Collagen Cross-Linking

ISOLATION OF CROSS-LINKED PEPTIDES FROM COLLAGEN OF CHICKEN BONE

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Cross-linked peptides were isolated from chicken bone collagen that had been digested with CNBr or with bacterial collagenase. Analyses of ^3H radioactivity in disc electrophoretic profiles of the CNBr peptides from bone collagens that had been treated with NaB^3H indicated that a major site of intermolecular cross-linking in chicken bone collagen is located between the carboxy-terminal region of an $\alpha 1$ chain and a small CNBr peptide, probably situated near the amino-terminus of an $\alpha 1$ or $\alpha 2$ chain in an adjacent collagen molecule. A small amount of this cross-linked CNBr peptide was isolated from a CNBr digest of chicken bone collagen by column chromatography. Amino acid analysis showed that the CNBr peptide, $\alpha 1\text{CB6B}$, the carboxy-terminal peptide of the $\alpha 1$ chain, was the major CNBr peptide in the preparation, and the reduced cross-linking components were identified as hydroxylysino-hydroxynorleucine (HylOHNle), with a smaller amount of hydroxylysino-norleucine (HylNle). However, the composition and the low recovery of the cross-linking amino acids suggested that the preparation was a mixture of CNBr peptides $\alpha 1\text{CB6B}$ and $\alpha 1\text{CB6B}$ cross-linked to a small CNBr peptide whose identity could not be determined. A small cross-linked peptide was isolated from chicken bone collagen that had been reduced with NaB^3H_4 and digested with bacterial collagenase. This peptide was the major cross-linked peptide in the digest and contained a stoichiometric amount of the reduced cross-linking compounds. A peptide which had the same amino acid composition, but contained the cross-linking compounds in their reducible forms, was isolated from a collagenase digest of chicken bone collagen that had not been treated with NaBH_4 . The absence of the reduced cross-links from this peptide indicates that, at least for the cross-linking site from which the peptide derives, natural reduction is not a significant pathway for biosynthesis of stable cross-links. However, most of the reducible cross-linking component in the peptide appeared to stabilize in the bone collagen by rearrangement from aldimine to ketoamine form.

Recent work on the nature of cross-links in collagen has emphasized the central role of the aldehydes derived from lysyl and hydroxylysyl residues, and their subsequent reaction with each other to form aldols, or with unmodified lysyl and hydroxylysyl residues to form aldimines (see Traub & Piez, 1971; Gallop *et al.*, 1972). Each collagenous tissue, such as skin, tendon, bone, cartilage etc., reveals a unique and characteristic profile of the reducible cross-linking compounds on analysis after reduction with NaB^3H_4 (Bailey *et al.*, 1970a; Eyre & Glimcher, 1972a). In soft-tissue collagens major components also involve histidine residues which apparently participate in the formation of more complex reduc-

ible cross-linking compounds (Tanzer *et al.*, 1973), whereas in bone collagen these compounds are not major reducible components (Mechanic *et al.*, 1971). The mineralized collagens of bone and dentine (Mechanic *et al.*, 1971; Davis & Bailey, 1971), the collagen of cartilage (Bailey, 1971), and also the collagens of several invertebrate tissues (Bailey, 1971; Eyre & Glimcher, 1971), have the aldimine cross-link, dehydro-hydroxylysino-hydroxynorleucine (dehydro-HylOHNle), as their major reducible cross-link, with a smaller amount of dehydro-hydroxylysino-norleucine (dehydro-HylNle). The concentration of these reducible compounds in collagen apparently falls as connective tissues age, suggesting that they are modified to other, more stable cross-links (Bailey *et al.*, 1970a; Bailey & Shimokomaki, 1971). Evidence has been presented suggesting that the aldimine cross-links of bone and dentine collagens

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are stabilized by reduction *in vivo* to secondary amines (Mechanic *et al.*, 1971). However, the major reducible cross-link of the mineralized collagens, dehydro-HylOHNle, itself appears to be relatively stable, resisting heat- and acid-treatment (Davis & Bailey, 1971; Bailey *et al.*, 1970b).

Despite the relative abundance of information on the chemical structure of intermolecular cross-links in collagen, little is known about the relative contributions of these reducible compounds to the structural stability of collagen fibrils, nor is much information available on the precise locations of these bonds within the collagen molecules. It is, however, generally agreed that the two ends of the collagen molecule are important sites of the precursor aldehydes that participate in cross-link formation.

