Downloaded from UvA-DARE, the institutional repository of the University of Amsterdam (UvA)
http://dare.uva.nl/document/1465

File ID 1465

SOURCE (OR PART OF THE FOLLOWING SOURCE):
Type Dissertation
Title Reactions of the organic matrix in dentin caries
Author G.A. Kleter
Faculty Faculty of Dentistry
Year 1997
Pages 105
ISBN 9053566694

FULL BIBLIOGRAPHIC DETAILS:
http://dare.uva.nl/record/106832

Copyright

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other then for strictly personal, individual use.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)

REACTIONS OF THE ORGANIC MATRIX IN DENTIN CARIES

Omslagontwerp en ontwerp binnenwerk: René Staelenberg, Amsterdam

ISBN 90 5356 669 4
NUR 887

© Amsterdam University Press, G.A. Kleter, 2003

Alle rechten voorbehouden. Niets uit deze uitgave mag worden verveelvoudigd, opgeslagen in een geautomatiseerd gegevensbestand, of openbaar gemaakt, in enige vorm of op enige wijze, hetzij elektronisch, mechanisch, door fotokopieën, opnamen of enige andere manier, zonder voorafgaande schriftelijke toestemming van de uitgever.

Voorzover het maken van kopieën uit deze uitgave is toegestaan op grond van artikel 16B Auteurswet 1912 j0 het Besluit van 20 juni 1974, St.b. 351, zoals gewijzigd bij het Besluit van 23 augustus 1985, St.b. 471 en artikel 17 Auteurswet 1912, dient men de daarvoor wettelijk verschuldigde vergoedingen te voldoen aan de Stichting Reprorecht (Postbus 882, 1180 AW Amstelveen). Voor het overnemen van gedeelte(n) uit deze uitgave in bloemlezingen, readers en andere compilatiewerken (artikel 16 Auteurswet 1912) dient men zich tot de uitgever te wenden.

REACTIONS OF THE ORGANIC MATRIX IN DENTIN CARIES

Academisch Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam,
op gezag van de Rector Magnificus,
Prof. Dr. J.J.M. Franse
ten overstaan van een door het college van dekanen
ingestelde commissie in het openbaar te verdedigen
in de Aula der Universiteit
op maandag 29 september 1997 te 15.00 uur

door

Gijsbertus Anthonius Kleter

geboren 1 november 1965 te Ede

Amsterdam University Press

Promotor:	Prof. Dr. J.M. ten Cate
Co-Promotor:	Dr. J.J.M. Damen
Beoordelingscommissie:	Prof. Dr. W. Beertsen (Universiteit van Amsterdam) Prof. G. Embery (University of Wales, Cardiff) Prof. Dr. A. van Nieuw Amerongen (Vrije Universiteit Amsterdam)
Paranimfen:	A. de Bie RA Dr. A.J.P. van Strijp

The research of this thesis was completed in the Department of Cariology Endodontology Pedodontology of the Academic Center for Dentistry Amsterdam (ACTA) under the auspices of the Netherlands Institute for Dental Sciences (IOT).

CONTENTS

Chapter		Page
1	Introduction	7
2	The influence of the organic matrix on demineralization of bovine root dentin in vitro GA Kleter, JJM Damen, V Everts, J Niehof and JM ten Cate Journal of Dental Research 73 (1994) 1523-1529	17
3	Discoloration of dental caries lesions: a review submitted for publication	33
4	The Maillard reaction in demineralized dentin in vitro GA Kleter, JJM Damen, MJ Buijs and JM ten Cate European Journal of Oral Sciences 105 (1997) 278-284	43
5	Modification of amino acid residues in carious dentin matrix GA Kleter, JJM Damen, MJ Buijs and JM ten Cate submitted for publication	57
6	A novel pyrroleninone cross-link from bovine dentin GA Kleter, JJM Damen, JJ Kettenes-van den Bosch, RA Bank, JM te Koppele, R Fokkens, JR Veraart and JM ten Cate submitted for publication	73
7	Concluding remarks	95
	Samenvatting (Dutch summary)	99
	Nawoord (Dutch epilog)	103

