Biochemical Characterization of Guanidinium Chloride-Soluble Dentine Collagen from Lathyrctic-Rat Incisors

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α - and β -Chains were isolated by sequential ion-exchange and gel-filtration chromatography of guanidinium chloride-soluble dentine collagen obtained from Tris/NaCl-extracted EDTA-demineralized lathyrctic-rat incisors. The α -chains were identified as α_1 I and α_2 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and amino acid analysis of the intact chains and their CNBr peptides. The dentine α -chains exhibited higher lysine hydroxylation and phosphate content, but lower hydroxylysine glycosylation, than α -chains from skin. Increased lysine hydroxylation was observed in the helical sequences. The α_1 I/ α_2 ratio was approx. 3:1, and was presumably due to the presence of $(\alpha_1$ I)₃ molecules along with $(\alpha_1$ I)₂ α_2 molecules as shown recently for neutral-salt-soluble dentine collagen [Wohllebe & Carmichael (1978) *Eur. J. Biochem.* **92**, 183–188]. In the borohydride-reduced β_{11} - and β_{12} -chains from guanidinium chloride-soluble dentine collagen, the reduced cross-links hydroxylysino-hydroxy-norleucine and hydroxylysino-norleucine were present. A higher proportion of hydroxylysino-norleucine in the reduced β_{12} -chain probably reflects differences in extent of hydroxylation of specific lysine residues of the α_1 I- and α_2 -chains.

The major component of the organic matrix of dentine is collagen. The insolubility of dentine collagen, attributed to a high degree of intermolecular cross-linking and tight mechanical weave (Veis & Schleuter, 1964), has presented formidable difficulties for the biochemical characterization of this protein. It has been possible to characterize the genetic species of collagen in rat (Butler & DeSteno, 1971) and bovine (Volpin & Veis, 1973; Scott & Veis, 1976) dentine by cleavage by CNBr of insoluble dentine. These studies showed dentine collagen to be essentially type I, albeit with unique post-translational modifications, notably covalent attachment of phosphate groups and increased lysine hydroxylation and intermolecular cross-linking, distinguishing it from the type I collagen of skin.

Aside from their role as structural components of teeth and bones, the collagen molecules of these tissues may be actively involved as heterogeneous catalysts for nucleation and deposition of hydroxyapatite (Glimcher & Krane, 1968; Neuman & Neuman, 1958). Previous studies on collagen-mineral interactions *in vitro* have been carried out with purified collagens from soft tissues that do not normally calcify, or with insoluble-collagen preparations from bone and dentine. More recent work (Veis *et al.*, 1977) on the non-collagenous proteins associated with bone and dentine collagens indicates that these proteins are most likely involved in the

mineralization of these tissues. Although most of these proteins are removed from collagen during or after demineralization of bones and teeth, there appears to be a fraction that is only released after collagen degradation. Hence the assessment of a role for collagen in mineralization of bone and dentine requires that a highly purified soluble hard-tissue collagen preparation be available for studies of collagen-mineral interactions.

Experimental lathyrism (for a review see Tanzer, 1965), which results in enhancement of tissue-collagen solubility by inhibition of cross-linking, has been used successfully to obtain soluble collagens from chick bone (Miller *et al.*, 1967), chick cartilage (Miller, 1971) and rat bone (Stolz *et al.*, 1973). Previous work (Carmichael *et al.*, 1974) demonstrated the partial solubilization of dentine collagen by guanidinium chloride extraction of acetic acid-demineralized lathyrctic-rat incisors. In subsequent work we carried out demineralization of the lathyrctic-rat incisors with EDTA at neutral pH to minimize the proteolytic cleavage of non-helical extensions from the collagen chains shown to occur under acidic conditions (Rauterberg, 1973). Extraction of the EDTA-demineralized matrix with Tris-buffered NaCl yielded an undenatured dentine collagen fraction, which we have partially characterized (Wohllebe & Carmichael, 1978). In the present paper we report the isolation and characterization

of the guanidinium chloride-soluble collagen obtained from the Tris/NaCl-extraction residue, as well as further characterization of the undenatured neutral-salt-soluble collagen, of lathyritic-rat incisors.

Experimental

Materials

The following chemicals were obtained from the indicated commercial sources: Tris (Trizma) and pepsin (twice-recrystallized) from Sigma Chemical Co., St. Louis, MO, U.S.A.; guanidinium chloride (Sequal grade) from Pierce Chemical Company, Rockford, IL, U.S.A.; dithiothreitol from Calbiochem, LaJolla, CA, U.S.A.; DEAE-cellulose (Whatman DE-52) and CM-cellulose (Whatman CM-52) from Mandel Scientific Co., Ville St. Pierre, Que., Canada; Bio-Gel A1.5m (200–400 mesh) and Bio-Gel P-2 (100–200 mesh) from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ont., Canada; CNBr from Eastman-Kodak Co., Rochester, NY, U.S.A. All other chemicals used were of the highest analytical grade available from local chemical-supply houses.