Much of our knowledge on the primary structure of collagen stems from analysis of the peptides released by digestion of the collagen chains with CNBr. For example, in chicken bone the collagen molecule has the chain composition $(\alpha 1)_2\alpha 2$. The $\alpha 1$ chain gives ten CNBr peptides which were originally assigned numbers, such as $\alpha 1CB1$, $\alpha 1CB2$, etc., according to their order of elution during column chromatography, and are now known to have the sequence 0-1-2-5-8-3-7-6A-6B in the $\alpha 1$ chain (Piez *et al.*, 1969). Similarly, the $\alpha 2$ chain has six CNBr peptides with the sequence 1-0-4-2-3-5 (Vuust *et al.*, 1970).

The present paper describes the isolation and analysis of cross-linked peptides from chicken bone collagen after digestion with CNBr or with bacterial collagenase. The results indicate the location of a major cross-linking site in the collagen molecule and illustrate the high stability of the non-reduced, dehydro forms of the reducible cross-links of bone collagen.

Materials and Methods

Preparation of bone collagen

Shafts of metatarsal bones from 12–14-week-old chickens were cleaned, dried, powdered and demineralized in 0.2M-EDTA, pH 7.9, as described (Eyre & Glimcher, 1973a). Bone collagen from mid-diaphyseal regions of tibiae and femora from foetal calves (5–6 months) was prepared in a similar manner.

The demineralized collagen was reacted with NaB^3H_4 (New England Nuclear Corp., Boston, Mass., U.S.A.) for 45 min in 0.1M-sodium phosphate, pH 7.4, at a ratio of 3mg of NaB^3H_4 to 100 mg of collagen. For analyses of ^3H radioactivity in CNBr peptides separated by disc electrophoresis, about 5mg of collagen was reduced with NaB^3H_4 (sp. radioactivity, 200 Ci/mol); a lower specific radioactivity (usually 1 Ci/mol) was used for reduction before other analyses. After reduction, the suspension

was adjusted to pH 3.0–4.0 by addition of acetic acid, and the collagen was thoroughly washed with water.

Cleavage with CNBr

NaB^3H_4 -treated, demineralized bone powder was stirred in 70% (v/v) formic acid under N_2 at 30°C for 4h with a 150:1 molar ratio of CNBr/methionine. The resulting solution was diluted tenfold with water and freeze-dried.

Disc electrophoresis of CNBr peptides in sodium dodecyl sulphate-polyacrylamide gels

The method of Furthmayr & Timpl (1971) was followed by using 7.5% (w/v) acrylamide in 10cm gels. Gels were stained with Coomassie Brilliant Blue or serially sliced (Gilson Instruments Co., Gel Fractionator) for measurement of ^3H -radioactivity. The minced gel slices (0.5mm) were treated with 0.5ml of Protosol (New England Nuclear Corp.) in capped scintillation vials for 4h and, after acidifying the digests by addition of 0.05ml of acetic acid, 10ml of Aquasol (New England Nuclear Corp.) was added and shaken vigorously for 30min to give a clear solution.

The molecular weights of cross-linked peptides were assessed from a calibration plot of the distance travelled in the gel versus the logarithm of the molecular weight of the known CNBr peptides of collagen. These peptides were identified in the stained gels by comparison with previous analyses (Furthmayr & Timpl, 1971). Although molecular weights below about 15000 cannot be estimated reliably by this technique alone (Williams & Gratzer, 1971), the elution position of the peptides on molecular-sieve chromatography in comparison with the CNBr peptides of known size confirmed the estimated values.

Molecular-sieve chromatography of CNBr peptides

Complete CNBr digests of reduced collagen from chicken bone (about 40mg) were fractionated on a column (200 cm $\times$ 2 cm) of Bio-Gel agarose (1.5 m, 200–400 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.), and eluted with 1M- CaCl_2 –0.05M-Tris-HCl, pH 7.4, at a flow rate of 10 ml/h.

The CNBr peptides isolated by phosphocellulose chromatography were further resolved by elution from a column (100 cm $\times$ 1.6 cm) of Bio-Gel P-150 (100–200 mesh) in 1M- CaCl_2 –0.05M-Tris-HCl, pH 7.4, at a flow rate of 12 ml/h. The peptide fractions eluting from this column were desalted by gel filtration on a column of Bio-Gel P-2 (100–200 mesh) and eluted with 0.1M-acetic acid.

Chromatography of CNBr peptides on phosphocellulose

The fraction of cross-linked peptide isolated by gel filtration on agarose was applied to a column (15 cm × 2 cm) of phosphocellulose (Whatman P-11; W. and R. Balston, Maidstone, Kent, U.K.) at 42°C and eluted with a linear gradient of 0–0.5 M-NaCl in 800 ml of 1 M-sodium acetate, pH 3.6, at a flow rate of 100 ml/h. The column was then flushed with starting buffer containing 1 M-NaCl and 8 M-urea, and the peptides in the voided fraction were collected.

