Simultaneous Determination of the Reducible and Nonreducible Cross-Links of Connective Tissue. Analysis of Mineralized and Nonmineralized Bone Collagen†

Lila Graham*,‡ and Gerald L. Mechanic

Departments of Biochemistry and Oral Biology, Dental Research Center, University of North Carolina, Chapel Hill, North Carolina 27514

Received January 6, 1989; Revised Manuscript Received May 8, 1989

ABSTRACT: Secondary amine cross-links occur in collagen and elastin from a number of tissue sources. Quantification of these cross-links by amino acid analysis is complicated by the problem of separating cross-links, which are often minor components, from the more common amino acids and also because relatively large amounts of a cross-link are required to determine a color factor. A specific radioactive labeling method has been developed and used to quantify cross-links in bone collagen. Primary amines such as lysine and hydroxylysine are first guanidinated with 3,5-dimethylpyrazole-1-carboxamidine nitrate (DMPC). Secondary amines, which are unreactive with DMPC, are then quantitatively cyanoethylated with [^{14}C]acrylonitrile. This procedure can be used to detect any secondary amine cross-link, with higher sensitivity than ninhydrin analysis, in peptide form as well as in acid hydrolysates. It is applied here in conjunction with [^{3}H]$NaBH_4$ reduction to simultaneously quantify Schiff base cross-links and amounts of in vivo reduction of Schiff bases in mineralized versus nonmineralized bovine bone.

Cross-linking of collagen is essential for the mechanical function of connective tissue; a cross-link-deficient collagen matrix does not have sufficient strength to hold an organism together (Levene & Gross, 1959). The initial cross-links formed in a new collagen fibril are Schiff bases, which can be detected by reduction with [^{3}H]$NaBH_4$ (Tanzer & Mechanic, 1968; Tanzer, 1976; Eyre et al., 1984). In addition to provide a radioactive label, borohydride reduction converts a Schiff base to a secondary amine, thereby stabilizing the cross-link to acid hydrolysis. This method has yielded considerable information about the structure (Bailey & Peach, 1968; Mechanic et al., 1971; Tanzer et al., 1973a,b), sequence location (Kuboki et al., 1981a,b; Stimler & Tanzer, 1979; Yamauchi et al., 1986; Henkel et al., 1976), and pathways of formation (Franzblau et al., 1970; Bernstein & Mechanic, 1980) of borohydride-reducible cross-links and also about changes in cross-linking in pathological conditions (Mechanic et al., 1972, 1975; Mechanic & Bullough, 1974; Russell et al., 1975; Banes et al., 1978).

Not all cross-links are susceptible to borohydride reduction; the class of nonreducible cross-links includes hydroxypyridinium compounds, which are easily detectable by their fluorescence (Eyre et al., 1984), and a number of other structures that can only be detected by amine-modifying reagents such as ninhydrin. Most of the nonreducible, nonfluorescent cross-links have at least one secondary amine moiety. These include lysinoalanine in dentin (Fujimoto et al., 1981), merodesmosine and lysinonorleucine in elastin (Gallop et al., 1972), and histidinohydroxylysinonorleucine (HHL)[1] in skin collagen (Yamauchi et al., 1987). The latter cross-link is of particular interest, since it appears exclusively in skin collagen, due to the unique orientation of skin collagen fibrils (Mechanic et al., 1987). HHL is also the only known collagen cross-link that is more abundant in aging tissues (Yamauchi et al., 1988).

Investigations of the tissue distribution and sequence locations of the nonreducible, nonfluorescent cross-links are encumbered by the necessity of subjecting protein or peptide samples to acid hydrolysis and then identifying and resolving minor peaks found by amino acid analysis. A radioactive labeling method specific for secondary amines would therefore be of value in pursuing such studies, since, in addition to providing higher sensitivity, it would facilitate detection of cross-links in peptide linkage. Ideally, secondary amine cross-links should be labeled quantitatively and specifically, and the product of the labeling reaction should be stable to acid hydrolysis. A ^{14}C reagent to modify secondary amines, such as [^{14}C]acrylonitrile (Graham & Mechanic, 1986), would allow simultaneous determination of reducible cross-links with [^{3}H]$NaBH_4$ in the same sample.

One application of a simultaneous labeling technique for reducible cross-links and secondary amines would be direct determination of in vivo reduction of cross-links. In vivo reduction is an important process in elastin (Gallop et al., 1972), but in vivo reduced cross-links are present in only trace amounts in acid hydrolysates of unreduced collagen (unpublished observations). An early result indicating 20–25% in vivo reduction in bone (Mechanic et al., 1971) was not supported by later papers (Robins et al. 1973; Eyre & Oguchi, 1980) and was subsequently reinterpreted as an artifact of the reduction procedure (Eyre et al., 1984).

The hydroxypyridinium cross-links in bone are confined almost exclusively to the nonmineralized compartment (Banes et al., 1983; Mechanic et al., 1985). Since it has been suggested that reducible cross-links might be precursors of hydroxypyridinium cross-links (Fujimoto & Moriguchi, 1978; Eyre, 1981), it is of interest to determine whether amounts of reducible cross-links are correspondingly lower in nonmineralized bone. Also, significant in vivo reduction in nonmineralized bone collagen cannot be ruled out at this point.

