaluated. The small peaks eluting at approximately 200 and 600 ml of effluent volume in Figures 4 and 5 represent peptides derived from the $\alpha 1$ portion of β_{12} which contaminates most preparations of $\alpha 2$ to the extent of 5–10%.

Figure 6 illustrates the phosphocellulose elution pattern of peptides obtained by methionyl bond cleavage of β_{12}. Peptides $\alpha 1$-CB-2, $\alpha 1$-CB-3, and $\alpha 2$-CB-2 were present in the proportion expected from the fact that β_{12} is a dimer of $\alpha 1$ and $\alpha 2$. Their identity with their counterparts from $\alpha 1$ and $\alpha 2$ was established by comparison of their amino acid compositions. Those peptides which varied quantitatively in digests of $\alpha 1$ and $\alpha 2$ ($\alpha 1$-CB-1a, $\alpha 1$-CB-1, $\alpha 2$-CB-1a, and $\alpha 2$-CB-1) were absent in digests of β_{12} while a new peptide, β_{12}-CB-1, eluting between 60 and 90 ml of effluent volume, was present. The initial peak at the start of the ionic strength gradient again consisted in part of nonpeptide material, but a small amount of a peptide with an amino acid composition identical with that of β_{12}-CB-1 could be isolated from it by chromatography on Bio-Gel P-2. The reasons for its chromatographic separation on phosphocellulose may be similar to those suggested for $\alpha 1$-CB-1b. The nature of the peak eluting at 130 ml of effluent volume is discussed below.

The β_{11} fraction, containing a substantial proportion of $\alpha 1$ (see Methods), was treated with CNBr and the resulting peptides were chromatographed on phosphocellulose (Figure 7). Although no peaks unique to β_{11} were noted, both the size of the peak eluting in the position of $\alpha 1$-CB-2 (relative to $\alpha 1$-CB-3, for example) and its amino acid composition suggested the presence of an additional peptide in this peak. Its isolation and identification as β_{11}-CB-1 is described below.

Although precise quantitation of the recovery of

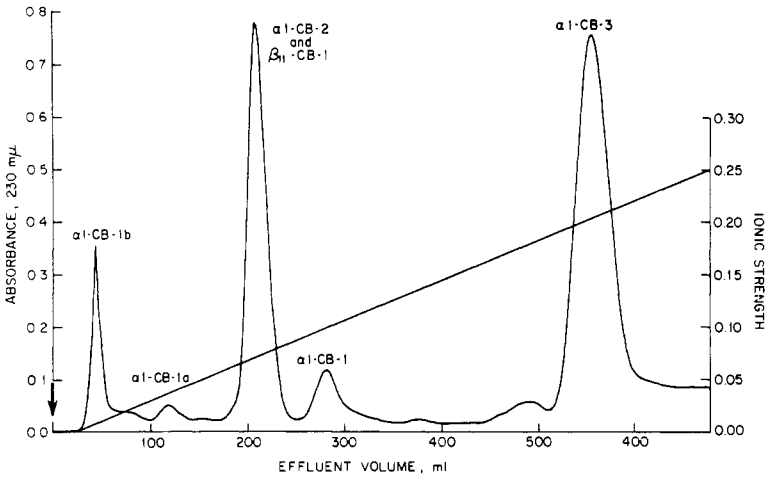

FIGURE 7: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the β_{11} fraction (100 mg) of acid-extracted collagen from normal rats. This fraction contained approximately 50% α1. The peak eluting at 200 ml consists of a mixture of the two peptides indicated. See legend to Figure 1 for further details.

these peptides from collagen chains after CNBr cleavage and phosphocellulose chromatography was difficult, in several experiments the uncorrected recovery of peptide β_{12}-CB-1 from β_{12} was 0.3–0.4 equiv of peptide/equiv of β component. In these calculations the equivalents of starting material were obtained from the weight of β_{12} used and the recovery of β_{12}-CB-1 was judged by an amino acid analysis of a portion of the desalted peptide. When corrections were made for the probable water content of β_{12}, incomplete cleavage with CNBr, and chromatographic and other handling losses, the yield approximated 1 equiv of peptide/β component. Similar calculations for the sum of α1-CB-1b, α1-CB-1a, and α1-CB-1 also indicated a corrected recovery of approximately 1 equiv of peptide/α1 chain.

Amino Acid Composition of Peptides Derived from α1, α2, β_{11}, and β_{12}. The peak fractions from phosphocellulose chromatograms containing the peptide in question were lyophilized and the residue was chromatographed on a column of Bio-Gel P-2. This step served both to separate the peptide from buffer salts and to further purify the peptide on the basis of its molecular size. The amino acid compositions of the peptides are listed in Table I. The amounts of all amino acids present in low concentration were used to calculate an average factor for the conversion of micromoles to residues. The total number of residues per peptide was determined on the assumption that each peptide contained 1 equiv of homoserine with the exception of peptides derived from double-chain components, which were assumed to contain two homoserines per peptide. In every case homogeneity was indicated by the finding that all amino acids were present in amounts consistent with small whole number equivalents.