Digestion with bacterial collagenase

This was carried out as previously described on samples (1 g) of demineralized bone collagen by using relatively crude bacterial collagenase (Schwarz/Mann, Orangeburg, N.Y., U.S.A.) (Eyre & Glimcher, 1973a).

Molecular-sieve chromatography of the collagenase digest of bone collagen

Cross-linked peptides in the collagenase digest of bone collagen were partially resolved by elution from a column (2.6 cm × 30 cm) of Bio-Gel P-2 (100–200 mesh). The void fraction recovered from this column was then applied to a column (1.6 cm × 90 cm) of Bio-Gel P-10 (200–400 mesh). Both columns were eluted with 0.1 M-acetic acid.

Ion-exchange chromatography of collagenase peptides on phosphocellulose

The partially purified, cross-linked peptides were fractionated by elution from a column (2 cm × 15 cm) of phosphocellulose (Whatman, P-11) at 42°C with a linear gradient of 0.2–0.6 M-NaCl in 800 ml of 1 M-sodium acetate, pH 3.6, at a flow rate of 100 ml/h.

Purification of peptides on Bio-Gel P-6

The cross-linked collagenase peptide in the major peak of ³H radioactivity that eluted from the phosphocellulose column near the end of the NaCl gradient was finally purified by elution from a column (1.6 cm × 100 cm) of Bio-Gel P-6, 200–400 mesh, in 0.1 M-acetic acid at a flow rate of 20 ml/h.

Oxidation with periodate

The peptide containing the reduced cross-linking compounds was dissolved in 1 ml of 0.02 M-acetic acid and reacted with 1 ml of 0.02 M-sodium metaperiodate in the dark at 25°C for 3 h. Excess of periodate was destroyed by addition of one drop of ethylene glycol, and aldehydic reaction products were reduced with

NaBH₄ after addition of 0.1 M-phosphate buffer, pH 7.4 (Bailey *et al.*, 1970b). The reaction mixture was adjusted to pH 3–4 with acetic acid, evaporated to dryness and applied to the Bio-Gel P-6 column (see above). The region of effluent containing the two partially resolved peptide fragments (Eyre & Glimcher, 1973b) was pooled, dried and hydrolysed in 6 M-HCl for amino acid analysis.

Amino acid analysis

Peptides were hydrolysed in 6 M-HCl under N₂ in a sealed tube at 105°C for 24 h. Phenol (10 mm) was included in the HCl to prevent loss of tyrosine during hydrolysis. Amino acid compositions were determined on an automated amino acid analyser with a single column eluted by a complex gradient. The cross-linking compounds, HylOHNle and HylNle, were eluted as separate peaks between NH₃ and hydroxylysine in this system. By using a split-stream device, 50% of the column effluent could be monitored for ³H radioactivity by mixing it with Aquasol (New England Nuclear Corp.) and pumping the mixture through a Teflon coil in a specially constructed well of a scintillation counter (Intertechnique Instruments, Model SL 20) (Eyre, 1973). The reduced cross-links, HylOHNle and HylNle, were quantitated in large samples of hydrolysed collagen by chromatography on the 60 cm analyser column eluted at 54°C with 0.35 M-sodium citrate, pH 5.2, and by reacting the effluent with ninhydrin.

Results

Fractionation of CNBr peptides

In the following experiments, reducible cross-links were labelled by reduction with NaB³H₄ before digestion of the bone collagen with CNBr in 70% (v/v) formic acid. When, however, a CNBr digest of non-reduced bone collagen was treated with NaB³H₄, labelled hydroxylysino-hydroxy-norleucine was found in about the same concentration as in the reduced, native collagen (Fig. 1), showing that the dehydro form of this compound is not disrupted in relatively strong acid or during degradation of the collagen by CNBr. A fraction of the other reducible cross-link in bone collagen, dehydro-HylNle, also appeared to resist destruction during digestion of the collagen with CNBr (Fig. 1).

After labelling the reducible cross-links in bone collagen by reduction with NaB³H₄, a profile of the molecular sizes of the major cross-linked CNBr peptides was derived by disc electrophoresis of the peptides in sodium dodecyl sulphate–polyacrylamide gels and then by measuring ³H radioactivity in serial slices of the polyacrylamide gel. By this technique, chicken bone collagen gave a different

Fig. 1. Ion-exchange column chromatography of ^3H -labelled cross-linking components

These were derived by reduction with NaB^3H_4 (10Ci/mol) of the peptides resulting from CNBr digestion of chicken bone collagen. Peptides were reacted with NaB^3H_4 as a solution in 0.1M-phosphate, pH 7.4, and desalted on a column of Bio-Gel P-2. The peptide mixture was hydrolysed in 3M-HCl for 48h at 105°C , and 10mg was applied to the column.