INTRODUCTION

TOOTH PHYSIOLOGY

A tooth consists of pulp tissues encased in hard, mineralized tissues. Dentin-forming cells (odontoblasts) are found at the border of the pulp. Odontoblasts have extensions running centripetally through narrow channels (tubules) in predentin and dentin, a bone-like tissue. Predentin is deposited by odontoblasts and consists of organic matrix. Mineralization takes place some time after the predentin deposition, thus at some distance from the odontoblast. Mineralization of the organic matrix is induced by enzymes and proteins secreted by the odontoblast extensions into the predentin at the level of the mineralization front. The tubules end in the outer part of dentin (mantle dentin), the composition of which deviates from bulk dentin. Dentin consists of mineral (70 wt%), water (10 wt%) and organic matrix (20 wt%). At the tooth's crown, dentin is lined with enamel, a mineralized tissue with far less of an organic matrix than dentin. Normally only a part of the crown is exposed to the oral cavity. Root dentin is lined with cementum, a thin layer of partially mineralized tissue containing organic material. The cementum near the root apex is of a cellular nature, but becomes increasingly acellular towards the crown. The tooth is set in the alveolar ridge of the jaw, and is encircled by the periodontal ligament, which helps absorb the mechanical forces exerted on the tooth. Above the alveolar bone, the tooth is covered by soft, gingival tissue.

Dentin organic matrix. The organic matrix of dentin consists of collagen for 90 wt%. Non-collagenous compounds comprise proteoglycans, highly-phosphorylated proteins, osteonectin, osteocalcin, dentin sialoprotein, and lipids (Boskey, 1989; Butler et al., 1992; Goldberg and Septier, 1985; Linde, 1989). Several non-collagenous compounds play a role in dentin formation and mineralization.

Collagen is the most prevalent protein in the human body. Nineteen types of collagen have been described. The common feature is the triple helix: three polypeptide chains (α) coiled around each other. It is essential that glycine occupies each third position in the α chains. High contents of hydroxyproline stabilize the structure. Type I collagen prevails in dentin. Its molecules have 300-nm-long rod shapes, containing triple helices (1011 residues per α chain) flanked by short non-helical ends (6-25 residues per α chain). Fibrils are evenly-spaced, linearly-aligned, cylindrical groupings of molecules. The fibrils show alternating bands in electron microscopy due to the overlapping of negatively charged

molecule segments stained by heavy metal ions, yielding a specific "banding pattern". Parallel fibrils are gathered into fibres (Van der Rest and Bruckner, 1993).

Type V collagen has been found in dentin (Lukinmaa and Waltimo, 1992) and is involved in fibril formation. Some peculiarities have been noted for dentinal type I collagen. For example, a high content of α_1 chains in rat dentin indicates the presence of molecules composed of three α_1 chains apart from the usual $(\alpha_1)_2\alpha_2$ composition (Sodek and Mandell, 1982). Some collagen fibres show a deviating banding pattern in electron microscopy, indicating an unusual packing of collagen molecules (Waltimo, 1996). Contrary to other tissues, type I collagen from dentin is degraded by trypsin, but not by pepsin (Carmichael et al., 1977; Scott and Leaver, 1974).

The major non-collagenous components of the dentin matrix are highly-phosphorylated proteins, phosphoryns, with many phosphoserine and aspartate residues (Butler et al., 1992). Dentin contains fewer proteoglycans than predentin. The proteoglycans from predentin are degraded upon mineralization, while small proteoglycans and phosphoryns are excreted by odontoblasts and incorporated into dentin (Goldberg et al., 1987; Linde, 1989).

The organic matrix of the highly-mineralized tubule walls (peritubular) differs from the bulk intertubular matrix. It consists of proteoglycans, which are not degraded upon mineralization (Takagi et al., 1990).

Collagen cross-links. Besides amide bonds between amino acids in the same α chain, bonds between amino acid side chains of different α chains can form "cross-links". These bonds originate from enzymatically-oxidized side chains of lysine and hydroxylysine residues. The oxidized residues react with other lysine and hydroxylysine residues, forming difunctional products. Reactions of such products with oxidized lysine or hydroxylysine yield trifunctional cross-links (Reiser et al., 1992).

Cross-linking protects collagen against proteolysis (Vater et al., 1979) and thermal denaturation (Flandin et al., 1984).