Peptide α1-CB-1 contained 15 amino acids of which three were glycine, two were proline, and one was lysine. Hydroxyproline was absent. Peptide α1-CB-1a contained 14 amino acids and differed from α1-CB-1 only in that it lacked the lysyl residue. Similarly, α2-CB-1a contained all the amino acids in α2-CB-1 with the exception of lysine. The amino acid composition of α2-CB-1 differed from α1-CB-1 only in that it contained one less valine. Despite the similarities in their composition, the amino acid sequences of α1-CB-1 and α2-CB-1 differed considerably as shown by the enzymatic cleavage studies presented below.

Peptides α1-CB-2, α1-CB-3, and α2-CB-2 contained precisely one-third of their residues as glycine and their imino acid content was 20% or greater. This composition is compatible with the existence of the portions of the collagen molecule represented by these peptides in the polyproline-like collagen helix. Preliminary data on the sequence of α1-CB-2 indeed indicate that glycine is present as every third amino acid in this 36 amino acid peptide. However, the amino acid composition of peptides α1-CB-1a, α1-CB-1, α2-CB-1a, and α2-CB-1 differs significantly from that of typical collagen peptides suggesting that a structure unlike that of the main body of the collagen helix exists in the region of the molecule giving rise to these peptides. Although the composition of these peptides does not resemble collagen, direct evidence that they constitute integral parts of collagen chains was obtained by the isolation, in small amounts, of three peptides that have amino acid compositions consistent with their identification as α1-CB-2 joined by a methionyl bond to either α1-CB-1, α1-CB-1a, or β_{12}-CB-1. These peptides are designated α1-CB-(1+2) (eluting between α1-CB-1 and α1-CB-2, Figures 1–3), α1-CB-(1a+2) (eluting just after α1-CB-1a, Figures 1 and 2), and (β_{12}-CB-1) + (α1-CB-2) (eluting after β_{12}-CB-1, Figure 6) in Table I. The presence of such peptides in small amounts is to

be expected since cleavage of methionyl bonds by CNBr under the conditions employed was only approximately 90% complete.

The Position of the Lysyl Residues in α1-CB-1 and α2-CB-1. The position of the lysyl residue in α1-CB-1 was investigated by tryptic cleavage of this peptide. The enzymatic digest was chromatographed on phosphocellulose in a manner identical with that described for CNBr-produced peptides. Under these conditions trypsin does not elute from phosphocellulose. Two widely separated peaks were eluted. The fractions containing these peaks were pooled, lyophilized, and desalted on Bio-Gel P-2, and the amino acid compositions of the two peptides were determined (Table II). The presence of lysine in a pentapeptide (α1-CB-1-T-1) together with the known specificity of trypsin demonstrated that lysine was present as the fifth amino acid from the N-terminal end in α1-CB-1.

TABLE II: Amino Acid Composition of Peptides Resulting from Enzymatic Cleavage of α1-CB-1 and α2-CB-1.[a]

	α1-CB-1			α2-CB-1		
	T-1	T-2	C-1	C-2	C-2-T-1	C-2-T-2
Aspartic acid	1.0	—	—	1.1	1.0	(0.13)
Serine	—	2.0	—	2.0	1.0	1.0
Homoserine	—	0.9	—	0.9	—	0.9
Glutamic acid	1.0	—	1.0	—	—	—
Proline	—	1.8	—	1.9	—	1.9
Glycine	1.1	2.1	(0.15)	3.0	—	3.0
Alanine	—	1.0	—	1.0	—	1.0
Valine	—	2.1	—	1.0	—	1.1
Tyrosine	0.9	—	1.1	—	—	—
Lysine	1.0	—	—	1.0	1.0	—

[a] The peptides designated T and C were obtained by tryptic and chymotryptic digestion, respectively. C-2-T-1 and C-2-T-2 refer to the tryptic peptides from C-2. Homoserine includes homoserine lactone. A dash indicates less than 0.1 residue. Residues in parentheses are fractional residues present as impurities. The values given are residues per peptide.

Trypsin also cleaved α2-CB-1 but the two resulting peptides were not easily separable on either phosphocellulose, Bio-Gel P-2, or Sephadex G-10. Chymotrypsin digestion, however, produced two peptides, one a dipeptide (α2-CB-1-C-1) and the other containing 12 amino acids (α2-CB-1-C-2), which could be separated from the enzyme and from each other by chromatography on Sephadex G-25. Their amino acid compositions are listed in Table II. Cleavage of α2-CB-1-C-2 with trypsin yielded two peptides which could now be separated on Sephadex G-25. The amino acid composition of the small peptide (α2-CB-1-C-2-T-1) was

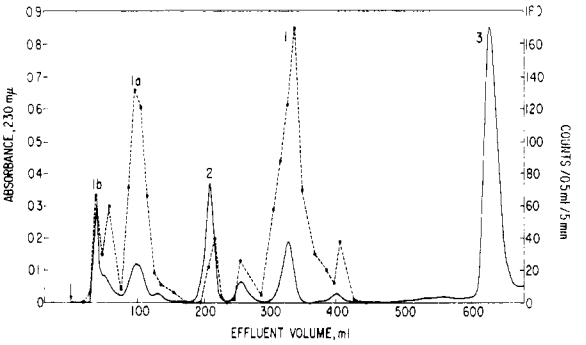

FIGURE 8: Phosphocellulose elution pattern of peptides obtained by CNBr cleavage of the α1 fraction (90 mg) of salt-extracted lysine-^{14}C-labeled collagen from normal rats. The solid trace indicates absorbance at 230 mμ. The dashed trace indicates radioactivity. Fractions beyond 440 ml of effluent volume were not counted in this experiment. See legend to Figure 1 for further details.