profile of tritiated cross-linked peptides from that of calf bone collagen (Plate 1). An obvious difference between the peptides from the two species is the presence of a major zone of ^3H radioactivity migrating near the $\alpha 1\text{CB}3$ peptide in the electrophoretic pattern derived from chicken bone collagen, and corresponding to a molecular size of about 10000 to 13000. This peak of activity was not present on analysis of peptides derived from calf bone collagen (Plate 1), nor in CNBr peptides derived from samples of human bone collagen analysed by the same technique. Compared with the profile for chicken bone collagen, more of the total ^3H radioactivity, and hence of cross-linked peptides, was detected in the peptide profiles of calf and human bone collagens in the region of the polyacrylamide gel containing the CNBr peptides $\alpha 1\text{CB}7$, $\alpha 1\text{CB}8$ and $\alpha 2\text{CB}4$, and corresponding to a molecular weight of about 20000 to 25000. Another major band of ^3H radioactivity was detected on electrophoresis of chicken bone collagen in the region of the uncleaved peptide, $\alpha 1\text{CB}3$ -7 (Plate 1). This peak was also absent from the profiles of calf and human CNBr peptides, being replaced by extra ^3H radioactivity in the region of the gel that would contain collagen CNBr peptides having a molecular weight about 10000 larger.

Molecular-sieve chromatography on agarose confirmed the presence in the CNBr digest of reduced chicken bone collagen of a major ^3H -labelled, cross-

linked fraction having a molecular weight of about 10000 (the peak marked with a bar in Plate 2). Disc electrophoresis of peptides recovered from this region of the chromatogram showed several stained bands, two of which migrated in the polyacrylamide gels where the ^3H radioactivity was previously detected after gel-slicing (Plate 2). The slower, and probably larger, of these two peptides was tentatively identified as peptide $\alpha 1\text{CB}3$. In an attempt to purify the cross-linked peptide, the material partially separated on the agarose column was chromatographed on phosphocellulose by elution with a salt gradient (not shown). Some ^3H radioactivity did elute with the gradient, but not as a discrete peak or peaks; nor were the recoveries of ^3H radioactivity from the column consistent in several experiments. It appeared that the cross-linked peptides were precipitating on the phosphocellulose column, thus preventing their elution, even with 1M-NaCl. However, elution of the column with 1M-NaCl in 8M-urea did remove the bound peptides containing the remaining ^3H radioactivity. Peptide peaks that were detected by u.v. absorption at 230nm during gradient elution of the phosphocellulose column were identified by their disc electrophoretic mobilities and amino acid compositions as the single-chain peptides $\alpha 1\text{CB}3$, $\alpha 1\text{CB}6\text{A}$, $\alpha 1\text{CB}7$ and $\alpha 1\text{CB}8$ (Miller *et al.*, 1969). However, the peptide $\alpha 1\text{CB}6\text{B}$ was not detected by amino acid analysis in any of the fractions eluted with the NaCl gradient.

In a final attempt to isolate a pure CNBr peptide containing a cross-link, the radioactive peptides that were stripped from the phosphocellulose column by urea were chromatographed on Bio-Gel P-150 (Fig. 2). The major peak of radioactivity chromatographed in the position expected for a collagen peptide of about 10000 molecular weight. Amino acid analysis of the peptide recovered from the region of effluent corresponding to this peak revealed the reduced cross-links, HylOHNle and HylNle, but assuming a molar colour yield with ninhydrin twice that of leucine, only 20% of the amount of these cross-linking compounds was detected compared with the amount that would be expected for a peptide of 10000 molecular weight that contained one cross-linking residue per molecule (Table 1). Disc electrophoretic analysis revealed one broad band of stained peptide material. The amino acid composition which includes almost one residue of 3-hydroxyproline, but only 0.2 residue of homoserine, indicated that the major CNBr peptide in the material was the carboxy-terminal peptide $\alpha 1\text{CB}6\text{B}$.

However, the amino acid composition of peptide $\alpha 1\text{CB}6\text{B}$ shown in Table 1 is of the peptide isolated from the $\alpha 1$ chain of acid-soluble chicken bone collagen (Miller, 1969), which has lost a C-terminal extra-helical sequence (Eyre & Glimcher, 1972b). Although the composition of the whole additional

EXPLANATION OF PLATE I

Disc electrophoresis in sodium dodecyl sulphate-polyacrylamide gels of CNBr digests of NaB^3H_4 -treated bone collagen

For each analysis one gel was stained with Coomassie Brilliant Blue and another, unstained gel, was serially sliced (0.5mm) for measurement of 3H radioactivity in the peptides. CNBr digest ($100\mu g$) was applied to each gel. (a) CNBr digest of chicken bone collagen, (b) CNBr digest of calf bone collagen. The positions of various peptides are marked on the gel scan.

