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# BASIC BIOLOGICAL SCIENCES

## Comparative Collagen Biochemistry of Bovine Periodontium, Gingiva, and Dental Pulp

YOSHINORI KUBOKI,\* TOHRU TAKAGI,\* SATOSHI SASAKI,\* SHIGERU SAITO,\*\* and GERALD L. MECHANIC\*\*\*

\*Department of Biochemistry, School of Dentistry, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113, Japan; \*\*Department of Oral Biochemistry, Kanagawa Dental College, Yokosuka-shi 238, Japan; and \*\*\*Dental Research Center and Department of Biochemistry and Nutrition, University of North Carolina, Chapel Hill, North Carolina 27514

*Cross-linking patterns of the collagen from bovine gingiva, periodontium, and dental pulp were analyzed chromatographically. The ratios of two main cross-links, dihydroxylysinoxorleucine to hydroxylysinoxorleucine, were 0.18, 0.31, and 0.49 for the bovine gingiva, periodontium, and dental pulp collagen, respectively. These ratios are similar to that of skin collagen rather than that of bone and dentin collagen.*

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### Introduction.

Collagen is the major organic component of connective tissue. Although it has been generally accepted that the biological role of collagen is the mechanical support of tissue, recent observations suggest that this protein might also be related to morphogenesis in certain tissues.<sup>1</sup> However, the elucidation of the direct role of collagen fiber on the morphogenic process will require more detailed knowledge of the chemical structure and function of collagen. For this purpose collagens in oral connective tissues, such as dentin, pulp, periodontium, and gingiva, are especially interesting. Since all these tissues are produced by cells of the same mesenchymal origin, a comparative biochemical analysis of collagen from differentiated tissues might provide useful information for understanding the biological role of collagen in tooth development.

Recent studies have established that the stability of the collagen fiber depends, in part, upon the formation of intermolecular cross-links between tropocollagen molecules

in the fiber.<sup>2</sup> The collagen cross-linking of the various tissues such as skin, tendon, bone and cartilage has been characterized, each tissue revealing a unique distribution of reducible cross-links.<sup>3,4</sup> However, with the exception of dentin,<sup>5-7</sup> there is a lack of data on the cross-links of collagen from oral tissues.

The purpose of this study was to compare the cross-link patterns and the amino acid compositions of the collagens obtained from bovine periodontium, dental pulp, and gingiva to that obtained from other connective tissues such as skin, dentin, and bone.

### Materials and methods.

*Reagents.* — The NaB<sup>3</sup>H<sub>4</sub> (100 mCi/mM) was purchased from New England Nuclear. One percent solution of NaB<sup>3</sup>H<sub>4</sub> in dimethylformamide was used for the reduction of collagens.

*Preparation of insoluble collagen.* — Fresh bovine periodontal ligament (periodontium), dental pulp, gingiva, dentin, and femoral bone were obtained from a slaughterhouse. Animals used were four yr old. All procedures were performed at 4°C. Preparation of periodontium collagen followed the method already reported,<sup>8</sup> and a similar method was applied for the preparation of other soft tissue collagens. The fresh soft tissues were cut into small pieces, washed thoroughly with cold distilled water, and then extracted with 0.05 M Tris-HCl buffer (pH 7.4) containing 1 M NaCl for two d. The insoluble residues were washed exhaustively with cold distilled water and lyophilized. The bone and dentin were powdered in a stainless steel mortar and pestle after being frozen in liquid nitrogen and then were decalcified with 0.5 M EDTA (pH 7.4, 0.05 M Tris-HCl) as previously reported.<sup>9</sup> After decalcification they were extracted

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in the same manner as described above for soft tissues.

**Characterization of the reducible components.** — The reduction of collagen was carried out with a tritiated  $\text{NaB}^3\text{H}_4$  solution as described previously by Kuboki *et al.*<sup>5</sup> Hydrolysis of reduced collagen was carried out in 6 N HCl at 110°C for 24 h under nitrogen and then dried by flash evaporation. Hydrolyzed samples (4 mg each) were analyzed chromatographically on a 0.9 x 50 cm column of a Hitachi 034 amino acid analyzer eluted with citrate buffer 0.35 M in  $\text{Na}^+$  ion, pH 5.28 at 60°C. By using a split-stream device, 10% of the column effluent was monitored for  $^3\text{H}$  radioactivity by mixing it with PCS scintillation cocktail† and pumping the mixture through a Teflon coil in a well of a scintillation counter.‡