(Asp-Ser)-Lys, whereas the larger peptide (α2-CB-1-C-2-T-2) contained the remaining nine amino acids in α2-CB-1-C-2 including homoserine (Table II). Since the mechanism of action of CNBr restricts the location of homoserine to the C-terminal position, lysine must also have been the fifth amino acid in α2-CB-1. The total sequences of α1-CB-1 and α2-CB-2 will be reported subsequently (A. H. Kang, P. Bornstein, and K. A. Piez, in preparation).

The Characterization of α1-CB-1a and α2-CB-1a and Their Relation to α1-CB-1 and α2-CB-1, Respectively. The reciprocal source-dependent variation in the quantities of α1-CB-1 and α1-CB-1a derived from α1, and α2-CB-1 and α2-CB-1a derived from α2, suggested a transformation in a portion of each α chain during or preceding the process of collagen cross-linking. The presence of a derivative of lysine in peptide α1-CB-1a was demonstrated by cleaving isolated α1 chains, labeled with lysine-^{14}C, with CNBr. The resulting peptides were chromatographed on phosphocellulose and examined for radioactivity. Figure 8 reveals that peptides α1-CB-1b and α1-CB-1a which lack lysine were highly radioactive with a specific activity approximately equal to that of α1-CB-1. The small amount of activity in peptide α1-CB-2 was due to a contaminant in this peak, probably a small amount of peptide β_{11}-CB-1. Continuous-flow scintillation counting of hydrolysates of these peptides demonstrated that no amino acid other than lysine contained significant radioactivity. Similar experiments indicated that peptide α2-CB-1a when derived from lysine-^{14}C-labeled α2 was also radioactive.

Recent studies by Gallop and his associates have focused attention on the presence of aldehydes in collagen (Gallop, 1964; Rojkind *et al.*, 1964, 1966). Because of their reactivity numerous suggestions have been made that these groups are involved in the cross-

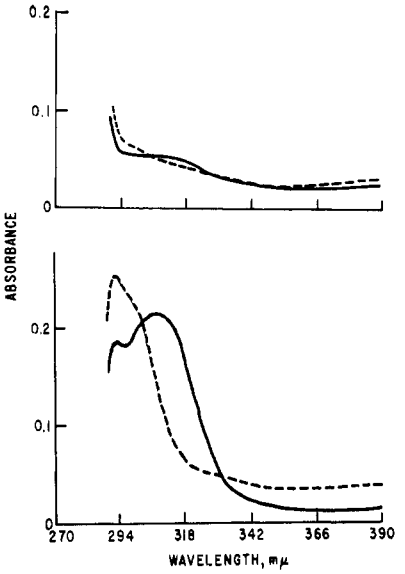

FIGURE 9: Absorption spectra of the azine derivatives of peptides α1-CB-1 (above) and α1-CB-1a (below) with MBTH, at pH 4 (solid line) and pH 1 (dashed line). The spectra below 294 mμ are unreliable owing to the high absorbance of the excess MBTH. The cuvet contained 0.035 μmole of α1-CB-1a in 1 ml of H_2O, 1 ml of 0.1 M glycine–HCl, pH 4.0, and 0.2 ml of 0.1% MBTH. HCl (6 N, 0.04 ml) was added to obtain spectra at pH 1.

linking of collagen. The possibility that peptides α1-CB-1a and α2-CB-1a contained aldehydic groups was investigated by the spectrophotometric method of Paz *et al.* (1965). Figure 9 illustrates the ultraviolet absorption spectra of the products of reaction of peptide α1-CB-1a and peptide α1-CB-1 (examined as a control) after 10-hr incubation at 40° with MBTH. The azine derivative of peptide α1-CB-1a at pH 4.0 had an absorption maximum at 308 mμ. The maximum shifted to 294 mμ at pH 1.0 in a manner characteristic of MBTH derivatives. The approximate half-time of the reaction was 4 min, and the molar extinction coefficient was 14×10^3 assuming one aldehyde group per peptide. These data, as well as the absorption spectrum, are consistent with the presence in peptide α1-CB-1a of a saturated aldehyde (Paz *et al.*, 1965). Peptide α1-CB-1 did not form an azine derivative as evidenced by the lack of absorption in the range of 300–380 mμ (Figure 9). Experiments with peptides α2-CB-1a and α2-CB-1 revealed analogous results.