Acid-soluble rat skin collagen was prepared by the method of Piez *et al.* (1963). EDTA-demineralized rat incisor dentine matrix was prepared as previously described (Wohllebe & Carmichael, 1978).

Methods

Soluble dentine collagen preparations. EDTA-demineralized rat incisor dentine matrix was sequentially extracted at 4°C with (a) 0.05M-Tris/HCl, pH 7.4, containing 1M-NaCl, for 3×24h, and (b) 0.05M-Tris/HCl, pH 7.4, containing 5M-guanidinium chloride and 1mM-dithiothreitol, for 1 week. Extraction residues and pooled extracts were dialysed against water to remove buffer salts, freeze-dried and weighed. The non-diffusible collagenous materials solubilized by Tris-NaCl and Tris/guanidinium chloride were freed from non-collagenous acidic macromolecules by DEAE-cellulose chromatography (Miller, 1971), and are subsequently referred to as neutral-salt-soluble dentine and guanidinium chloride-soluble dentine respectively.

Limited pepsin digestion. To determine the extent of denaturation of the soluble collagens, both neutral-salt- and guanidinium chloride-soluble dentines were subjected to limited proteolysis by pepsin by the method of Layman *et al.* (1971), with minor modifications (Wohllebe & Carmichael, 1978), and examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Isolation of collagen chains from soluble dentines. Electrophoretically homogeneous α - and β -chains were obtained from neutral-salt- and guanidinium chloride-soluble dentines by sequential ion-exchange

and gel-filtration chromatography (Wohllebe & Carmichael, 1978).

CNBr cleavage. Collagenous samples were stirred for 4h at room temperature (23°C) in stoppered N₂-flushed flasks with deaerated 70% (w/w) formic acid containing CNBr (13mg/ml) and dithiothreitol (1.54mg/ml), at a ratio of 2mg of collagen to 1ml of the formic acid solution of CNBr. Reaction was stopped by 15-fold dilution of the reaction mixture with water, followed by freeze-drying. The freeze-dried digests were dissolved in 0.15M-acetic acid, filtered, and desalted on a Bio-Gel P-2 column (2.6cm×45cm), and freeze-dried.

Isolation of CNBr peptides. Desalted CNBr peptides were chromatographed on CM-cellulose under conditions indicated in the legend to Fig. 4. Individual CM-cellulose fractions were desalted on a Bio-Gel P-2 column (2.6cm×60cm) in 0.15M-acetic acid, freeze-dried and rechromatographed on a Bio-Gel A1.5m column (2.6cm×106cm) in 0.05M-Tris/HCl, pH 7.4, containing 2M-guanidinium chloride. Bio-Gel A1.5m column fractions were desalted on Bio-Gel P-2 and freeze-dried.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. For collagen chains, electrophoresis was carried out on 5% (w/v) acrylamide gels (Furthmayr & Timpl, 1971). For CNBr peptides, electrophoresis was carried out on 7.5% (w/v) acrylamide gels by the method of Scott & Veis (1976). In both cases, gels were stained with Coomassie Blue, destained with 10% (v/v) acetic acid and scanned at 560nm.

Amino acid analysis. Samples were hydrolysed in 6M-HCl for 20h at 108°C in N₂-flushed evacuated tubes and analysed by a single-column method on a JEOL JLC-5AH amino acid analyser.

Hydroxylysine glycoside analysis. Samples were hydrolysed in 2M-NaOH in Teflon screw-capped bottles under N₂ for 24h at 105°C. After acidification with dilute HCl, the hydrolysates were immediately chromatographed on a Jeol LC-R-2 column (0.8cm×45cm). Elution was carried out at a flow rate of 0.8ml/min with 0.033M-sodium citrate buffer, pH 4.95, for 150min to elute glucosylgalactosylhydroxylysine (retention time 101min), and with 0.266M-sodium citrate buffer, pH 4.5, for 100min to elute galactosylhydroxylysine (retention time 178min). The initial column temperature was 47°C, and 110min after the start of the chromatographic run, the column temperature was 70°C.

Reduced-cross-links analysis. Samples were reduced with calibrated (Paz *et al.*, 1970) NaB³H₄, hydrolysed in acid and analysed for reduced cross-links (Carmichael *et al.*, 1975).