EXPLANATION OF PLATE 2

Molecular-sieve chromatography of a CNBr digest of NaB^3H_4 -treated chicken bone collagen on Bio-Gel Agarose

The column (1.5 m, 200–400 mesh; 200 cm $\times$ 2 cm), was eluted with 1 M- CaCl_2 –0.05 M-Tris-HCl, pH 7.4, at a flow rate of 10 ml/h. The inset shows the results of disc electrophoretic analysis in sodium dodecyl sulphate–polyacrylamide gel of peptides isolated after dialysis against water of the regions of column effluent marked by the bars. Peptides in the region marked by the bar on the chromatograph were taken for ion-exchange chromatography on phosphocellulose. —, E_{230} ; ----, ^3H radioactivity.

Fig. 2. Molecular-sieve chromatography on Bio-Gel P-150 (100–200 mesh; 100 cm × 1.6 cm) of the CNBr peptide fraction of chicken bone collagen recovered from the phosphocellulose column by elution with 1 M-NaCl in 8 M-urea

The peptide in the region of column effluent marked by a bar was desalted on Bio-Gel P-2 before hydrolysis in acid and amino acid analysis. —, E_{230} ; - - - -, ^3H radioactivity.

Table 1. Amino acid composition of a CNBr peptide that contains cross-links from NaBH_4 -treated chicken bone collagen

The values in parentheses denote the measured values from which nearest integers are derived. The composition of the peptide fraction isolated in the present study is compared with the sum of published analyses for peptide $\alpha 1\text{CB6B}$ plus an extrahelical C-terminal sequence of $\alpha 1$.

Amino acid composition (residues/molecule)			
	Peptide	$\alpha 1\text{CB6B}^*$	C-Terminal extrahelical region of $\alpha 1^\dagger$
3Hyp	1 (0.7)	1	—
4Hyp	9 (8.7)	9	—
Asp	5 (4.7)	3	2
Thr	2 (1.8)	1	—
Ser	6 (5.8)	5	1
Glu	8 (7.9)	5	3
Pro	11 (10.8)	13	3
Gly	31 (31.0)	28	3
Ala	9 (9.4)	6	1
Val	2 (2.0)	2	—
Ile	1 (1.1)	1	—
Leu	3 (2.6)	3	2
Tyr	1 (0.8)	—	1
HylOHNle‡	0.13	—	—
HylNle‡	0.06	—	—
Hyl	0.4	0.6	—
Lys	2.1	1.4	1§
His	1 (1.0)	1	1
Arg	4 (4.2)	4	1
Hse	0.2	—	—
Total	98	85	20

* Values in this column from Miller (1969).

† Data for the $\alpha 1$ chain of calf skin collagen (Stark *et al.*, 1971).

‡ Assuming a molar colour yield twice that of leucine.

§ Present mainly as lysine aldehyde.

|| Homoserine + homoserine lactone.

Fig. 3. Molecular-sieve chromatography of a collagenase digest of 1g of NaB^3H_4 -treated chicken bone collagen

(a) Elution of the complete digest from Bio-Gel P-2 (100–200 mesh; 2.6cm×30cm) in 0.1M-acetic acid. Peptides in the column effluent marked by a bar were freeze-dried. (b) Elution of the peptides recovered from the Bio-Gel P-2 column from Bio-Gel P-10 (200–400 mesh; 1.6cm×90cm) in 0.1M-acetic acid. Peptides in the region of effluent marked by a bar were fractionated on phosphocellulose (Fig. 4). —, E_{230} ; - - - -, ^3H radioactivity.

sequence is not known for chicken bone collagen, it is apparently similar to that of calf skin collagen (Eyre & Glimcher, 1972b), which is given in Table 1. The sum of this composition and that of peptide $\alpha 1\text{CB6B}$ from acid-soluble bone collagen is similar to that found in the present preparation which probably contains peptide $\alpha 1\text{CB6B}$ that has the C-terminal extra-helical extension. The low recovery of the cross-linking amino acids and of homoserine suggests that

Fig. 4. Ion-exchange column chromatography on phosphocellulose of the collagenase peptides of chicken bone collagen isolated by gel filtration on Bio-Gel P-10 (Fig. 3)

The phosphocellulose column (2cm×15cm) was eluted at 42°C with a linear gradient of 0.2–0.6M-NaCl in a total volume of 800ml of 1mM-sodium acetate, pH3.6, at a flow rate of 100ml/h. At the arrow the column was eluted with 1M-NaCl in starting buffer. The fractions marked by a bar were further analysed by molecular-sieve chromatography on Bio-Gel P-6. —, E_{230} ; - - - -, ^3H radioactivity.

the preparation is a mixture of free $\alpha 1\text{CB6B}$ with a smaller amount of peptide $\alpha 1\text{CB6B}$ cross-linked to a short CNBr peptide that does not significantly increase the molecular size.