† This work was supported in part by NIH Grants AR 30587 and AR 19969, and NASA Grant NAG-2-181.

* To whom correspondence should be addressed.

‡ Present address: Laboratory for Human Biochemistry, Children's Hospital Medical Center, 300 Longwood Ave., Boston, MA 02115.

[1] Abbreviations: HHL, histidinohydroxylysinonorleucine; DMPC, 3,5-dimethylpyrazole-1-carboxamidine nitrate; HLNL, hydroxylysinonorleucine; DHLNL, dihydroxylysinonorleucine; HHMD, histidinohydroxymerodesmosine; CEHLNL, carboxyethyl-HLNL; CEDHLNL, carboxyethyl-DHLNL; CEHHMD, carboxyethyl-HHMD.

0006-2960/89/0428-7889$01.50/0

It would not be apparent on analysis of unfractionated bone, because the nonmineralized compartment represents only about 20% of the total. Therefore, as a first application of secondary amine cross-link analysis, we have elected to simultaneously quantify reducible and in vivo reduced cross-links to determine their relative abundance in mineralized versus nonmineralized bone collagen.

Materials and Methods

Reagents. Most starting materials were from Aldrich. Solvents were dried for several hours over a 4-Å molecular sieve, $^1/_{16}$-in. diameter, 8–12 mesh (Aldrich). 3,5-Dimethylpyrazole-1-carboxamidine nitrate was from Research Organics (Cleveland OH). *N*-α-Acetyl-L-lysine methyl ester was from Sigma.

Analytical Instrumentation. Amino acid analysis was done on a Varian 5560 HPLC with an AA911 Interaction column and a postcolumn ninhydrin derivatization system with color development at 135 °C.

Fast atom bombardment mass spectrometric analyses were performed on a VG ZAB-4F four-sector instrument of B_1E_1–E_2B_2 configuration (VG Analytical, Manchester, U.K.) (Hass et al., 1984). Ion generation, collision conditions, and data acquisition were as described by Yamauchi et al. (1987).

Collagen Preparation. Bone was lyophilized and powdered to a −400 mesh powder in a Spex mill. The powder was then demineralized by extraction three times over 24 h in 0.5 M EDTA and 0.05 M Tris-HCl, pH 7.5 at 4 °C. The bone collagen was then washed extensively with cold deionized water and lyophilized (Kuboki & Mechanic, 1982). For isolation of nonmineralized and mineralized compartments, procedures were as described by Banes et al. (1983) (see below).

Guinea pig skin was shaved, scraped free of fascia and hair in the cold, minced, and extracted as previously described (Fukae & Mechanic, 1976), but without enzyme inhibitors in the buffers. Fibrils from soluble collagen were reconstituted by dialysis of about 200 mL of collagen solution (2 mg of collagen/mL of 1% acetic acid) against three changes of 4 L of low ionic strength phosphate buffer (0.02 M, pH 7.4) over 24 h in the cold. In some experiments, an alternative procedure was used to reconstitute fibrils. Collagen solution (as above) was dialyzed against three changes of 4 L of 0.02 M sodium acetate buffer containing 0.15 M NaCl, pH 5.6, at 4 °C over 24 h and then against the same volume of 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.4, at 4 °C over 24 h, followed by incubation at 37 °C for 24 h.

Trypsin Digestion/Isolation of Mineralized and Nonmineralized Bone Collagen. Bone powder was suspended in 0.05 M NH_4HCO_3, pH 7.4, containing 0.01 M cysteine to irreversibly inactivate collagenase, and then washed three times and resuspended in bicarbonate buffer to remove cysteine. The material was then digested with 0.2 mg of TPCK trypsin/100 mg of collagen (collagen weight was roughly estimated as half the weight of the powder) for 4 h at 37 °C to remove noncollagenous protein. (Trypsin was stored in the freezer in a 10 mg/mL solution of 0.01 M HCl and added to bone powder suspensions in appropriate aliquots.) The digests were then washed four times with bicarbonate buffer to remove trypsin.

Nonmineralized collagen in the bone powder was then denatured by heating at 65 °C for 20 min. Insoluble nonmineralized collagen is denatured under these conditions (Becker et al., 1975), while mineral protects collagen from denaturation (Bonar & Glimcher, 1970). After the suspension was cooled to 37 °C, 0.4 mg of trypsin/100 mg of collagen was added and the digest continued for 6–8 more hours. Undigested material was washed three times with bicarbonate buffer, and the supernatants were combined, lyophilized, dialyzed against cold deionized water, and relyophilized. Precipitates were demineralized as described above and washed with bicarbonate buffer.