Phosphate analysis. Samples were hydrolysed in 6M-HCl as described under 'Amino acid analysis', evaporated to dryness *in vacuo*, and analysed for phosphate by the method of Ames (1966).

Results and Discussion

Extraction of soluble dentine collagens

The solubilization and recovery results are summarized in Table 1. The difference in the amounts of material solubilized from lathyritic and normal dentine matrices is almost entirely due to the presence of soluble collagen in the lathyritic matrix. The non-diffusible Tris/NaCl and Tris/guanidinium chloride extracts were passed through DEAE-cellulose (Miller, 1971) to remove residual non-collagenous acidic macromolecules not extracted by EDTA, yielding neutral-salt-soluble dentine and guanidinium chloride-soluble dentine respectively.

The total amount of soluble collagen obtained from EDTA-demineralized lathyritic-rat incisors was

significantly greater than that previously obtained by Carmichael *et al.* (1974) from acetic acid-demineralized lathyritic-rat incisors. The removal of associated non-collagenous proteins during EDTA demineralization probably rendered the collagen more accessible to the extraction solvents. Indeed, when acetic acid-demineralized lathyritic-rat incisors were first extracted with Tris/NaCl to remove phosphoproteins (Butler *et al.*, 1972) before guanidinium chloride extraction, the amount of soluble collagen subsequently recovered was increased (M. Wohlbe & D. J. Carmichael, unpublished work) to almost the same concentrations as that obtained from EDTA-demineralized material. It thus appears that interactions between collagen and associated non-collagenous macromolecules of den-

Table 1. Solubilization and recovery results for lathyritic and normal EDTA-demineralized rat incisor dentine matrices. The recoveries were obtained by difference between the freeze-dried weights of the EDTA-demineralized rat dentine matrices before and after extraction with each solvent.

Rat	Recovery (% of EDTA-demineralized matrix)				
	Tris/NaCl-soluble		Tris/guanidinium chloride-soluble		Insoluble remainder
	Total	Non-diffusible	Total	Non-diffusible	
Lathyritic	20.3	6.2	35.0	33.8	44.7
Normal	10.6	—	3.7	1.0	84.7

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of soluble dentine collagens before and after limited pepsin digestion

Electrophoresis was carried out by the method of Furthmayr & Timpl (1971). Migration of proteins was from the negative to the positive electrode. (a) Neutral-salt-soluble dentine; (b) pepsin digest of neutral-salt-soluble dentine; (c) guanidinium chloride-soluble dentine; (d) pepsin digest of guanidinium chloride-soluble dentine. The positions of the various chains are indicated above the scans.

tine render even poorly cross-linked dentine collagen from lathyritic rat less susceptible to solubilization.

Subunit composition of soluble dentine collagens

The sodium dodecyl sulphate/polyacrylamide-gel patterns of neutral-salt-soluble and guanidinium chloride-soluble dentines, both before and after limited pepsin digestion, are shown in Fig. 1. Both soluble dentines were predominantly made up of collagen α -chains. However, a greater proportion of β - and γ -chains and higher aggregates was present in guanidinium chloride-soluble dentine than in neutral-salt-soluble dentine. After limited pepsin digestion, the electrophoretic pattern was essentially unchanged for neutral-salt-soluble dentine, indicating that it was predominantly undenatured collagen. In contrast, guanidinium chloride-soluble dentine was almost completely degraded into fragments smaller than α -chains, and no material exhibiting mobilities lower than α -chains was observed after pepsin digestion. Electrophoretic banding patterns were unchanged under reducing conditions, indicating the absence of disulphide-linked collagen and procollagen chains of type III or IV, or of disulphide-linked procollagen type I.

Isolation of dentine collagen chains

Both neutral-salt-soluble and guanidinium chloride-soluble dentines were initially chromatographed on DEAE-cellulose to remove any procollagens (Byers *et al.*, 1974). The bulk of the neutral-salt-soluble and guanidinium chloride-soluble dentines were recovered as collagen chains in the unretarded fraction. The unretarded fractions were then chromatographed on CM-cellulose under denaturing conditions (Miller, 1971). A comparison of the CM-cellulose elution patterns (Fig. 2) of neutral-salt-soluble dentine collagen and guanidinium chloride-soluble dentine collagen with that of acid-soluble rat skin collagen showed that the α_1 - to α_2 -chain ratio, calculated from the total α - and β -chain content of the soluble collagens, was higher for the dentine collagens than for skin collagen. Individual fractions obtained from each soluble dentine collagen by CM-cellulose chromatography were rechromatographed on Bio-Gel A1.5m (Piez, 1968) to yield individual α - and β -chains for further characterization.