Fractionation of collagenase peptides

About one-third of the total ^3H radioactivity incorporated into demineralized chicken bone by NaB^3H_4 was recovered by molecular-sieve chromatography of a collagenase digest on columns of Bio-Gel P-2 and Bio-Gel P-10 (Fig. 3), in peptides which accounted for 2% of the collagen by weight. Thus, the cross-linked peptides had been concentrated approximately 15-fold in the recovered peptide fraction. Chromatography of this material on phosphocellulose (Fig. 4) gave two major peaks of ^3H radioactivity. One of these, the void peak, could be resolved into several radioactive peptides by rechromatography on the phosphocellulose column with a NaCl gradient starting at a lower concentration. None of these peptides was analysed in the present study. The peak of ^3H radioactivity eluting at the end of the NaCl gradient contained essentially one cross-linked peptide when purified by elution from Bio-Gel P-6 (Fig. 5), and was clearly the major peptide in the digest that contained reduced cross-links. This peptide had an identical amino acid composition, apart from the relative proportion of the reduced cross-linking

amino acids, HylOHNle and HylNle, to a peptide recently isolated by a similar procedure from calf bone collagen (Eyre & Glimcher, 1973b). After cleavage of the cross-links in the peptide by oxidation with NaIO_4 and reduction of the reaction products with non-radioactive NaBH_4 , hydroxylysine and hydroxynorvaline were recovered by amino acid analysis (Table 2). As previously found with the peptide from calf bone collagen (Eyre & Glimcher, 1973b), most of the ^3H radioactivity originally incorporated into the peptide by reduction of the cross-link with NaB^3H_4 remained associated with the peptide chains that were released after cleavage of the cross-link with NaIO_4 , and was recovered as radioactive hydroxynorvaline and chlornorvaline (derived from hydroxynorvaline on hydrolysis in HCl) on amino acid analysis. After hydrolysis of the intact cross-linked peptide in 2M-KOH , HylOHNle and HylNle were not detected on the amino acid analyser, instead new ninhydrin-positive peaks with coincident ^3H radioactivity appeared in the chromatogram, the major one eluting just before NH_3 .

These findings indicate that most of the tritiated cross-links in the peptide fraction from chicken bone collagen had derived by NaB^3H_4 reduction of the keto-amine-rearranged forms of the dehydro cross-links. Scheme 1 illustrates the rearrangement from aldimine to keto-amine for dehydro-HylOHNle and

the degradation of the reduced compound by periodate when the remaining hydroxyl group, the one not derived by reduction, is glycosylated. The recovery of hydroxylysine after oxidation of the cross-linked peptide with periodate and hydrolysis in acid suggests that hydroxylysyl residues whose

Fig. 5. Purification of the cross-linked collagenase peptide in the peak marked with a bar on the phosphocellulose chromatogram (Fig. 4), by molecular-sieve chromatography

A column of Bio-Gel P-6 (200–400 mesh; $1.6\text{cm} \times 100\text{cm}$) was eluted with 0.1M -acetic acid at a flow rate of 20ml/h . —, E_{230} ; - - -, ^3H radioactivity. The fractions marked by a bar were pooled and freeze-dried for amino acid analysis.

Table 2. Amino acid composition of the cross-linked peptide from collagenase-digested chicken bone collagen

The values in parentheses denote the measured values from which the nearest integers are derived. Samples of peptide for analysis were isolated from (a) NaBH_4 -treated collagen and (b) untreated collagen.

	Amino acid composition (residues/molecule)		
	(a) Reduced collagen	(b) Non-reduced collagen	Reduced peptide + NaIO_4 + NaBH_4
Asp	1 (1.3)	1 (1.3)	1.3
OHNva*	—	—	0.6
Glu	3 (3.1)	3 (3.2)	3.4
Pro	3 (2.9)	3 (3.0)	3.4
Gly	5 (5.2)	5 (4.9)	5.0
Ala	1 (1.2)	1 (1.1)	1.4
Met	1 (0.9)	1 (1.0)	0.8
Leu	1 (0.9)	1 (1.0)	1.1
Tyr†	0.6	0.5	—
Phe	1 (0.9)	1 (0.9)	0.9
HylOHNle‡	1 (0.7)	—	—
HylNle‡	1 (0.2)	—	—
Hyl	—	—	0.9
His	2 (1.8)	2 (1.8)	2.0
Arg	2 (2.1)	2 (2.0)	2.0

* The sum of hydroxynorvaline and chlornorvaline.