The identification of the reduced radioactive cross-links and their precursors was confirmed by their elution position relative to the amino acids in collagen by Ninhydrin reaction, as well as to those contained in hydrolysates of reduced bovine bone and dentin collagen.<sup>10</sup>

## Results.

**Intermolecular cross-links of periodontium, gingiva, dental pulp, dentin, and bone.** — As shown in the Fig., there are three major reducible cross-links (A, B, and C) in the soft tissue collagens and two (A and B) in the hard tissue collagens. These cross-links are identified as dihydroxylysinoxorleucine (DHLNL), hydroxylysinoxorleucine (HLNL), and histidinohydroxymerodesmosine (His-HMD). Two cross-links, DHLNL and HLNL, are common among all the collagens examined. It is interesting to note that the former is higher in amount than the latter in the hard tissue collagen (Fig. e and f), and is lower in the soft tissue collagen (Fig. a, b, c, and d).

Table 1 summarizes the distribution of the radioactivity of the major cross-links in collagen examined. The ratios of DHLNL to HLNL in gingiva, periodontium, dental pulp, skin, bone, and dentin collagen were 0.18, 0.31, 0.49, 0.66, 2.3, and 6.0 in the mean value of two separate analyses.

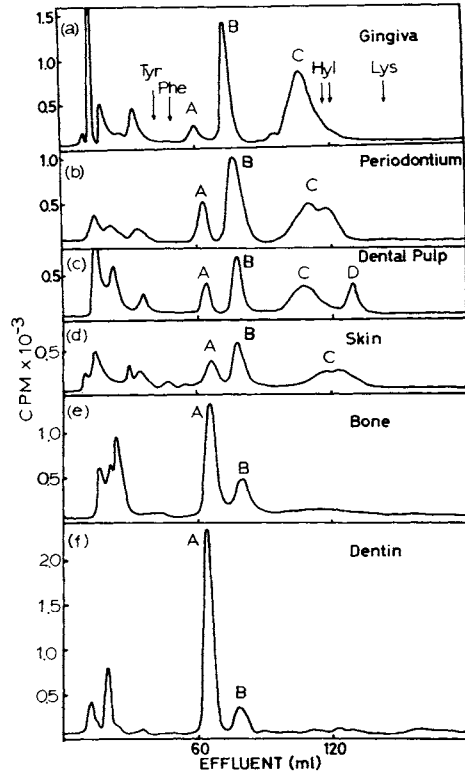


Fig. — Chromatographic patterns of the radioactive cross-links in acid hydrolysates of reduced collagen from bovine gingiva (a), periodontium (b), dental pulp (c), skin (d), bone (e), and dentin (f). Four mg of each hydrolysate were applied to the amino acid analyzer. The conditions are described in the text. Peaks are: A) dihydroxylysinoxorleucine; B) hydroxylysinoxorleucine; C) histidinohydroxymerodesmosine; D) unknown.

Direct quantitation of cross-links by Ninhydrin reaction on an amino acid analyzer was attempted under the same conditions described in the method. There is a significant correlation between the ratios of DHLNL/HLNL obtained from the radiochromatograms (Fig. and Table 1) and those ratios obtained from a direct quantitation by Ninhydrin reaction (Table 2). Partial amino acid compositions, including cross-links, are shown in Table 2. Cross-links in bone and dentin collagen could be quantitated and expressed as the residues per total 1000 residues, where the color yield of the DHLNL and HLNL was assumed to be 1.35 fold of that of leucine.<sup>11</sup> In the case of skin collagen, however, only HLNL was signif-

† Amersham

‡ Radio Analyzer, Aloka Co., Ltd., Tokyo, Japan

TABLE 1  
THE RELATIVE AMOUNTS OF THE THREE MAJOR CROSS-LINKS IN VARIOUS COLLAGENS

	Gingiva	Periodontium	Pulp	Skin	Bone	Dentin
DHLNL	6.6	12.8	17.7	23.3	69.9	85.7
HLNL	36.0	41.7	36.3	35.1	30.3	14.3
HHMD	57.4	45.5	45.9	41.6	—	—
DHLNL/HLNL	0.18	0.31	0.49	0.66	2.3	6.0

Data expressed as the percent of the total radioactivity in three major reducible cross-links.