Borohydride Reduction. [^{3}H]$NaBH_4$ was dissolved in dimethylformamide or dimethylacetamide (15 mg/mL) and stored at −20 °C. Sodium borohydride stored in this manner was stable for several months. Insoluble collagen was suspended in 0.02 M phosphate buffer, pH 7.4, and reduced with a 100-fold molar excess of tritiated sodium borohydride. Reconstituted fibrils were reduced at the reconstitution temperature and in the same buffer. Bovine bone, which was divided into mineralized and nonmineralized collagen, was reduced prior to the first denaturation step to label the nonmineralized collagen and then after trypsinization and demineralization to label the remaining collagen. After reduction, collagen was dialyzed against cold, deionized water until the dialysate was free of radioactivity and lyophilized. The specific activity of [^{3}H]$NaBH_4$ was generally about 1.0 Ci/mmol for experiments done with nonradioactive acrylonitrile and 10–20 mCi/mmol for double-label experiments.

Guanidination with 3,5-Dimethylpyrazole-1-carboxamidine Nitrate (DMPC). Lysine and hydroxylysine residues of borohydride-reduced or unreduced collagen were guanidinated with 0.4 M DMPC, pH 9.5, at 4 °C for 7 days. Collagen was then dialyzed against cold deionized water and lyophilized.

Cyanoethylation with Acrylonitrile. Nonradioactive acrylonitrile reactions were carried out a 40 °C for 24 h at pH 8.5 in 0.08 M sodium borate at various reagent concentrations. The modified collagen was dialyzed against cold deionized water and lyophilized.

[^{14}C]Acrylonitrile was synthesized as described previously (Graham & Mechanic, 1986). The specific activity of [^{14}C]acrylonitrile was typically 3–5 mCi/mmol. Reactions with radioactive acrylonitrile were carried out in a minimum volume of buffer to conserve the radioactive reagent. For insoluble collagen, 100 mg of collagen was allowed to hydrate in 600 μL of borate buffer for 48 h at 4 °C, and then 200 μL of 0.08 M [^{14}C]acrylonitrile was added and the reaction carried out for 48 h at 40 °C. Reaction vessels were 1-mL glass screw cap micro reaction vials with Teflon-lined caps. Modified collagen was then dialyzed and lyophilized as above.

Determination of [^{3}H]$NaBH_4$-Reduced Cross-Links. Labeled collagen was hydrolyzed in 6 N HCl in vacuo at 110 °C for 24 h. Hydrolysates were filtered in a medium porous fritted glass funnel. Exact aliquots of 0.4–1.0 mL of hydrolysate containing 5–25 mg of collagen were analyzed for cross-links as described by Mechanic (1974a). Cross-link peaks of interest were pooled, diluted three times with water, acidified to pH 1 with concentrated HCl, and brought to 25 mL in a volumetric flask. Exact portions of the pooled fractions (4–6 mL) were then analyzed on a second column isocratically with citrate buffer that was 0.35 M sodium ion, pH 5.28 (Mechanic, 1974b).

Determination of Carboxyethylated Cross-Links. The chromatographic procedure for separating and quantifying carboxyethylated cross-links was a modification of the procedure for secondary amine (reduced) cross-links. The same buffer gradient was used, except the salt ratio was held constant from the elution point of hydroxynorleucine for 160 min or longer to separate [^{14}C]carboxyethylated cross-links from [^{14}C]carboxyethylated single amino acids and then continued as before to elute the more basic components. Fractions from this first column were collected, pooled with 1 mL of concentrated HCl to acidify them to pH 1, and brought to 25 mL

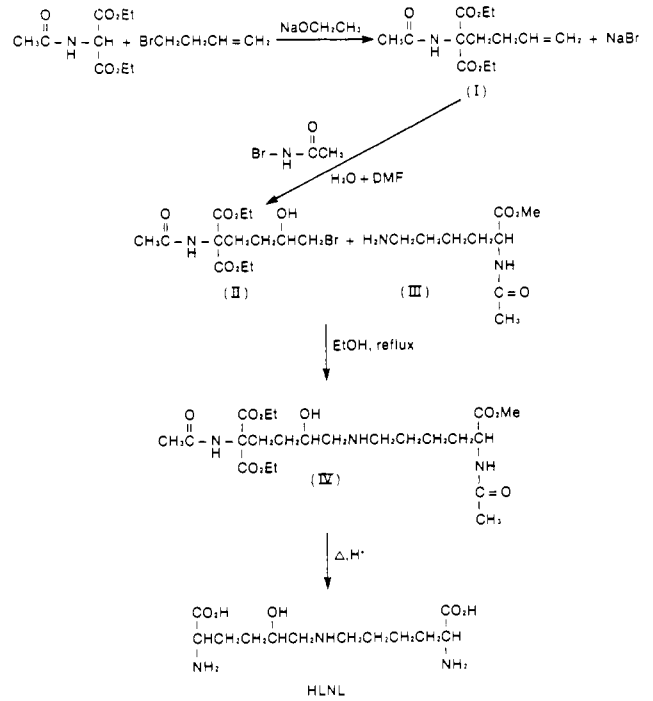

FIGURE 1: Synthesis of a derivative of HLNL that is blocked on the α-amino and α-carboxyl functions (IV). On hydrolysis, IV gives a compound that cochromatographs with HLNL by amino acid analysis.

in a volumetric flask. Exact aliquouts (4–6 mL) were then loaded onto a second column and eluted isocratically with 0.27 M sodium ion in citrate buffer at pH 3.9 Fractions of interest from the second column were transferred quantitatively to scintillation vials containing 15 mL of Aquasol, 1 mL of column eluent, and 1 mL of water and counted.