Amino acid composition and hydroxylysine glycoside content of dentine α -chains

The amino acid compositions of the isolated dentine α -chains are shown in Table 2, in comparison with those of the corresponding α -chains from rat bone and rat skin (Stolz *et al.*, 1973). There was no significant difference between the neutral-salt-soluble and guanidinium chloride-soluble dentine collagen

Fig. 2. CM-cellulose chromatography of soluble dentine collagens

Each of the soluble dentine collagens, as well as acid-soluble rat skin collagen, was passed through a DEAE-cellulose column to remove any procollagens. The unretarded material was then chromatographed on a CM-cellulose column (1.6cm $\times$ 12cm) at 45°C. Elution was carried out with 0.03M-acetate buffer, pH4.8, containing 1M-urea, superimposed with a linear NaCl gradient from 0 to 0.12M-NaCl over a total volume of 400ml at a flow rate of 80ml/h. Horizontal bars indicate fractions pooled for further purification by gel-filtration chromatography. (a) Acid-soluble rat skin collagen; (b) neutral-salt-soluble dentine; (c) guanidinium chloride-soluble dentine.

α -chains. Although the methionine values were low for the dentine α -chains, these chains gave CNBr peptides characteristic of α_1 I- and α_2 -chains, as discussed in detail below. The low values for methionine probably arose from some oxidation of methionine to methionine sulfoxide and methionine sulphone during acid hydrolysis. In our analytical system, methionine sulfoxide is detected as a discrete peak, but methionine sulphone is not,

Table 2. Amino acid compositions of collagen α -chains from lathyritic-rat dentine in comparison with the corresponding chains from lathyritic-rat bone and rat skin

Methionine values for the dentine collagen α -chains are approximate, as explained in the text. Abbreviations used: NSS, neutral-salt-soluble; GS, guanidinium chloride-soluble.

	Amino acid content (residues/1000 residues)							
	α_1 I-chain				α_2 -chain			
	NSSrat dentine	GSrat dentine	Rat bone*	Rat skin*	NSSrat dentine	GSrat dentine	Rat bone*	Rat skin*
Hyp	100	97	97	94	79	84	83	85
Asp	57	57	46	46	53	45	44	44
Thr	21	21	21	21	21	21	21	21
Ser	43	45	41	44	45	40	40	43
Glu	79	78	81	78	72	77	74	74
Pro	125	125	120	128	108	114	103	103
Gly	313	310	323	326	330	338	332	331
Ala	111	110	108	103	99	99	103	100
$\frac{1}{2}$ Cys	—	—	—	—	—	—	—	—
Val	19	18	20	20	31	28	31	32
Met	3.1	4.1	6.6	7.7	1.2	2.0	2.8	3.8
Ile	6.6	6.6	6.6	6.8	19	18	19	20
Leu	20	20	21	21	36	34	35	35
Phe	14	14	14	14	13	12	12	12
Tyr	2.6	3.8	3.8	2.9	3.0	2.4	3.8	3.8
Hyl	16.1	17.6	14.2	4.8	14.0	15.8	13.2	7.6
Lys	18	19	22.7	31.8	17.0	16.1	19.9	26.0
His	1.9	1.9	1.8	1.9	8.5	7.2	9.4	9.5
Arg	51	51	52	51	50	46	52	50

* From the results of Stolz *et al.* (1973).

because it co-elutes with aspartic acid. Any sulphone present would therefore be masked by and included in the aspartic acid peak.

Rat dentine α -chains, like rat bone α -chains, exhibited higher lysine hydroxylation than rat skin α -chains. This suggests a role for lysine hydroxylation in cross-linking and mineralization. Thus the extent of lysine hydroxylation of the soluble collagen chains appears to correlate well with the amounts of stable hydroxylysine-derived reducible cross-links (Mechanic *et al.*, 1971) and the degree of insolubility of intact dentine, bone and skin. Bone and dentine collagens both mineralize *in vivo*, and they exhibit similar amounts of lysine hydroxylation. A correlation between the increased hydroxylysine content of bone collagen and its increased affinity *in vitro* for hydroxyapatite over skin collagen (Nawrot *et al.*, 1976; Nawrot & Campbell, 1977) has been suggested. It is plausible that groups responsible for enhanced binding to hydroxyapatite *in vitro* may also be involved in mineral binding *in vivo* by participating in specific arrays of reactive groups that act as mineral-nucleation sites.