Collagens from different tissues do not necessarily contain the same cross-links. In dentin, cross-links occur between two collagen molecules with two or three peptide chains involved. The difunctional didehydrohydroxylysinonorleucine and didehydro-dihydroxylysinonorleucine, and the trifunctional hydroxylysylpyridinoline and lysylpyridinoline have been demonstrated in human and animal dentin (fig. 1). Predentin, on the other hand, contains more difunctional and fewer trifunctional cross-links than dentin (Linde and Robins, 1988; Walters and Eyre, 1983; Yamauchi et al., 1992). These cross-links connect the non-helical extension

of one molecule with the adjacent helical part of another molecule (Kuboki et al., 1981; Kuboki et al., 1993). A yet unidentified cross-link from polymerized terminal molecule segments is thought responsible for the marked stability of dentin collagen (Barnard et al., 1987; Light and Bailey, 1985).

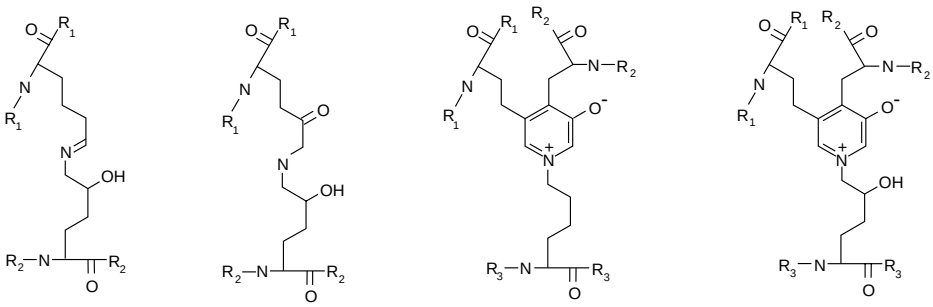


Figure 1
Collagen cross-links in dentin. ΔHLNL, didehydro-hydroxylysinonorleucine; ΔDHLNL, didehydro-dihydroxylysinonorleucine; LP, lysylpyridinoline; HP, hydroxylysylpyridinoline.

Contradictory reports on different cross-link contents of mineralized and non-mineralized collagen from mineralized tissues have appeared (Banes et al., 1983; Wu and Eyre, 1988). A role for cross-linking in inhibiting mineralization has been postulated (Yamauchi and Katz, 1993).

Pathology and prevalence

Pathology. Tooth plaque produces acids during the fermentation of dietary carbohydrates, causing the underlying tooth mineral to solubilize (demineralization). Upon restoration of a neutral plaque pH, mineral can reprecipitate (remineralization). When this equilibrium is lost, net demineralization occurs, causing dental caries.

In coronal caries, the enamel of the tooth crown is affected. With lasting caries, the lesion deepens and acquires a conical shape. In polarized light microscopy, zones with different mineral densities can be distinguished, such as the lesion body and the mineralized surface layer

(Thylstrup and Fejerskov, 1994). If the surface layer of advanced lesions is ruptured, a cavity is formed.

When the lesion front reaches the dentin, a widening along the enamel-dentin border can be observed. This phenomenon is caused by acids diffusing through seemingly intact overlying enamel rather than by lateral progression (Bjørndal and Thylstrup, 1995). This process is referred to as dentin caries. The tubules provide access to penetrating acids, which are followed by bacteria (Frank, 1990). Underneath the demineralized zone, sclerotic dentin forms, a substance transparent to the eye. Here a reprecipitated mineral (whitlockite) occludes the tubules, protecting the extensions of odontoblasts (Fusuyama, 1992). Reactive dentin forms in the pulp beneath these infiltrated tubules (Karjalainen, 1984). In a later stage, bacteria infiltrate and degrade the intertubular dentin (Frank, 1990). If this situation worsens, even the pulp can become infected.

With respect to collagen degradation, two zones can be distinguished within a lesion. The innermost layer is partially demineralized and still recalcifiable, and contains intact collagen fibrils. In the outermost layer, however, the integrity of the fibrils and the capacity for remineralization are lost (Ohgushi and Fusayama, 1975).