Synthesis of Standard N^{ϵ}-(Carboxyethyl)hydroxylysinonorleucine. Hexenoate (compound I, Figure 1) was synthesized from 4-bromo-1-butene and diethyl acetamidomalonate. Sodium (7.06 g) was dissolved in 50 mL of ethanol. Diethyl acetamidomalonate (69.71 g) was added and dissolved by refluxing, followed by addition of 47.52 g of 4-bromobutene. This solution was then refluxed for 18 h, giving a yellow solution, which was concentrated on a rotary evaporator, and a white precipitate, which was dissolved in 25 mL of deionized water and extracted three times with an equal volume of methylene chloride, and the combined extracts were backwashed once with 50 mL of deionized water. The concentrated yellow solution was mixed with methylene chloride and extracted in the same manner. The combined methylene chloride extracts were dried over molecular sieve, filtered, concentrated on a rotary evaporator, and dissolved in cyclohexane. On addition of petroleum ether, remaining diethyl acetamidomalonate oiled out, leaving hexenoate in solution. The hexenoate solution was concentrated and the remaining oil slowly crystallized at room temperature (Weisiger, 1950).

Bromohydrin (II) was synthesized by the reaction of *N*-bromoacetamide with hexenoate. *N*-Bromoacetamide (8.28 g) was dissolved in a solution of 32 mL of deionized water, 30 mL of dimethylformamide, and 0.6 mL of 6 N H_2SO_4. Hexenoate (13.55 g) was added and the mixture stirred for 2 h in the cold room. The reaction mixture was extracted (in the cold) three times with 50 mL of methylene chloride, and the combined extracts were washed twice with an equal volume of 5% $NaHCO_3$ and then twice with equal volumes of deionized water. The methylene chloride solution was dried over molecular sieve, filtered, and concentrated. The remaining oil was dissolved in boiling ethanol, decolorized with carbon, filtered through a medium porous fritted glass funnel, are concentrated to an oil. The oil was stored under vacuum in the cold room and was stable under these conditions for several months. *N*-Bromoacetamide gives an anti-Markovnikov addition product (Schmidt et al., 1926).

A derivative of hydroxylysinonorleucine modified at the α-amino and α-carboxyl moieties (IV) was synthesized by a substitution reaction with the bromohydrin and N-α-acetyl-L-lysine methyl ester (III). Bromohydrin (250 mg) was dissolved in 4 mL of ethanol, followed by addition of 1 g of III. This mixture was stirred for 15 min, and the 1.5 g of molecular sieve was added to remove any water. The reaction was refluxed for 6 h, cooled to room temperature, and diluted with 2 mL of deionized water. The aqueous solution was acidified to Congo Red with concentrated HCl, washed twice with equal volumes of ethyl acetate, and then loaded directly onto a P2 column.

Compound IV was separated from lower molecular weight compounds on a Bio-Gel P2 column, −400 mesh, that was 100 × 1.5 cm. The buffer was 20% acetic acid with a flow of 2 mL/h. Two-milliliter fractions were collected, and the eluent was monitored at 230 nm. Fractions were pooled, dried in a Speed Vac, hydrolyzed, and subjected to amino acid analysis. The peak containing α-modified HLNL was the first, eluting from fractions 34–44. This was further purified on a phosphocellulose column (Whatman P11) that was 8 × 1.5 cm. A linear gradient was used, consisting of 125 mL of 0.17 M HCl in the first chamber and 125 mL of deionized water in the mixing chamber. The flow rate was 80–100 mL/h, maintained by a peristaltic pump, and 4–5-mL fractions were collected. The eluent was monitored at 230 nm. Fractions were pooled, and acetic acid was added to a final concentration of 20%. Pooled fractions were dried on a Speed Vac. α-Modified HLNL was the major peak, eluting at 15–25 min.

Purified α-modified HLNL was subjected to FAB mass spectral analysis. Both proton and sodium adducts were observed, at m/e 490 and 512, respectively. A minor peak at 177 was also present. Fragmentation of the m/e 490 peak resulted in peaks consistent with losses of water, methyl formate, acetamide, and other neutral fragments from the parent peak.

Purified α-modified HLNL was cyanoethylated with acrylonitrile (Figure 2) by using the same conditions as for collagen modification (above). The cyanoethylated compound (V) was separated from buffer salts on the P2 column described above after acetic acid was added to the reaction mixture to give a final concentration of 20%. Compound V was hydrolyzed to give (carboxyethyl)hydroxylysinonorleucine.

Thin-Layer Chromatography of Amide Compounds. Formation of amide compounds in the synthesis of (carboxyethyl)hydroxylysinonorleucine was monitored by thin-layer chromatography. Silica plates were dried overnight at 110 °C. Solvent systems were 90/10 benzene/methanol for diethyl acetamidomalonate (R_f = 0.4), hexenoate (R_f = 0.6), and bromohydrin (R_f = 0.2) and 75/25 benzene/methanol for bromohydrin (R_f = 0.5) and compound IV (R_f = 0.1). Amide compounds on the dried plates were detected by application of Chlorox diluted 5:1 with water, followed after drying by 95% ethanol, followed after drying by a solution of 1% soluble starch and 1% potassium iodide.