The presence of a lysine-derived aldehyde in peptides α1-CB-1a and α2-CB-1a was further demonstrated by reduction of these peptides with borohydride. In one experiment, 0.08 μmole of peptide α1-CB-1a, derived from lysine-^{14}C-labeled collagen, was reduced with $NaBH_4$ and the reduced peptide was hydrolyzed in 6 N HCl under nitrogen at 108° for 24 hr. The hydrolysate was placed on the amino acid analyzer and the effluent was monitored in the flow counter. A radioactive peak eluting between proline and glycine was present after reduction with $NaBH_4$ but was absent both in peptide α1-CB-1a before reduction, and in peptide α1-CB-1 before and after reduction. It was identified as ε-hydroxynorleucine (ε-hydroxy-α-aminocaproic acid) by comparison of its chromatographic position with that of the authentic compound (Cyclo Chemical Co.). Using the color value obtained for ε-hydroxynorleucine, the recovery in this experiment was 0.02 μmole or 25%. In a similar experiment employing 0.13 μmole of α2-CB-1a, 0.05 μmole of ε-hydroxynorleucine or 38% of an equivalent was recovered. These recoveries must be approximately doubled to correct for losses of the amino acid during acid hydrolysis. When 0.25 μmole of authentic ε-hydroxynorleucine was hydrolyzed under the same conditions, recoveries of 36, 53, and 56% were obtained in three separate experiments. An additional unknown peak was observed in the region of tyrosine. All the missing ε-hydroxynorleucine could be accounted for if a color value equal to that of leucine was assumed for this product. There was evidence that a similar product, derived from ε-hydroxynorleucine, was present in hydrolysates of the reduced peptides. Radioactivity was observed in the region of tyrosine and recoveries of "tyrosine" were in excess of 1 equiv in these hydrolysates.

In support of these results, oxidation with performic acid of peptide α1-CB-1a, derived from lysine-^{14}C-labeled collagen, yielded a radioactive peak which chromatographed between proline and glycine in a position identical with that of authentic α-aminoadipic acid. In one experiment, the oxidation of 0.098 μmole of α1-CB-1a resulted in a recovery of 0.033 μmole of α-aminoadipic acid or 34% of an equivalent. All amino acids in α1-CB-1a were recovered in close to 100% yield with the exception of tyrosine which was almost totally destroyed. Performic acid oxidation of peptide α1-CB-1 produced no α-aminoadipic acid.

These data indicate that a lysyl residue in the portion of each α chain represented by α1-CB-1 and α2-CB-1 is converted to the δ-semialdehyde of α-aminoadipic acid in peptide linkage. The presence of this derivative gives rise to peptides α1-CB-1a and α2-CB-1a. The loss of a positive charge in this conversion accounts for the chromatographic separation of the two forms on phosphocellulose. Studies to be presented elsewhere (P. Bornstein, A. H. Kang, and K. A. Piez, in preparation) indicate that peptides α1-CB-1 and α2-CB-1 (and therefore their derivatives α1-CB-1a and α2-CB-1a) are derived from the N-terminal regions of the α1 and α2 chains, respectively.

The Characterization of β_{12}-CB-1. The presence of an interchain cross-link in peptide β_{12}-CB-1 was suggested by the following. (a) β_{12}-CB-1 was limited to CNBr digests of β_{12}. (b) The amino acid composition of β_{12}-CB-1 was consistent with its structure as a dimer of α1-CB-1a and α2-CB-1a, and the latter peptides as well as their precursors α1-CB-1 and α2-CB-1 were absent in CNBr digest of β_{12}. Like α1-CB-1a and α2-CB-1a, β_{12}-CB-1 was radioactive when derived

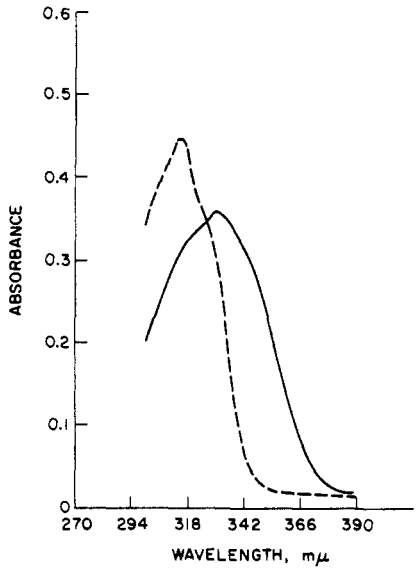

FIGURE 10: Absorption spectra of the azine derivative of β_{12}-CB-1 with MBTH at pH 4 (solid line) and pH 1 (dashed line). The cuvet contained 0.038 μmole of β_{12}-CB-1 in 1 ml of H_2O, 1 ml of 0.1 M glycine–HCl, pH 4.0, and 0.2 ml of 0.1% MBTH. The reaction was performed at 40° for 24 hr. HCl (6 N, 0.04 ml) was added to obtain a spectrum at pH 1.

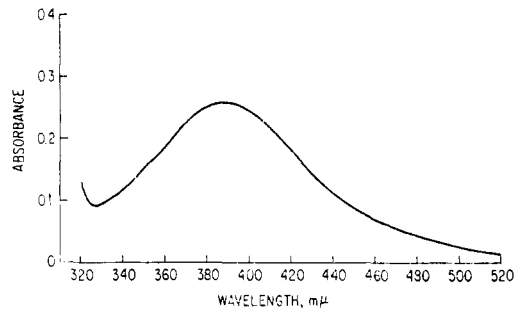

FIGURE 11: Ultraviolet absorption spectrum of the 2,4-DNP-hydrazone of β_{12}-CB-1 obtained by CNBr cleavage and phosphocellulose chromatography of 2,4-DNP-hydrazine-treated acid-extracted collagen. The cuvet contained 0.06 μmole of peptide in 3.5 ml of H_2O. The pH was adjusted to 3.5 with acetic acid.

from lysine-^{14}C-labeled collagen even though no lysine could be demonstrated by amino acid analysis.