There is no clarity yet on a possible causative bacterial species for dentin caries. An increase in the proportion of Gram-positive bacteria has been noted. Especially lactobacilli have been isolated from the most advanced parts of dentin lesions (Edwardsson, 1987).

In root surface caries, the root is affected after it has become exposed by gingival recession. The cement is damaged first with sequential destruction of laminated cementum layers. When the caries reaches the dentin, the lesion becomes wedge-shaped (Nyvad and Fejerskov, 1990; Schüpbach et al., 1989). The histochemical changes of root surface caries correspond with those of dentin caries (Frank, 1990). Mineralized surface layers have been reported for both dentin and root surface caries (Mellberg, 1986).

Although *Actinomyces* has been linked with root surface caries (Edwardsson, 1987), contradictory reports on the microbiology of root surface caries still appear (Beighton and Lynch, 1995; Schüpbach et al., 1995; Schüpbach et al., 1996; Van Houte et al., 1994). A longitudinal study, however, failed to prove a role for *Actinomyces* (Ellen, 1993). Actually, lactobacilli and mutans streptococci are considered risk factors for root surface caries (Banting, 1991; Ravald and Birkhed, 1992). Generally, Gram-positive species predominate in the initial attacks on cementum and root dentin (Edwardsson, 1987).

The inactive, arrested caries lesion is black, remineralized, and hard on probing, whereas the active lesion is brownish and soft. Increased oral

hygiene and fluoride therapy arrest root surface caries (Nyvad and Fejerskov, 1986; Billings et al., 1985).

Prevalence. Root surface caries are prevalent in the elderly, since gingival tissue recession occurs more frequently with age, exposing tooth roots. Reported prevalences can be as high as 100% in groups of elderly subjects (Banting, 1991; Fejerskov and Nyvad, 1986). It occurs also in patients suffering from parodontitis. The root surface caries index expresses caries experience as a percentage of exposed root surfaces affected. Root surface caries has been correlated with high sugar intake and decreased salivary flow, and inversely with water fluoridation (Newbrun, 1986).

Dentin caries is inversely correlated with oral hygiene (Axelsson et al., 1994; Øgaard et al., 1994) and fluoride intake (Frencken et al., 1991). It can be expected that an improved oral status will decrease the incidence of dentin caries, but will increase root surface caries because the elderly are more dentate. A higher number of teeth retained, however, is associated with fewer root surface caries (Vehkalahti and Paunio, 1994).

PROTEOLYSIS IN MODEL DENTIN CARIES

The relationship between the degradation of organic matrix and dentin lesion formation has been studied both in vitro and in situ. Several authors employed matrix destruction to assess the role of the matrix in de- and remineralization. For example, Apostolopoulos and Buonocore (1966) reported facilitated demineralization of dentin at pH<5.5 after treatment with ethylene diamine. Inaba and coworkers (1996) found that removal of matrix from dentin lesions by hypochlorite promotes remineralization, consistent with a larger crystal surface available for mineral deposition after ashing (McCann and Fath, 1958). Hypochlorite-mediated destruction also increases the permeability of mineralized dentin (Barbosa et al., 1994).

In addition to chemical methods, dentin has been treated enzymatically in some studies. For example, Klont and Ten Cate (1991a) found that proteolytic degradation of the organic matrix is possible only after demineralization and does not affect dentin remineralization in vitro. The quantity of collagen degraded is not necessarily linearly correlated with the quantity of dissolved mineral (Klont and Ten Cate, 1991b). In addition, subsurface lesions and erosive lesions differ in their capacity for remineralization (Klont and Ten Cate, 1991a). Treatment of dentin lesions with collagenase causes surface erosion (Clarkson et al., 1986; Kawasaki and Featherstone, 1997). Proteolysis of tubular organic matter by a collagenase-protease mixture enhances the permeability of mineralized

dentin (Lindén et al., 1995).

Modifications of the organic matrix other than degradation have been studied for their effect on dentin de- or remineralization. Glutardialdehyde cross-linking of matrix in dentin lesions inhibits progressive demineralization (Boonstra et al., 1993). Removal of soluble phosphoproteins promotes calcification of demineralized dentin (Clarkson et al., 1991).

In an in situ study, specimens combining demineralized and mineralized dentin were exposed intraorally (Van Strijp et al., 1997). No correlation was found between demineralization of the mineralized dentin and collagen degradation in the demineralized dentin.