Cross-Link Analysis of Double-Labeled Collagen. Collagen that was reduced with [^{3}H]$NaBH_4$ and modified with [^{14}C]acrylonitrile was analyzed on the system described above for carboxyethylated cross-links. The ratio of ^{3}H cpm to ^{14}C cpm was determined for carboxyethyl-HLNL and carboxy-

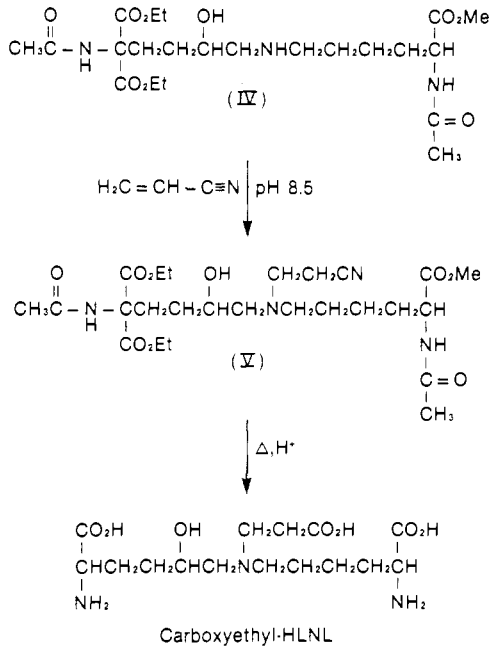

FIGURE 2: Synthesis of carboxyethyl-HLNL. Compound IV was subjected to acrylonitrile modification under the same conditions used for modification of collagen. After hydrolysis, the product cochromatographed with radioactive carboxyethyl-HLNL on the second cross-link column. The synthetic compound was detected by reaction with ninhydrin (Mechanic, 1974a).

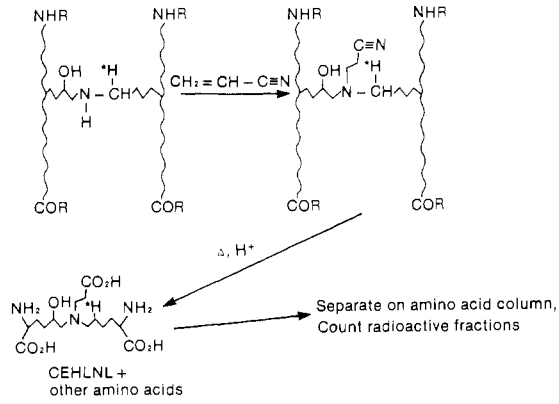

FIGURE 3: Illustration of the reaction of acrylonitrile with reduced cross-links in collagen. The peptide-bound cyanoethylated cross-link gives a labeled carboxyethylated free cross-link after acid hydrolysis.

ethyl-DHLNL from each sample; the highest counting fraction of peaks from the second column was counted in a liquid scintillation spectrometer to a counting error of less than 1% in both channels.

RESULTS

Acrylonitrile Modification of Cross-Links. The products of acrylonitrile modification of peptidyl amines are cyanoethylated amines, which are converted to carboxyethylated amines after acid hydrolysis (Riehm & Scheraga, 1966). Schiff base cross-links in collagen can be reduced with $[^3H]NaBH_4$ to give radioactively labeled secondary amines, which were used here to study acrylonitrile modification of secondary amines cross-links; the reaction with HLNL is illustrated in Figure 3. In guinea pig skin neutral salt soluble collagen, reconstituted to fibrils by incubation at 37 °C, both HLNL and HHMD were quantitatively converted to apparent derivative peaks, which eluted earlier than the cross-link peaks on the cross-link analyzer column (Figure 4). These earlier elution times are consistent with the expected structure of the derivatives, which includes an additional carboxyl group. The

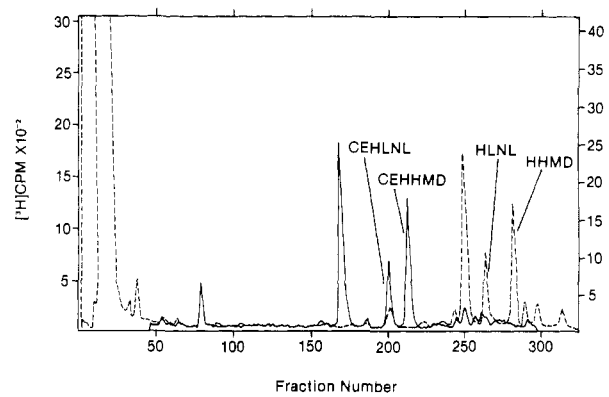

FIGURE 4: $[^3H]NaBH_4$-reduced collagen fibrils ± acrylonitrile (neutral salt soluble collagen). Fibrils were reconstituted from guinea pig skin collagen, reduced, and analyzed for cross-links with or without modification with acrylonitrile prior to hydrolysis. The dotted line shows the cross-links present in unmodified collagen. The solid line shows carboxyethylated amino acids present after hydrolysis of acrylonitrile-modified collagen. The chromatographic shift of the peaks is in the direction expected for the derivatives, which have an additional carboxyl function. The unlabeled peak in both cases is probably reduced *N*-hexosyllysine.