The hydroxylysine glycoside contents of rat dentine α -chains are compared in Table 3 with those of rat bone and rat skin α_1 I-chains. The increased hydroxylation of lysine of the rat dentine and rat bone α_1 I-chains was not accompanied by a

Table 3. Hydroxylysine glycoside content of α -chains from lathyritic-rat dentine, bone and skin

	Glc-Gal-Hyl (units/1000 amino acid residues)	Gal-Hyl (units/1000 amino acid residues)	Glycosylation (%)
Rat dentine			
α_1 I-chain	0.76	0.54	8.2†
α_2 -chain	0.82	0.93	12.5‡
Rat bone*			
α_1 I-chain	2.7	1.3	27
Rat skin*			
α_1 I-chain	2.1	0.7	58

* Data for rat bone and rat skin α_1 I-chains are from Stolz *et al.* (1973).

† Based on a total hydroxylysine content of 16.1 residues/1000 amino acid residues for the rat dentine α_1 I-chain.

‡ Based on a total hydroxylysine content of 14.0 residues/1000 amino acid residues for the rat dentine α_2 -chain.

corresponding increase in glycosylation of hydroxylysine. Thus, whereas lysine hydroxylation increased in the order skin < bone < dentine, hydroxylysine glycosylation decreased in the order skin > bone > dentine. Rat dentine α_1 I-chains were less glycosylated than rat dentine α_2 -chains. Volpin & Veis (1973) have shown that bovine dentine α_2 -chains exhibited

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the CNBr peptides of the α -chains from rat dentine and rat skin

Electrophoresis was carried out by the method of Scott & Veis (1976). Migration of peptides was from the negative to the positive electrode. Numbers above peaks represent known CNBr peptides. CNBr peptides are from rat skin α_1 I-chains (a), rat skin α_2 -chains (b), neutral-salt-soluble dentine α_1 I-chains (c), neutral-salt-soluble dentine α_2 -chains (d), guanidinium chloride-soluble dentine α_1 I-chains (e) and guanidinium chloride-soluble dentine α_2 -chains (f).

higher hydroxylysine glycosylation than bovine dentine α_1 I-chains. The role of hydroxylysine glycosides is not clear, although they have been suggested to play a role in collagen fibre morphology (Morgan *et al.*, 1970) and cross-link regulation (Robins & Bailey, 1974). It has been suggested that calcification may be regulated by the nature of the hydroxylysine glycosides in bone collagen, the diglycosylated

hydroxylysine perhaps preventing mineral deposition due to steric hindrance (Toole *et al.*, 1972), but Royce & Barnes (1977) have shown that there is no correlation between the nature of hydroxylysine glycosides and mineralization in chick bone. Nevertheless it is plausible that hydroxylysine glycosides may influence mineralization of dentine collagen by its possible involvement as the site of covalent

attachment of a non-collagenous phosphoprotein moiety to dentine collagen (Carmichael *et al.*, 1971), since these non-collagenous phosphoproteins associated with dentine collagen have been suggested to be actively involved in the mineralization process (Veis *et al.*, 1977).

CNBr peptides of soluble dentines

The CNBr-peptide patterns of the dentine α -chains after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis are compared with those of acid-soluble rat skin α -chain CNBr peptides in Fig. 3. It is clear that the soluble dentine α -chains exhibited peptide patterns identical with those of rat skin α_1 I- and α_2 -chains.

The large CNBr peptides were isolated from guanidinium chloride-soluble dentine. CM-cellulose chromatography (Fig. 4) yielded peptide fractions that were further chromatographed on Bio-Gel A1.5m to give electrophoretically homogeneous peptides α_1 I CB3, α_1 I CB6, α_1 I CB7, α_1 I CB8 and α_2 CB4. Electrophoretically homogeneous peptide α_2 CB3.5 was more easily obtained by Bio-Gel A1.5m chromatography of the first major peak initially obtained by Bio-Gel A1.5m chromatography of the guanidinium chloride-soluble dentine CNBr digest.

The amino acid compositions of the rat dentine

α_1 I-chain CNBr peptides are compared in Table 4 with those of the corresponding peptides from rat bone (Stolz *et al.*, 1973). The composition of the corresponding rat or calf skin peptides obtained from sequence data (Fietzek & Kühn, 1976) is also shown for further comparison. The dentine peptides α_1 I CB3, α_1 I CB7 and α_1 I CB8 all showed higher amounts of lysine hydroxylation compared with skin peptides, and similar lysine hydroxylation compared with the bone peptides.

The amino acid composition of rat dentine peptides α_2 CB4 and α_2 CB3.5 are compared in Table 5 with the corresponding peptides from rat skin and bovine dentine (Volpin & Veis, 1973). The greater lysine hydroxylation of the dentine peptides compared with the skin peptides was also observed for both peptides α_2 CB4 and α_2 CB3.5.