† Tyrosine content varied in different preparations of the peptide. Chains with 0, 1 or 2 carboxy-terminal tyrosines apparently could be isolated (Eyre & Glimcher, 1973b).

‡ Assuming a molar colour yield twice that of leucine.

Scheme 1. Rearrangement from aldimine to keto-amine for (glycosylated) dehydro-HylOHNle and the degradation of the reduced compound by periodate
 Glucosylgalactosyl-dehydro-HylOHNle is depicted as an example. Galactosyl-dehydro-HylOHNle would behave similarly.

Fig. 6. Analysis of the reduced cross-linking amino acids, HyLOHNle and HyLNle, in an acid hydrolysate of the cross-linked peptides recovered by molecular-sieve chromatography of a collagenase digest of chicken bone collagen (Fig. 3)

The 60cm column of the amino acid analyser was eluted at 54°C with 0.35M-sodium citrate, pH 5.2, and the column effluent was reacted with ninhydrin. (a) Analysis of 1 mg of peptides from NaBH_4 -treated chicken bone collagen. (b) Analysis of 1 mg of peptides from untreated chicken bone collagen.

hydroxyl groups were substituted had participated in the biosynthesis of most of the cross-linking component in this peptide fraction. The normally susceptible side chains of such residues would resist cleavage by NaIO_4 and acid hydrolysis would release free hydroxylysine from the NaIO_4 -treated cross-links. The absence of HyLOHNle and HyLNle and the appearance of new ninhydrin-positive peaks with coincident ^3H radioactivity on analysis of the cross-linked peptide after hydrolysis in 2M-KOH, conditions under which glycosidic attachments to hydroxylysine are stable, suggest that most of the cross-linking compounds in this peptide were glycosylated, as

found for the similar peptide from calf bone collagen (Eyre & Glimcher, 1973b).

A collagenase digest of chicken bone collagen that had not been treated with NaBH_4 was fractionated by a similar chromatographic procedure, by pooling appropriate regions of the various column effluents. A peptide with the same amino acid composition, apart from the absence of HyLOHNle and HyLNle, was recovered in a similar yield to the peptide isolated from the reduced bone collagen (Table 2). Some hydroxylysine and unidentified ninhydrin-positive peaks were now detected (not included in Table 2), which probably had derived by decomposition of the dehydro cross-links during acid hydrolysis. This conclusion was supported by the absence of the hydroxylysine and the unknown peaks on amino acid analysis of a sample of the peptide that had been treated with NaBH_4 and then hydrolysed in acid, when HyLOHNle and HyLNle were recovered in the same concentrations as in the peptide isolated from reduced bone collagen.

Samples of the mixture of cross-linked peptides isolated from reduced chicken bone collagen and from non-reduced chicken bone collagen by elution from the Bio-Gel P-10 column (Fig. 3), were analysed directly for the cross-links HyLOHNle and HyLNle (Fig. 6). The result indicated that at the most only 6% of the HyLOHNle and 2% of the HyLNle detected in NaBH_4 -treated bone collagen could be present as the naturally reduced cross-links in the native bone collagen.

Discussion

The location of a major cross-linking site in the collagen molecule can be predicted from the results of disc electrophoretic analyses of the CNBr peptides of bone collagen that contain NaBH_4 -reduced cross-links. Thus the presence in a CNBr digest of NaB^3H_4 -reduced chicken bone collagen of a ^3H -labelled peptide with a molecular weight of about 10000, and its absence from similar digests of calf and human bone collagens, probably reflects the basic difference between the CNBr peptides of chicken bone collagen and those of calf and human bone collagens. The CNBr peptides derived from $\alpha 1$ and $\alpha 2$ chains of calf skin collagen (Rautenberg & Kühn, 1971; Fietzek *et al.*, 1970) appear identical with those of human skin collagen (Click & Bornstein, 1970) with respect to number and size. However, in skin and bone collagen of chicken there is an extra methionine residue in the carboxy-terminal region of the $\alpha 1$ chain, giving two peptides, $\alpha 1\text{CB6A}$ and $\alpha 1\text{CB6B}$, in place of one peptide, $\alpha 1\text{CB6}$, from calf and human collagens (Miller *et al.*, 1969; Piez *et al.*, 1969; Lane & Miller, 1969; Kang *et al.*, 1969a,b). Now peptides $\alpha 1\text{CB6A}$ and $\alpha 1\text{CB6B}$ (Miller *et al.*, 1969; Kang *et al.*, 1969a) are each about