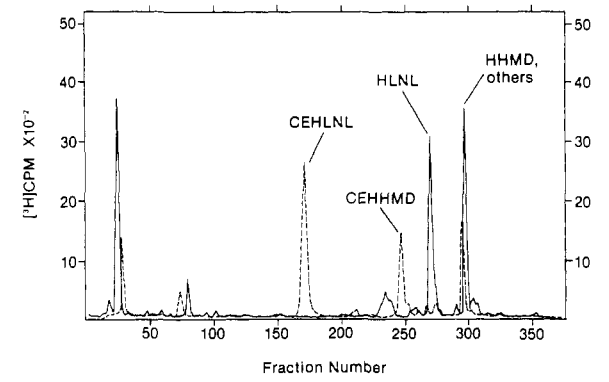

FIGURE 5: $[^3H]NaBH_4$-reduced collagen fibrils ± acrylonitrile (acetic acid soluble collagen). Fibrils were reconstituted from guinea pig skin collagen, reduced, and analyzed for cross-links with or without modification with acrylonitrile prior to hydrolysis. The solid line shows the cross-links present in unmodified collagen. The dotted line shows carboxyethylated amino acids present after hydrolysis of acrylonitrile-modified collagen. The peak containing HHMD is heterogeneous in this sample.

predictable chromatographic properties, as well as the quantitative conversion of the cross-link peaks to their apparent derivatives, strongly suggest the chemical identity of the peaks obtained after reaction of these cross-links with acrylonitrile, i.e., they are carboxyethyl derivatives of the original secondary amines. Further evidence for their structure was obtained by unequivocal synthesis of the carboxyethyl derivative of HLNL, which was found to cochromatograph with the radioactive peak from collagen on the second column of the cross-link analyzer (see Materials and Methods).

Acetic acid soluble guinea pig skin collagen, reconstituted from solution by dialysis against low ionic strength phosphate buffer at 4 °C, was also subjected to acrylonitrile modification, and HLNL was converted to the same earlier eluting peak (Figure 5). HHMD in acetic acid soluble collagen coeluted with an unidentified radioactive peak. Elution times of derivative peaks were variable, changing with slight differences of buffer composition. The derivative peaks from neutral salt soluble collagen were used as standards, and coelution with these peaks was confirmed in subsequent samples. Amino acid elution times were also routinely determined by qualitative ninhydrin reactions on column fractions to confirm radioactive

Table I: Conversion of HLNL in DMPC-Modified Collagen to CEHLNL by Reaction with Acrylonitrile[a]

	1	2	3	av ± SD
HLNL (CPM/μmol HYP) (DMPC only)	13783	14308	14885	14315 ± 536
CEHLNL (CPM)/μmol HYP) (DMPC, acrylonitrile)	14340	14450	14630	14473 ± 146

[a] Reduced reconstituted fibrils (sample shown in Figure 5) were first guanidinated with DMPC and then subjected to cross-link analysis with or without modification by acrylonitrile (three separate reactions) prior to hydrolysis. Quantitative conversion of radioactivity from the HLNL to the CEHLNL peak was observed after acrylonitrile modification. Prior guanidination had no effect on the acrylonitrile reaction with cross-links.

peak identities (Mechanic, 1974a).

Acetic acid soluble collagen was used to determine the degree of modification of secondary amines in collagen as a function of acrylonitrile concentration. The amount of HLNL remaining after the reaction was determined and normalized to the hydroxyproline content of the sample. About 14% of HLNL remained when incubated with 0.05 M acrylonitrile for 24 h at 40 °C. HLNL remaining was 5% or less for 0.2–0.4 M acrylonitrile.

Specific Modification of Lysine and Hydroxylysine. Since there are roughly 5 residues of hydroxylysine per 1000 in type I collagen, and roughly 30 residues of lysine, compared to less than 1 residue per 1000 of Schiff base cross-links, it was necessary to block these primary amine residues prior to modification of secondary amines with a radioactive reagent. The radioactive peaks from lysine and hydroxylysine would otherwise create an exceptionally difficult separation problem, as well as a high base line. Therefore, the criteria for a suitable primary amine modifying reagent are at least 90% modification of lysine and hydroxylysine, as determined by amino acid analysis; quantitative recovery of secondary amine cross-links after modification of the primary amines, as determined by cross-link analysis; and accessibility of the secondary amines to a derivatizing reagent after the primary amines have been blocked.

The reagent 3,5-dimethylpyrazole-1-carboxamidine nitrate (DMPC) has been reported to be a quantitative modifier of amines in collagen (Bethiel & Gallop, 1960). In the present study, the degree of modification of primary amines by DMPC was monitored by amino acid analysis of insolube tendon collagen after the modification reaction. Lysine and hydroxylysine were essentially eliminated, with no significant losses of other amino acids.