The increased lysine hydroxylation of the peptides from helical sequences is significant because of the potential involvement of such regions in the formation of intermolecular cross-links (Zimmermann *et al.*, 1973). Thus the increase in stable hydroxyllysine-derived reducible cross-links, which is reflected in a decrease of solubility of bone and dentine over skin, may be due to the availability of the hydroxyllysines in the helical sequence for additional intermolecular cross-linking.

Reduced cross-link content of β -chains

β_{11} - and β_{12} -chains obtained from guanidinium chloride-soluble dentine showed the presence of both hydroxylysino-hydroxynorleucine and hydroxylysino-norleucine after borohydride reduction. Hydroxylysino-hydroxynorleucine was the predominant cross-link in both β -chains, and although the sum of hydroxylysino-hydroxynorleucine and hydroxylysino-norleucine was the same for both, the hydroxylysino-hydroxynorleucine/hydroxylysino-norleucine ratios were slightly different, being 5.52 and 3.02 for β_{11} - and β_{12} -chains respectively. This probably reflects differences in the extent of lysine hydroxylation at specific loci in the α_1 I- and α_2 -chains.

Phosphate content

Table 6 shows the phosphate content of the α -chains from rat dentine, bone and skin. These results for rat dentine α -chains are similar to those reported by Volpin & Veis (1973) for bovine dentine. The manner in which the phosphate groups are attached to dentine collagen is not at present known. Volpin & Veis (1973) suggested that in bovine dentine the phosphate groups may be present as hexose phosphate, since they detected phosphate only in those CNBr peptides that contained hexose as well. The results for rat bone α -chains are in close agreement with those reported by Miller & Martin (1968)

Fig. 4. CM-cellulose chromatography of the CNBr digest of guanidinium chloride-soluble dentine

Guanidinium chloride-soluble dentine was digested with CNBr in 70% (w/w) formic acid. The digest was freeze-dried, dissolved in 0.15M-acetic acid and filtered. The 0.15M-acetic acid-soluble CNBr peptides were chromatographed at 42°C on a CM-cellulose column (1.6cm $\times$ 12cm). Elution was carried out with 0.02M-citrate buffer, pH 3.6, superimposed with a linear NaCl gradient from 0.01 to 0.16M-NaCl over a total volume of 1000ml at a flow rate of 100ml/h. Horizontal bars represent fractions pooled for further purification by gel-filtration chromatography. Peaks 1, 2, 3, 4 and 5 yielded peptides α_1 I CB3, α_1 I CB6, α_1 I CB7, α_1 I CB8 and α_2 CB4 respectively.

Table 4. Amino acid compositions of rat dentine collagen $\alpha_1(I)$ CNBr peptides in comparison with homologous peptides from rat bone and calf or rat skin

Source	Peptide ...	Amino acid content (residues/peptide)											
		$\alpha_1(I)$ CB3			$\alpha_1(I)$ CB6			$\alpha_1(I)$ CB7			$\alpha_1(I)$ CB8		
		Rat dentine	Rat bone*	Rat skin†	Rat dentine	Rat bone*	Calf skin†	Rat dentine	Rat bone*	Calf skin†	Rat dentine	Rat bone*	Rat skin†
Hyp	15	15	15	17	18	19	21	25	25	28	26	30	32
Asp	7	7	7	7	10	11	10	10	12	12	9	12	11
Thr	2	2	2	2	4	4	4	7	7	5	5	5	5
Ser	3	3	3	3	10	12	12	8	9	7	12	11	11
Glu	16	16	16	16	15	16	16	17	18	16	20	22	19
Pro	14	14	14	12	33	30	31	38	35	35	32	32	30
Gly	52	50	50	50	74	69	68	92	89	89	96	91	94
Ala	19	20	20	20	19	21	19	29	31	35	38	39	38
†Cys	—	—	—	—	—	2	—	—	7	6	5	6	5
Val	4	4	4	4	2	2	2	8	—	—	—	—	—
Met	—	—	—	—	—	—	—	—	—	—	—	—	—
Ile	1	3	3	1	3	3	3	3	3	3	2	1	1
Leu	3	3	3	3	5	4	6	4	4	4	4	4	4
Phe	3	3	3	3	3	3	2	3	3	3	3	3	3
Tyr	—	—	—	—	1	2	3	—	—	—	—	—	—
Hyl	1	1	1	—	3	3	2	5	5	1	4	3	—
Lys	4	4	4	5	4	4	4	5	6	10	6	7	10
His	—	—	—	—	1	1	2	—	—	—	—	—	—
Arg	6	6	6	6	12	12	12	13	13	13	16	15	15
Homoserine	1	1	1	1	—	—	—	1	1	1	1	1	1
Total	151	152	150	150	217	216	217	268	268	268	279	282	279

* From the results of Stolz *et al.* (1973).
† From the results of Fietzek & Kühn (1976).