Objectives

From the studies summarized above, it is clear that the breakdown of the dentin matrix plays an important role in the pathology of dentin- and root surface caries. In addition, the demineralized dentin can be modified by a number of reactions, with consequences for its degradability. The studies described in this thesis were designed to address the role of degradation and modification of the dentin collagen.

Chapter 2 describes in vitro experiments to establish the role of proteolysis in dentin demineralization. Chapter 3 reviews the possible mechanisms for the discoloration of caries lesions. This phenomenon is particularly interesting since it may correspond with changes in the organic matrix that contribute to caries arrestment. The nonenzymatic glycosylation of proteins, known as the Maillard reaction, seems a likely cause of the discoloration. In Chapters 4 and 5, research is focused on the Maillard reaction by studying its effect on in vitro proteolysis of dentin (Chapter 4) and by analysing products formed during caries in vivo (Chapter 5). These two studies also included the analysis of physiological collagen cross-links, since any change in their levels presumably influences matrix stability. In an attempt to identify potential additional effectors of dentin matrix stabilization, two novel collagen cross-links were purified from dentin in Chapter 6. Chapter 7 reviews the results of Chapters 2-6 and additional results before reaching a final conclusion.

References

Apostolopoulos AX and Buonocore MG (1966) Comparative dissolution rates of enamel, dentin, and bone. I. Effect of the organic matter. J Dent Res 45, 1093-1100.

Axelsson P, Buischi YAP, Barbona MFZ, Karlsson R and Prado MCB (1994) The effect of a new oral hygiene training program on approximal caries in 12-15-year-old Brazilian children: results after three years. Adv Dent Res 8, 278-284.

Banes AJ, Yamauchi M and Mechanic GL (1983) Nonmineralized and mineralized compartments of bone: the role of pyridinoline in nonmineralized collagen. Biochem Biophys Res Comm 113, 975-981.

Banting DW (1991) Management of dental caries in the older patient. In: Geriatric dentistry (eds. Papas AS, Niessen LC and Chauncey HH), Chap. 9, pp. 141-167. Mosby Year Book, St. Louis MO, USA.

Barbosa SV, Safavi KE and Spångberg LSW (1994) Influence of sodium hypochlorite on the permeability and structure of cervical human dentine. Int Endod J 27, 309-312.

Barnard K, Light ND, Sims TJ and Bailey AJ (1987) Chemistry of the collagen cross-links. Origin and partial characterization of a putative mature cross-link of collagen. Biochem J 244, 303-309.

Beighton D and Lynch E (1995) Comparison of selected microflora of plaque and underlying carious dentine associated with primary root caries lesions. Caries Res 29, 154-158.

Billings RJ, Brown LR and Kaster AG (1985) Contemporary treatment strategies for root surface dental caries. Gerodontics 1, 20-25.

Bjørndal L and Thylstrup A (1995) A structural analysis of approximal enamel caries lesions and subjacent dentin reactions. Eur J Oral Sci 103, 25-31.

Boonstra WD, De Vries J, Ten Bosch JJ, Ögaard B and Arends J (1993) Inhibition of bovine dentin demineralization by a glutardialdehyde pretreatment: an in vitro caries study. Scand J Dent Res 101, 72-77.

Boskey AL (1989) Noncollagenous matrix proteins and their role in mineralization. Bone Miner 6, 111-123.

Butler WT, D'Souza RN, Bronckers ALJJ, Happonen R-P and Somerman MJ (1992) Recent investigations on dentin specific proteins. Proc Finn Dent Soc 88(Suppl.1), 369-376.

Carmichael DJ, Dodd CM and Veis A (1977) The solubilization of bone and dentine collagens by pepsin. Effect of cross-linkages and non-collagen components. Biochim Biophys Acta 491, 177-192.

Clarkson BH, Feagin FF, McCurdy SP, Sheetz JH and Speirs R (1991) Effects of phosphoprotein moieties on the remineralization of human root caries. Caries Res 25, 166-173.

Clarkson BH, Hall DL, Heilman JR and Wefel JS (1986) Effect of proteolytic enzymes on caries lesion formation in vitro. J Oral Pathol 15, 423-429.