In order to obtain additional evidence for the structure of β_{12}-CB-1 as a cross-linked peptide, its molecular weight was determined by high-speed sedimentation equilibrium. The weight-average molecular weights of β_{12}-CB-1 and peptide α1-CB-2 (examined as a control) are summarized in Table III. The β_{12}-CB-1 preparation was run at two concentrations and α1-CB-2 at three different concentrations. Homogeneity of the peptide preparations was indicated by the close correspondence between the molecular weights obtained from the whole cell, by extrapolation to zero concentration (c = 0), and by extrapolation to the bottom of the cell (r = b). The data indicated molecular weights of about 2800 for β_{12}-CB-1 and 3200 for α1-CB-2. Another experiment with different samples, run under the same conditions, also indicated homogeneity. Molecular weights of 2700 for β_{12}-CB-2 and 3400 for α1-CB-2 were obtained. The calculated molecular weight of β_{12}-CB-1, assuming it to be a dimer of α1-CB-1a and α2-CB-1a linked by the aldehyde cross-link discussed below, is 2787. The molecular weight of α1-CB-2 calculated from its amino acid composition is 3277. The close agreement between the measured and calculated molecular weights together with the amino acid composition (Table I) demonstrate the presence of two residues of homoserine (or its lactone) per equivalent of β_{12}-CB-1 and therefore the presence of two polypeptide chains.

TABLE III: Molecular Weights of Peptides β_{12}-CB-1 and α1-CB-2.

		M_w		
Peptide	mg/ml	Whole Cell	at c = 0	at r=b (M_z)
β_{12}-CB-1	0.1	3010	3230	2730
	0.3	2650	2640	2710
	Av	2830	2960	2720
α1-CB-2	0.15	2930	2830	3190
	0.3	3390	3320	3520
	0.6	3360	3290	3540
	Av	3230	3150	3420

In view of the above results demonstrating the presence of lysine-derived aldehydes in the cross-link precursor peptides α1-CB-1a and α2-CB-1a, it was of interest to examine β_{12}-CB-1 itself for aldehydic groups. The use of the spectrophotometric method of Paz *et al.* (1965) also indicated the presence of an aldehyde in β_{12}-CB-1. However, the absorption spectrum of the azine derivative with MBTH, illustrated in Figure 10, differed from the spectra obtained with derivatives of α1-CB-1a and α2-CB-1a. The absorption maximum at pH 4.0 was 330 mμ shifting to 314 mμ at pH 1.0. The molar extinction at 330 mμ and pH 4.0 was 21 × 10^3 assuming 1 g-equiv of aldehyde/2800 g of peptide. The reaction occurred at a slower rate than that observed for peptides α1-CB-1a and α2-CB-1a. The half-time for completion of the reaction was approximately 2 hr. Similar kinetics and a similar absorption spectrum were obtained for the reaction of crotonaldehyde with MBTH. Similar data have also been reported by Paz *et al.* (1965) for crotonaldehyde as well as for 2-methyl-2-butenal. These data suggest the presence in β_{12}-CB-1 of an α,β-unsaturated aldehyde.

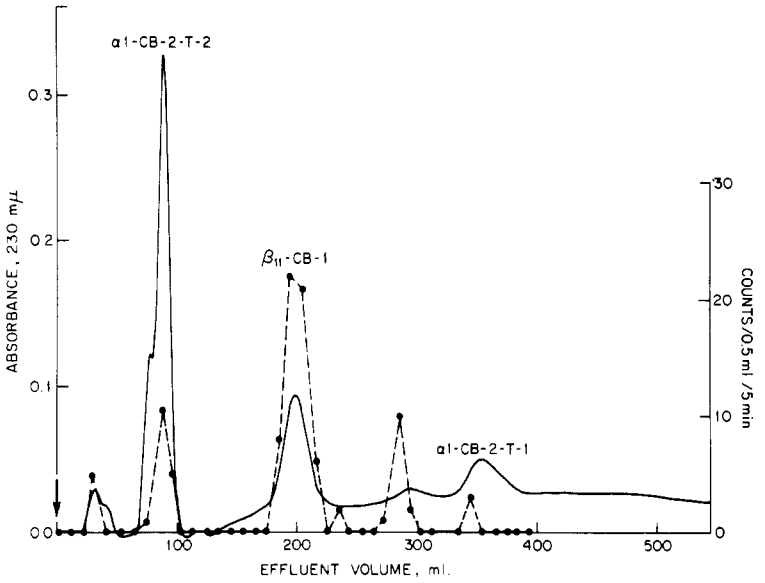

FIGURE 12: Phosphocellulose elution pattern of a tryptic digest of a mixture of β_{11}-CB-1 and α1-CB-2 (see Figure 7) from lysine-^{14}C-labeled collagen. The solid trace indicates absorbance at 230 mμ. The dashed trace indicates radioactivity. See legend to Figure 1 for conditions of chromatography.