Table 5. Amino acid compositions of rat dentine collagen α_2 CNBr peptides in comparison with homologous peptides from rat skin and bovine dentine

Peptide ...	Amino acid content (residues/peptide)					
	α_2 CB4			α_2 CB3.5		
	Rat dentine	Rat skin	Bovine dentine*	Rat dentine	Rat skin	Bovine dentine*
Hyp	30	29	30	42	44	50
Asp	9	13	14	29	30	33
Thr	6	7	6	10	10	11
Ser	11	12	9	25	22	22
Glu	23	21	22	48	46	44
Pro	32	33	39	74	72	71
Gly	114	111	109	212	218	210
Ala	36	33	37	64	63	64
$\frac{1}{2}$ Cys	—	—	—	—	—	—
Val	11	9	10	18	16	17
Met	—	—	—	1	1	1
Ile	4	6	4	12	12	9
Leu	11	11	9	19	19	18
Phe	4	3	4	7	5	7
Tyr	1	—	—	1	—	2
Hyl	5	3	4	8	4	7
Lys	5	7	6	10	12	12
His	2	3	2	6	5	6
Arg	17	17	17	33	36	34
Homoserine	1	1	1	—	—	—
Total	322	319	323	619	615	618

* From the results of Volpin & Veis (1973).

Table 6. Phosphate content of α -chains from lathyritic-rat dentine, bone and skin

	Phosphate (groups/1000 amino acid residues)
Rat dentine	
α_1 I-chain	0.33
α_2 -chain	2.78
Rat bone	
α_1 I-chain	0.16
α_2 -chain	0.40
Rat skin	
α_1 I-chain	0.06
α_2 -chain	0.06

for lathyritic-chick bone α -chains. However, much higher values for the phosphate content of chick bone collagen α_2 -chains have been reported by Francois *et al.* (1967) and Cohen-Salal & Glimcher (1978), who found 1.7 and 3.4 mol of phosphate per α_2 chain respectively. Cohen-Salal & Glimcher (1978) tentatively identified the organic phosphate moiety of chick bone α_2 -chain as glutamyl phosphate, an extremely labile phosphate ester. Thus the low phosphate values for the rat bone α_2 -chain reported

in the present study and for the chick bone α_2 -chain reported by Miller & Martin (1968) may be due to cleavage of labile phosphate groups during isolation and purification procedures that involve acidic pH. Differences in phosphate content between hard- and soft-tissue collagens may be of some significance in the biological mineralization process. However, it is difficult to evaluate the role of collagen-bound phosphate with regard to nucleating properties of hard-tissue collagens without first determining the exact nature and location of the phosphate groups.

Concluding remarks

On the basis of amino acid analysis of intact chains, as well as CNBr peptides from these chains, only α_1 I and α_2 -chains were present in both neutral-salt-soluble and guanidinium chloride-soluble lathyritic-rat dentine collagens. These results confirm previous work (Butler & DeSteno, 1971; Scott & Veis, 1976) that showed that dentine collagen is essentially type I. The α_1 I- to α_2 -chain ratios of the soluble dentine collagens were, however, greater than the characteristic 2:1 ratio for type I collagen. We have previously reported (Wohlbe & Car-

michael, 1978) that for the undenatured neutral-salt-soluble dentine, the excess of α_1 I-chains was due to the presence of $(\alpha_1\text{I})_3$ along with $(\alpha_1\text{I})_2\alpha_2$ molecules, which could be separated from each other by fractional salt precipitation. The same argument may be true for the guanidinium chloride-soluble dentine, but preferential solubilization of α_1 I-chains not involved in cross-linking from type I molecules cannot be precluded. Examination of the CNBr-peptide patterns of the guanidinium chloride-insoluble residue may shed light on this matter. The significance of the occurrence of $(\alpha_1\text{I})_3$ molecules in lathyrictic-rat dentine requires further study.

The availability of well characterized highly purified soluble collagen α -chains from dentine, as well as from bone (Stolz *et al.*, 1973), now paves the way for further work on collagen-mineral interactions *in vitro*, which may be expected to elucidate the role of hard-tissue collagens in the physiological mineralization process.