10000 molecular weight and peptide $\alpha 1\text{CB6}$ is the sum of these at about 20000. An intermolecular cross-link between this carboxy-terminal region of an $\alpha 1$ chain and a relatively small CNBr peptide in another chain would explain the recovery of a cross-linked CNBr peptide of about 10000 molecular weight from chicken bone collagen, but its absence from a CNBr digest of calf or human bone collagens. The carboxy-terminus of the $\alpha 1$ chain of collagen is now known to be an important site of a cross-linking aldehyde (Stark *et al.*, 1971), and peptide $\alpha 1\text{CB6}$ in rat (Rojkind *et al.*, 1970; Kang, 1972), and bovine collagen (Volpin & Veis, 1971), and peptide $\alpha 1\text{CB6B}$ in chicken (Miller, 1969) were indicated as important participants of intermolecular cross-linking in collagen. In the present study the molecular size of the cross-linked CNBr peptide indicates that it consists of a relatively small CNBr peptide linked to peptides $\alpha 1\text{CB6A}$ or $\alpha 1\text{CB6B}$. From the known structure of chicken bone collagen the small CNBr peptide could be $\alpha 2\text{CB1}$ or 2, or $\alpha 1\text{CB1}$, 4 or 5, all of which, apart from peptide $\alpha 2\text{CB2}$, are situated within or adjacent to the overlap region at the amino-terminus of the molecule. Scheme 2 illustrates the distribution of CNBr peptides in $\alpha 1$ and $\alpha 2$ chains and possible sites of intermolecular cross-linking between $\alpha 1$ chains of overlapping molecules packed in the Hodge-Petruska modification of a quarter-stagger arrangement (Hodge & Petruska, 1963).

The amino acid composition of the cross-linked CNBr peptide that was detected by disc electro-

phoresis and isolated by column chromatography, confirms that peptide $\alpha 1\text{CB6B}$ is the main component. However, it would appear from the recovery of the cross-linking compounds that the peptide preparation consisted of about 20% of cross-linked peptide mixed with free peptide $\alpha 1\text{CB6B}$. The results support the previous observation that the $\alpha 1$ chain of chicken bone collagen loses a carboxy-terminal region when extracted in non-denaturing solvents (Eyre & Glimcher, 1972b), as do the $\alpha 1$ chains of calf and rat skin collagens (Rauterberg & Kühn, 1971; Rauterberg *et al.*, 1972). The amino acid composition suggests that the extrahelical sequence at the carboxy-terminus of chicken $\alpha 1$ is similar to that of calf $\alpha 1$.

The cross-linked peptide that was isolated from collagenase-digested bone collagen could derive from the same site in the molecule as the cross-linked CNBr peptide described above. It is noteworthy that the cross-linked peptide isolated from chicken bone collagen after digestion with crude collagenase has an almost identical amino acid composition with a comparable peptide isolated from calf bone collagen (Eyre & Glimcher, 1973b), and presumably derives from a similar molecular site. It appeared from the composition of its component chains, that the peptide from calf bone collagen was derived from a sequence at the carboxy-terminus of an $\alpha 1$ chain that was cross-linked intermolecularly by a reducible bond to a glycosylated hydroxylysine residue located in the amino-terminal sequence of peptide $\alpha 1\text{CB5}$ in another $\alpha 1$ chain. In fact, assuming that the $\alpha 1$ chains of calf

Scheme 2. Distribution of CNBr peptides in $\alpha 1$ and $\alpha 2$ chains of chicken bone collagen and two of the possible sites of intermolecular cross-linking between $\alpha 1$ chains of overlapping molecules packed in a quarter-stagger arrangement

bone and skin have the same primary structure, the amino acid compositions of the peptide chains were so highly characteristic with all the rare amino acid constituents of collagen represented, that the peptides could not be ascribed to any other regions of the $\alpha 1$ chain (Eyre & Glimcher, 1973b). However, the results do not rule out their origin in an $\alpha 2$ chain which may contain homologous sequences to the $\alpha 1$ chain.

The major reducible component of bone collagen, dehydro-Hy(OHN)le, appears to be a stable intermolecular cross-link, even without reduction. It is not destroyed on digestion of collagen with CNBr in 70% formic acid, nor on digestion of the collagen to small peptides by bacterial collagenase. Rearrangement from an aldimine to a keto-amine form apparently explains the unusual stability of this major reducible compound of bone collagen (Tanzer, 1973; Eyre & Glimcher, 1973b). Further, there is no evidence from the present data, nor from direct analyses of the reduced cross-links in several species of bone collagen at various stages of maturity (Brickley, 1972; D. R. Eyre, D. M. Brierley & M. J. Glimcher, unpublished work), that reduction of these bonds occurs to a significant extent *in vivo*. However, since the number of reducible cross-links in collagen diminishes markedly with age and maturation of the tissues (Bailey *et al.*, 1970a; Bailey & Shimokomaki, 1971), it seems likely that the reducible bonds, including the keto-amine forms, are modified *in vivo*, but in a manner and to components as yet unidentified.

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