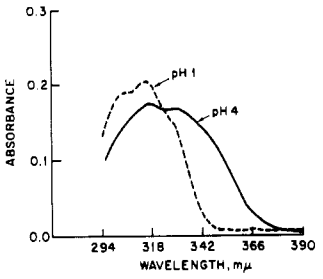

FIGURE 13: Absorption spectra of the azine derivative of β_{11}-CB-1 with MBTH at pH 4 (solid line) and pH 1 (dashed line). The cuvet contained 0.017 μmole of β_{11}-CB-1 in 1 ml of H_2O, 1 ml of 0.1 M glycine–HCl, pH 4.0, and 0.2 ml of 0.1% MBTH. The reaction was performed at 40° for 16 hr. HCl (6 N, 0.04 ml) was added to obtain a spectrum at pH 1.

The presence of an unsaturated aldehyde in β_{12}-CB-1 was also supported by the preparation and isolation of its 2,4-DNP-hydrazone. Acid-extracted collagen, treated with 2,4-DNP-hydrazine, was denatured and chromatographed on CM-cellulose. The β_{12} fraction was cleaved with CNBr and the resulting peptides were chromatographed on phosphocellulose. The fractions containing β_{12}-CB-1 were visibly yellow. The absorption spectrum (Figure 11) of the 2,4-DNP-hydrazone of peptide β_{12}-CB-1 at pH 3.5 had a maximum at 390 mμ and was similar to that described for the aldehyde-containing peptide from ichthyocol isolated by Rojkind *et al.* (1964).

The Characterization of β_{11}-CB-1. The material contained in the peak labeled β_{11}-CB-1 and α1-CB-2 (Figure 7), which was derived from acid-extracted lysine-^{14}C-labeled collagen, was desalted on Bio-Gel P-2 and treated with trypsin. The products were rechromatographed on phosphocellulose under the same conditions employed for the original peptides. Aliquots of the effluent fractions were counted in a planchet counter. Three main peaks were detected by their absorption at 230 mμ (Figure 12). The peak labeled β_{11}-CB-1 chromatographed in the position of the original material and contained most of the radioactivity. Its amino acid composition (Table I) revealed an absence of lysine and was consistent with its identification as the cross-linked dimer of α1-CB-1a. Since this peptide contains no arginine or lysine, its resistance to trypsin was expected. α1-CB-2, however, contains one arginyl residue (Table I) and was cleaved by trypsin, The tryptides chromatographed in new positions leaving β_{11}-CB-1 essentially pure. α1-CB-2-T-1 had the composition (Ser-Pro$_2$-Gly$_2$)-Arg while α1-CB-2-T-2 contained the remainder of the amino acids in α1-CB-2. Further details of the sequence of α1-CB-2 are presently under investigation. The amino acid composition of the radioactive material eluting at 280–300 ml of effluent volume (Figure 12) indicated that it was β_{11}-CB-1 linked by a methionyl bond to α1-CB-2-T-1. It presumably resulted from tryptic digestion at the arginyl bond of a small amount of the uncleaved peptide (β_{11}-CB-1) + (α1-CB-2) which contaminated the original preparation.

If peptide β_{11}-CB-1 is analogous to β_{12}-CB-1 and results from the covalent linkage of the α1-CB-1a portions of two α1 chains, it might be expected to contain an aldehyde similar to that present in β_{12}-CB-

1. Figure 13 shows the absorption spectra at pH 4 and pH 1 of the MBTH derivative of β_{11}-CB-1. The main features of the spectra were very similar to those observed for the MBTH derivative of β_{12}-CB-1 (Figure 10). The spectrum at pH 4 revealed a shoulder at 348 mμ and a double peak at 330 and 318 mμ. The expected shift to a lower wavelength at pH 1 was seen. The kinetics of the reaction and approximate molar extinction coefficient (22×10^3) were also similar to those found for β_{12}-CB-1.

Discussion

Evidence has been obtained which implicates the participation of specific lysyl side chains in both α1 and α2 in the formation of intramolecular covalent cross-links in collagen. Experiments leading to this conclusion were undertaken when it was observed that CNBr digests of α chains derived from different collagen preparations, when chromatographed on phosphocellulose, differed in their proportion of two peptides. In digests of both α1 and α2 the two peptides appeared to be derived from the same amino acid sequence in each chain. The more basic forms, peptides α1-CB-1 and α2-CB-1, predominated in the least cross-linked (lathyritic) collagens and differed from their counterparts, α1-CB-1a and α2-CB-1a, only in that they contained a lysyl residue. The latter peptides predominated in the more highly cross-linked (normal acid extracted) collagens and contained a derivative of lysine. The evidence that this derivative is the δ-semialdehyde of α-aminoadipic acid in peptide linkage is as follows. (1) The absorption spectrum and the kinetics of formation of the MBTH derivatives of α1-CB-1a and α2-CB-1a suggest the presence of a single saturated aldehyde per peptide. (2) α1-CB-1a and α2-CB-1a, derived from lysine-^{14}C collagen, can be reduced by $NaBH_4$ to a form which upon acid hydrolysis yields radioactive ϵ-hydroxynorleucine. (3) The same peptides, when oxidized with performic acid, yield radioactive α-aminoadipic acid after hydrolysis. The finding of peptides in small amounts that have amino acid compositions consistent with their identification as α1-CB-1 linked to α1-CB-2 by a methionyl bond (α1-CB-(1+2), Table I) or α1-CB-1a linked by a methionyl bond to α1-CB-2 (α1-CB-(1a+2), Table I) further supports the conclusion that peptides α1-CB-1 and α1-CB-1a are derived from the same portion of the α1 chain.