TABLE 2  
PARTIAL AMINO ACID COMPOSITIONS\* AND THEIR RATIOS OF VARIOUS COLLAGENS

	Gingiva	Periodontium	Pulp	Skin	Bone	Dentin
4-Hyp	77.8	76.7	77.9	78.6	119	112
Pro	125	125	104	126	129	123
Gly	315	308	288	332	331	330
Hyl	5.7	6.6	7.1	5.9	4.8	8.3
Lys	27.4	27.0	30.5	24.4	23.3	20.7
DHLNL	0.02	0.05	0.04	—	0.22	0.27
HLNL	0.10	0.16	0.08	0.16	0.10	0.05
DHLNL/HLNL	0.20	0.32	0.50	—	2.2	5.4
Hyl/Hyl+Lys	0.17	0.20	0.19	0.19	0.18	0.29

\*Figures expressed as residues per 1000.

icant because of the presence of unknown peaks and the scarcity of the cross-links.

## Discussion.

The present study revealed that the cross-linking patterns of collagen in oral soft tissues differed in some respects from the patterns of skin, bone, and dentin. In all the oral soft tissues examined, DHLNL, HLNL, and His-HMD represented the three major cross-links. The ratios of DHLNL to HLNL in oral soft tissue collagen varied slightly in each tissue and were significantly lower than those of hard tissue collagen as shown in Table 1. It has been established that DHLNL is the most abundant cross-link in bone and dentin collagen,<sup>10</sup> whereas normal adult skin collagen contains a much lesser amount of DHLNL than HLNL as shown in Table 1 and reported previously.<sup>12,13</sup> The present study showed that in terms of the ratio of two main cross-links, *i.e.*, DHLNL/HLNL, the collagens from oral soft tissue with their ratios between 0.5 and 0.2 can be ranked in the group of skin collagen (0.7), rather than that of bone (2.3) and dentin (6.0) collagen.

In a recent study histidinohydroxymerodesmosine (His-HMD) was found to be abundant in bovine skin collagen,<sup>24</sup> and in the present study it was identified in signif-

icant amounts in oral soft tissue collagens. This finding further supports the consideration that the presence of His-HMD is rather ubiquitous among the soft tissue collagens.<sup>25</sup> It may be interesting to note from the Fig. that dentin and bone collagen with much higher amounts of DHLNL contain negligible amounts of His-HMD, whereas gingiva collagen with the least amount of DHLNL contains the highest amount of His-HMD (57.4%).

Although the biological significance of these varied compositions of cross-links (among others, the ratio of DHLNL to HLNL) is unknown, circumstantial evidence has been accumulated by several authors<sup>12-14</sup> from comparative studies of various collagens. The DHLNL/HLNL ratio is fairly characteristic to the collagen of a specific tissue of adult animal, as evidenced in bone,<sup>10,15</sup> dentin,<sup>10</sup> tendon,<sup>4</sup> skin,<sup>12,13</sup> and various other tissues.<sup>12,14,16</sup> In several tissues the ratio is generally high in the young and decreases with age.<sup>12,17-19</sup> Even in the adult animal, the ratio will increase in fibrotic tissue such as in granulation tissue,<sup>20</sup> scar tissue,<sup>21,22</sup> and fibroma.<sup>23</sup> In the present study it was found that the oral soft tissue collagens of a four-year-old bovine are similar to the skin collagen in terms of the ratio of DHLNL to HLNL, and also of the His-HMD content. These collagens can

probably be categorized into the same group in these respects.

The biochemical characterization of periodontium collagen was first reported by Saito,<sup>8</sup> however, detailed structural studies had to await further advances in collagen chemistry. In this paper cross-link analysis was proved to be useful to characterize the collagen of oral tissues in normal adult animals. Developmental changes of the cross-link pattern of these collagens are now being studied.

## Conclusions.

The cross-link patterns found in each of these oral tissue collagens clearly differ from the cross-link patterns of collagen from bone and dentin. The patterns are similar to that of skin collagen, suggesting the characteristics of oral tissues in terms of the maturity, stability, and function of collagen. This observation may provide a key to understanding the biochemical mechanism of the development of the tooth and its supporting tissues.

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