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References

- Ames, B. N. (1966) *Methods Enzymol.* **8**, 116–118
- Butler, W. T. & DeSteno, C. V. (1971) *Abstr. Int. Assoc. Dent. Res.* **49th**, 87
- Butler, W. T., Finch, J. E., Jr. & DeSteno, C. V. (1972) *Biochim. Biophys. Acta* **257**, 167–171
- Byers, P. H., McKenney, K. H., Lichtenstein, J. R. & Martin, G. R. (1974) *Biochemistry* **13**, 5243–5248
- Carmichael, D. J., Veis, A. & Wang, E. T. (1971) *Calcif. Tissue Res.* **7**, 331–344
- Carmichael, D. J., Dodd, C. M. & Nawrot, C. F. (1974) *Calcif. Tissue Res.* **14**, 177–194
- Carmichael, D. J., Chovelon, A. & Pearson, C. H. (1975) *Calcif. Tissue Res.* **17**, 263–271
- Cohen-Salal, L. & Glimcher, M. J. (1978) *Colloq. Fed. Eur. Connect. Tissue Clubs 6th*, pp. 159–160, C.N.R.S., Paris
- Fietzek, P. P. & Kühn, K. (1976) *Int. Rev. Connect. Tissue Res.* **7**, 1–60
- Francois, C. J., Glimcher, M. J. & Krane, S. M. (1967) *Nature (London)* **214**, 621–622
- Furthmayr, H. & Timpl, R. (1971) *Anal. Biochem.* **41**, 510–516
- Glimcher, M. J. & Krane, S. N. (1968) in *Treatise on Collagen* (Gould, B. S., ed.), vol. 2, part B, pp. 67–251, Academic Press, New York
- Glimcher, M. J., Hodge, A. J. & Schmitt, F. O. (1957) *Proc. Natl. Acad. Sci. U.S.A.* **43**, 860–867
- Layman, D. L., McGoodwin, E. B. & Martin, G. R. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 454–458
- Mechanic, G., Gallop, P. M. & Tanzer, M. L. (1971) *Biochem. Biophys. Res. Commun.* **45**, 644–653
- Miller, E. J. (1971) *Biochemistry* **10**, 1652–1658
- Miller, E. J. & Martin, G. R. (1968) *Clin Orthop. Relat. Res.* **59**, 195–232
- Miller, E. J., Martin, G. R., Piez, K. A. & Powers, M. J. (1967) *J. Biol. Chem.* **242**, 5481–5489
- Morgan, P. H., Jacobs, H. G., Segrest, J. P. & Cunningham, L. W. (1970) *J. Biol. Chem.* **245**, 5042–5048
- Nawrot, C. F. & Campbell, D. J. (1977) *J. Dent. Res.* **56**, 1017–1022
- Nawrot, C. F., Mittelstadt, J. K. & Kuo, M. (1976) *J. Dent. Res.* **55**, 841–847
- Neuman, W. F. & Neuman, M. W. (1958) *The Chemical Dynamics of Bone Mineral*, pp. 169–187, University of Chicago Press, Chicago
- Paz, M. A., Hanson, E., Rombauer, R., Abrash, L., Blumenfeld, O. O. & Gallop, P. M. (1970) *Biochemistry* **9**, 2123–2127
- Piez, K. A. (1968) *Anal. Biochem.* **26**, 305–312
- Piez, K. A., Eigner, E. A. & Lewis, M. S. (1963) *Biochemistry* **2**, 58–66
- Rauterberg, J. (1973) *Clin. Orthop. Relat. Res.* **97**, 196–212
- Robins, S. P. & Bailey, A. J. (1974) *FEBS Lett.* **38**, 334–336
- Royce, P. M. & Barnes, M. J. (1977) *Biochim. Biophys. Acta* **498**, 132–142
- Scott, P. G. & Veis, A. (1976) *Connect. Tissue Res.* **4**, 117–129
- Stolz, M., Furthmayr, H. & Timpl, R. (1973) *Biochim. Biophys. Acta* **310**, 461–468
- Tanzer, M. L. (1965) *Int. Rev. Connect. Tissue Res.* **3**, 91–112
- Toole, B. P., Kang, A. H., Trelstad, R. L. & Gross, J. (1972) *Biochem. J.* **127**, 715–720
- Veis, A. & Schleuter, R. J. (1964) *Biochemistry* **3**, 1650–1657
- Veis, A., Sharkey, M. & Dickson, I. (1977) in *Calcium-Binding Proteins and Calcium Function* (Wasserman, R. H., Corradino, R. A., Carafoli, E., Kretsinger, R. H., MacLennan, D. H. & Siegel, F. L., eds.), pp. 409–418, Elsevier-North-Holland, New York
- Volpin, D. & Veis, A. (1973) *Biochemistry* **12**, 1452–1464
- Wohllebe, M. & Carmichael, D. J. (1978) *Eur. J. Biochem.* **92**, 183–188
- Zimmermann, B. K., Timpl, R. & Kühn, K. (1973) *Eur. J. Biochem.* **35**, 216–221