CNBr digests of β_{12}, the covalently linked dimer of α1 and α2, contain neither α1-CB-1 and α2-CB-1 nor their derivatives α1-CB-1a and α2-CB-1a. Instead, a new peptide, β_{12}-CB-1, is present. Additional evidence that β_{12}-CB-1 represents the dimer of α1-CB-1a and α2-CB-1a and therefore contains an interchain cross-link is as follows. (1) The amino acid composition of β_{12}-CB-1 is consistent with its proposed structure as the condensation product of α1-CB-1a and α2-CB-1a. When isolated from lysine-^{14}C-labeled collagen, β_{12}-CB-1 lacks lysine but is radioactive. (2) The molecular weight of β_{12}-CB-1 is approximately equal to the sum of α1-CB-1a and α2-CB-1a. (3) The MBTH derivative of β_{12}-CB-1 is clearly different from that obtained with α1-CB-1a and α2-CB-1a and suggests the presence of 1 equiv of an α,β-unsaturated aldehyde. An aldehyde of this nature could result from the condensation of the saturated aldehydes in α1-CB-1a and α2-CB-1a with a subsequent dehydration. The identification of the aldehyde component of β_{12}-CB-1 is further supported by the characterization of its 2,4-DNP-hydrazone. More limited experiments with peptide β_{11}-CB-1 indicate that two α1 chains condense in an analogous fashion to form the β_{11} component.

Although the outlines of the formation and nature of intramolecular cross-links in collagen appear to be drawn, the precise mechanism and structures involved must await further study. Furthermore, these studies do not exclude the presence of other carbonyl groups elsewhere in the collagen molecule (see Blumenfeld and Gallop, 1966). The finding of less than 1 equiv of ϵ-hydroxynorleucine after reduction of α1-CB-1a or α2-CB-1a with borohydride and the incomplete yield of α-aminoadipic acid after oxidation of α1-CB-1a with performic acid may indicate incomplete reaction or the presence of competing side reactions. However, the possibility that other structures may be present cannot be excluded. For example, the lysine-derived aldehyde may be present in the form of a Schiff's base with the N-terminal group of the α chain. This could complicate studies which involve reduction or oxidation of this carbonyl group.

The precise nature of the interchain cross-link itself also remains to be determined. Since α1-CB-1a and α2-CB-1a, the precursor peptides of β_{12}-CB-1 and β_{11}-CB-1, contain saturated aldehydes whereas the cross-linked peptides themselves contain a different aldehyde, probably an α,β-unsaturated aldehyde, it is possible that a condensation reaction of the aldol-type is responsible for the formation of the cross-link. Rojkind *et al.* (1964) have also reported the isolation from a collagenase digest of ichthyocol of an aldehyde-containing peptide which in many respects resembles β_{12}-CB-1. Recently this peptide was further characterized (Rojkind *et al.*, 1966) and was shown to contain a β-hydroxyaldehyde. α,β-Unsaturation could be promoted by incubation of the peptide in 1 N HCl at 50° for 5–30 min. It is therefore possible that *in vitro* dehydration of β_{12}-CB-1 and β_{11}-CB-1 occurred either during CNBr cleavage or during the subsequent isolation of the peptides. The aldehyde in β_{12}-CB-1 and β_{11}-CB-1 may also be involved in a Schiff's base with a free N-terminal group, thus increasing the complexity of the chemistry of this region of the molecule.

Recently Tanzer *et al.* (1966) studied the reaction of thiosemicarbazide with collagen and reported spectral evidence for the presence of aldehydes on α chains and β components. In agreement with the studies on peptides presented here, derivatives with β_{12} were found to have characteristics of unsaturated aldehydes. However, derivatives with α1 were reported to possess spectral characteristics of both saturated and unsaturated aldehydes. This finding together with the amount

of radioactive thiosemicarbazide bound to α chains and β components led these investigators to conclude that the intramolecular cross-link may not form by the condensation of aldehyde groups. Since their studies were performed on whole chains which may contain other reactive groups, the relation of these results to the peptides under consideration here, and to the intramolecular cross-link, will require further investigation.

During the course of these experiments, it was kept in mind that the two α1 chains of rat skin collagen might be two different chains (Bornstein and Piez, 1965). If all three α chains were different as shown for cod fish skin collagen (Piez, 1965), additional peptides from the cross-link region might have been found. However, evidence for this kind of heterogeneity was not obtained from these experiments suggesting that if differences exist they must be limited to regions of the α1 chain other than those considered here.