The specificity of DMPC for primary amines was established by modification of reduced reconstituted guinea pig skin (the sample shown in Figure 5). Cross-link analysis of this sample showed no loss or conversion of cross-links after guanidination (data not shown). The availability of cross-links for modification after guanidination was demonstrated by modifying collagen with DMPC prior to cyanoethylation of tritium-labeled HLNL with acrylonitrile. Cross-link analysis showed that HLNL in guanidinated collagen was quantitatively converted to a derivative peak by acrylonitrile (Table I), and therefore this cross-link was not derivatized in the DMPC reaction.

Quantification of Cross-Links in Bone Collagen. Total reducible cross-links in bone have been quantified (Mechanic et al., 1974) and in vivo reduction of cross-links is not apparent in total bone collagen (Robins et al., 1973; Eyre & Oguchi, 1980), but levels of reducible and in vivo reduced cross-links in isolated nonmineralized bone have not previously been

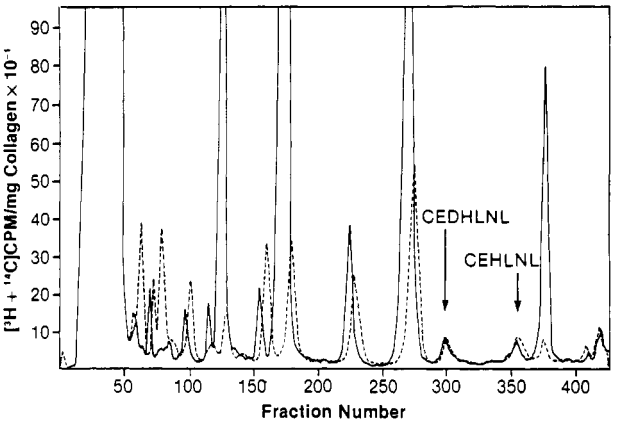

FIGURE 6: Mineralized and nonmineralized compartments of bone isolated as described under Materials and Methods and reduced with tritiated sodium borohydride, followed by modification with [^{14}C]-acrylonitrile. A comparison of total radioactive compounds in both samples is shown (solid line, mineralized; dotted line, nonmineralized). The cross-link peaks (CEDHLNL, fractions 295–310; CEHLNL, fractions 350–360) were further isolated on a second column, and the ratio of ^{3}H to ^{14}C in cross-links was determined to compare in vivo reduction. The larger peaks in the chromatogram are derivatives of lysine, hydrdoxylysine, and histidine (see text).

determined. To measure total cross-links and in vivo reduction in mineralized versus nonmineralized bone, these samples were first reduced with [^{3}H]$NaBH_4$ and then labeled with [^{14}C]-acrylonitrile, which was synthesized by a procedure described earlier (Graham & Mechanic, 1986). Cross-links existing in vivo in reducible form therefore have both tritium and ^{14}C labels, whereas those present in vivo as secondary amines would have a ^{14}C label only.

Figure 6 is an elution pattern of radioactive peaks from mineralized versus nonmineralized bovine bone collagen. The fractions were counted on a channel that excluded both ^{3}H and ^{14}C. Carboxyethyl-HLNL (at fraction 350) and carboxyethyl-DHLNL (at fraction 355) were well separated from the carboxyethylated single amino acid peaks. (Monocarboxyethyl)- and (dicarboxyethyl)lysine (eluting at fractions 370–380 and 160–180, respectively) and (monocarboxyethyl)- and (dicarboxyethyl)hydroxylysine (at fractions 260–280 and 120–130, respectively) were previously identified in acrylonitrile-modified tendon collagen (Graham & Mechanic, 1986). The major (carboxyethyl)histidine peak coelutes with (carboxyethyl)hydroxylysine. It is evident that this separation would have been impossible without prior blockage of lysine and hydroxylysine by guanidination, since the radioactive carboxyethylated lysine and hydroxylysine peaks would have been larger by at least a factor of 10.

There is no apparent difference in the cross-link derivative peaks from the two samples, although the lysine and hydroxylysine derivative peaks are greatly reduced in the nonmineralized sample. Since the nonmineralized collagen was in the form of trypsin peptides during the reaction, it may have been more efficiently modified by DMPC than the mineralized sample, which was reacted as insoluble collagen. The higher degree of modification of amine residues in insoluble collagen by acrylonitrile compared to DMPC may be attributed to the smaller size and lack of charge of acrylonitrile, which would allow it to penetrate fibrils more easily.

The cross-link derivative peaks from this first column were pooled and applied to a second column, described under Materials and Methods, and fractions from this column were counted in a liquid scintillation spectrometer to determine ratios of ^{3}H/^{14}C. The specific activities of the [^{3}H]$NaBH_4$ and [^{14}C]acrylonitrile were initially chosen to give a minimum

Table II: Cross-Links in Bone Collagen

	mineralized bovine bone		nonmineralized bovine bone		chick bone	
	HLNL	DHLNL	HLNL	DHLNL	HLNL	DHLNL
^{14}C dpm/nmol of collagen	412.5	430.2	713.2	616.7	793.4	1926.8
$^{3}H/^{14}C$ cpm ratio	2.30	2.06	2.11	2.19	2.20	2.15

of about 1000 cpm in both ^{3}H and ^{14}C channels to keep the error due to base-line variation in the column fractions below 1%. Specific activities were typically 3–5 mCi/mmol for [^{14}C]acrylonitrile and 10–20 mCi/mmol for [^{3}H]$NaBH_4$.