The amino acid compositions of the cross-link precursor peptides (Table I) differ significantly from that of whole collagen and presumably prohibit this region of the collagen molecule from assuming the triple-chain helical structure which characterizes the body of the collagen molecule. It is of interest that the amino acid composition of the peptide material recovered after cleavage of native collagen with pepsin and other enzymes (Rubin *et al.*, 1963, 1965; Drake *et al.*, 1966) as well as the composition of the aldehyde-containing peptide isolated by Rojkind *et al.* (1964, 1966) from collagenase digests of ichthyocol bear certain resemblances to that of the peptides described in this communication. It is therefore likely that they originate from similar regions of the collagen molecule. The absence of a protective helical structure in this region may account for its accessibility to proteolytic enzymes (Hodge *et al.*, 1960; Rubin *et al.*, 1963, 1965; Kühn *et al.*, 1963; Drake *et al.*, 1966; Martin *et al.*, 1966). This topic will be covered in more detail in a following publication (P. Bornstein, A. H. Kang, and K. A. Piez, in preparation). The configuration of this region may also permit the selective enzymatic oxidation of its lysyl residue *in vivo*, while the remaining lysyl residues are unaffected.

The inhibition of cross-linking in collagen by lathyrogenic agents was demonstrated a number of years ago (Levene and Gross, 1959; Martin *et al.*, 1961, 1963; Gross, 1963). It has been proposed that lathyrogens are active as carbonyl reagents and block preexisting aldehydic groups on collagen which normally function to form interchain cross-links (Levene, 1962). However, the inability to demonstrate ^{14}C-labeled BAPN bound to lathyritic collagen (Orloff and Gross, 1963) did not support this suggestion. Also in contradiction to the aldehyde-blocking concept is the evidence presented here which indicates that a single specific lysyl residue in each α chain of collagen is converted to an aldehyde prior to the formation of the cross-link and that this step is blocked in lathyritic rats. Since the lysine content of rat skin collagen is about 25 residues/1000, the loss of a single residue per chain of about 1000 amino acids would not be apparent in a comparison of the amino acid analysis of collagen from normal and lathyritic rats.

Additional evidence relating to the action of lathyrogens has come from studies on elastin. The inhibition by lathyrogens of cross-linking in this protein has been demonstrated (O'Dell *et al.*, 1965, 1966; Miller *et al.*, 1965). The cross-links in elastin consist of the polyfunctional amino acids desmosine and isodesmosine (Partridge *et al.*, 1963; Thomas *et al.*, 1963) and evidence for their derivation from four lysyl residues in peptide linkage has been obtained (Partridge *et al.*, 1964, 1966; Miller *et al.*, 1964, 1965). In these studies it has been shown that the normally low lysine content of elastin is elevated while conversion to the cross-link compounds, desmosine and isodesmosine, is depressed in the presence of a lathyrogen. In support of the suggestion that aldehydes may be intermediates in the formation of the desmosines (Partridge *et al.*, 1964), elastin from the aortas of lathyritic chicks has also been shown to be deficient in aldehydic groups when compared to normal elastin (Miller and Fullmer, 1966). These observations suggest that lathyrogenic agents block the conversion of lysine to an aldehyde intermediate and, in this manner, inhibit cross-linking in elastin.

From these studies on elastin, it is reasonable to suggest that the formation of the cross-link in collagen represents one or more intermediate steps in a series of reactions involving the transformation of lysine, and culminating in the formation of the desmosines in elastin. In collagen the reaction may, for steric or other reasons, be limited to a bimolecular condensation, or possibly in the case of the triple chain γ component to an occasional trimolecular condensation.

The data presented here pertain only to intramolecular cross-links. In view of the fact that intermolecular as well as intramolecular cross-linking is affected by lathyrism, it seems likely that the same functional groups are involved in both processes. However, the aldehyde groups discussed here are located near the end of the molecule and in general, based on electron microscopic evidence, molecules in the fibril are staggered. The possibility remains that these aldehydes react with other functional groups in the body of adjacent molecules. The latter may be ϵ-amino groups of lysine. A precedent for this may exist in elastin since N^{ϵ}-(5-amino-5-carboxypentanyl)lysine has recently been isolated from hydrolysates of this protein (Franzblau *et al.*, 1965). This compound, designated lysinonorleucine, could arise from reduction of a Schiff's base between the side chain of a lysyl residue and a residue of the δ-semialdehyde of α-aminoadipic acid.

If, as proposed, lathyrogens function in collagen to inhibit the conversion of lysine in peptide linkage to the δ-semialdehyde of α-aminoadipic acid, their action at the enzymatic level seems probable. The demonstration that several monoamine oxidase inhibitors were relatively poor lathyrogens in the chick embryo (Levene, 1961) does not exclude the inhibition by lathyrogens of a monoamine oxidase-like enzyme since the specificity of

these enzymes, and presumably their susceptibility to inhibitors, vary widely. On the other hand semicarbazide and other carbonyl reagents which are lathyrogens have been shown to be potent inhibitors of the amine oxidase of beef plasma (Tabor *et al.*, 1954; Yamada and Yasunobu, 1963) and of human plasma monoamine oxidase (McEwen, 1965).

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