From ^{3}H and ^{14}C cpm values, the percent in vivo reduction could be calculated if the specific activities for both radioactive reagents are known; however, this procedure would involve taking the ratio of measured specific activities, and assuming (optimistically) that each has an error of about 5%, the combined error would be 10%. A more direct method would be to use the $^{3}H/^{14}C$ cpm ratio as an index of in vivo reduction, which can be compared to the ratio for a parallel reaction on reconstituted fibrils or other collagen samples that are known to have no significant amount of in vivo reduction. The error for this method was estimated from the result of three parallel [^{14}C]acrylonitrile reactions on a sample of [^{3}H]$NaBH_4$-reduced reconstituted fibrils, which gave $^{3}H/^{14}C$ ratios with a standard deviation of less than 3% of the mean (3.45 ± 0.10). The cpm ratio method also obviates problems with variable counting efficiencies for tritium in different scintillation mixtures.

In Table II, the total cross-links in mineralized and nonmineralized adult bovine bone are indicated by the ^{14}C cpm values, normalized to nanomoles of collagen in the sample by assuming 300 nmol of hydroxyproline/nmol of collagen (100 residues/1000). As a consistency check, DHLNL and HLNL were also determined in chick bone by radioactive acrylonitrile modification, and their relative abundance (DHLNL/HLNL) was found to be comparable to previous results obtained by borohydride reduction (Mechanic et al., 1972, 1975). The amounts of HLNL and DHLNL in mineralized and nonmineralized bone collagen were similar, in contrast to the result for pyridinoline (Mechanic et al., 1985; Banes et al., 1983). The cpm ratio, and therefore the in vivo reduction, was not significantly different for any of the samples. This result rules out the possibility of elevated levels of in vivo reduction in nonmineralized bone collagen.

Discussion

To demonstrate the validity of secondary amine cross-link analysis, we have shown that (1) primary amines can be premodified with DMPC, making them unreactive with the radioactive labeling reagent [^{14}C]acrylonitrile; (2) secondary amines are unaffected by the first reaction and quantitatively modified by the second reaction; and (3) secondary amine residues in a protein react to give identifiable derivatives with the expected structures. The kinetics of the DMPC reaction with primary and secondary amines generally will be the subject of a future study.

Potential applications of secondary amine cross-link analysis include isolation of peptides containing lysinoalanine, merodesmosine, or lysinonorleucine. To date, no sequence locations have been assigned for these cross-links. Radioactive labeling of secondary amines in peptide form will not only facilitate isolation of cross-linked peptides for sequencing, but it will also confirm that the secondary amines exist as such in vivo, rather than as breakdown products generated from more complex structures by acid hydrolysis. This method can also be applied more generally to specifically label histidine residues in other proteins (collagen and elastin contain only around 1–6 residues of histidine per 1000). Finally, the determination of comparative in vivo reduction shows that Schiff base and secondary amine cross-link analysis can be applied simultaneously to the same collagen sample. This procedure is also applicable to determination of percent reduction in vivo of lysinonorleucine and merodesmosine in elastin. Collagen was chosen to demonstrate the general applicability of the method since collagen contains hydroxylysine, and about three times as many lysine residues as elastin, and therefore represents a more difficult technical problem. Results obtained by secondary amine analysis are consistent with results obtained by standard methodology (borohydride reduction only); the relative abundance of HLNL and DHLNL in chick bone determined by acrylonitrile modification is comparable to that found in previous studies (Mechanic et al., 1972, 1975).

Similar levels of HLNL and DHLNL were found in the mineralized and nonmineralized compartments of bone. This is a somewhat unexpected result, since nonmineralized bone contains much higher levels of pyridinoline than mineralized bone (Banes et al., 1983; Mechanic et al., 1985), and pyridinoline is thought to be derived from dehydro-DHLNL (Fujimoto & Moriguchi, 1978; Eyre, 1981). It has been suggested that mineralization blocks conversion of dehydro-DHLNL to pyridinoline (Eyre, 1981). In nonmineralized bone, some other mechanism is evidently operating to maintain levels of reducible cross-links.

At the present time, the only known source of reducible cross-links is the spontaneous reaction of lysyl oxidase derived aldehydes with lysine or hydroxylysine, although it is conceivable that some percentage of the reducible cross-link population represents breakdown products from more complex unidentified structures. The only mechanisms for loss of reducible cross-links from bone for which there is evidence at present are turnover and hydroxypyridinium formation. [Cross-linking pathways are different in skin and skeletal tissues; for example, the nonreducible cross-link HHL, found only in skin, is apparently derived from dehydro-HLNL (Mechanic et al., 1987; Yamauchi et al., 1987)]. The finding that the level of in vivo reduction of cross-links in nonmineralized bone is not different from that of mineralized bone shows that this process is not acting as an additional "sink" for reducible cross-links in the nonmineralized compartment.

Acknowledgments

We are indebted to M. Olivier Monbureau for his ingenious design of programmer/controllers for the cross-link analyzer.

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