Edwardsson S (1987) Bacteriology of dentin caries. In: Dentine and dentine reactions in the oral cavity (eds. Thylstrup A, Leach SA and Qvist V), pp. 95-102. IRL Press, Oxford UK.

Ellen RP (1993) Ecological determinants of dental root surface caries. In: Cariology for the nineties (eds. Bowen WH and Tabak LA), pp. 319-332. University of Rochester Press, Rochester NY, USA.

Fejerskov O and Nyvad B (1986) Pathology and treatment of dental caries in the aging individual. In: Geriatric Dentistry (eds. Holm-Pedersen P and Löe H), Chap. 19, pp. 238-262. Munksgaard, Copenhagen, Denmark.

Flandin F, Buffevant C and Herbage D (1984) A differential scanning calorimetry analysis of the age-related changes in the thermal stability of rat skin collagen. Biochim Biophys Acta 791, 205-211.

Frank RM (1990) Structural events in the caries process in enamel, cementum, and dentin. J Dent Res 69, 559-566.

Frencken JE, Truin GJ, Van't Hof MA, König KG, Kahabuka FKA, Mulder J and Kalsbeek H (1991) Fluoride in drinking water and caries progression in a Tanzanian child population. Commun Dent Oral Epidemiol 19, 180-181.

Fusuyama T (1992) Intratubular crystal deposition and remineralization of carious dentin. J Biol Bucc 19, 255-262.

Goldberg M and Septier D (1985) Improved lipid preservation by malachite green-glutaraldehyde fixation in rat incisor predentine and dentine. Arch Oral Biol 30, 717-726.

Goldberg M, Septier D and Escaig-Haye F (1987) Glycoconjugates in dentinogenesis and dentine. Progr Histochem Cytochem 17(2), 1-112.

Inaba D, Ruben J, Takagi O and Arends J (1996) Effect of sodium hypochlorite treatment on remineralization of human root dentine in vitro. Caries Res 30, 218-224.

Karjalainen S (1984) Secondary and reparative dentin formation. In: Dentin and dentinogenesis (ed. Linde A), Vol. 2, pp. 107-120. CRC Press, Boca Raton FA, USA.

Kawasaki K and Featherstone JDB (1997) Effects of collagenase on root demineralization. J Dent Res 76, 588-595.

Klont B and Ten Cate JM (1991a) Remineralization of bovine incisor root lesions in vitro: the role of the collagenous matrix. Caries Res 25, 39-45.

Klont B and Ten Cate JM (1991b) Susceptibility of the collagenous matrix from bovine incisor roots to proteolysis after in vitro lesion formation. Caries Res 25, 46-50.

Kuboki Y, Okuguchi M, Takita H, Kimura M, Tsuzaki M, Takakura A, Tsunazawa S, Sakiyama F and Hirano H (1993) Amino-terminal location of pyridinoline in dentin collagen. Connect Tissue Res 29, 99-110.

Kuboki Y, Tsuzaki M, Sasaki S, Liu CF and Mechanic GL (1981) Location of the intermolecular cross-links in bovine dentine collagen, solubilization with trypsin and isolation of cross-link peptides containing dihydroxylysinonorleucine and pyridinoline. Biochem Biophys Res Comm 102, 119-126.

Light N and Bailey AJ (1985) Collagen cross-links: location of pyridinoline in type I collagen. FEBS Lett 182, 503-508.

Linde A (1989) Dentin matrix proteins: composition and possible functions in calcification. Anat Rec 224, 154-166.

Linde A and Robins SP (1988) Quantitative assessment of collagen crosslinks in dissected predentin and dentin. Collagen Rel Res 8, 443-450.

Lindén LÅ, Källskog Ö and Wolgast M (1995) Human dentine as a hydrogel. Arch Oral Biol 40, 991-1004.

Lukinmaa PL and Waltimo J (1992) Immunohistochemical localization of types I, V, and VI collagen in human permanent teeth and periodontal ligament. J Dent Res 71, 391-397.

McCann HG and Fath EH (1958) Phosphate exchange in hydroxylapatite, enamel, dentin, and bone. J Biol Chem 231, 863-868.

Mellberg JR (1986) Demineralization and remineralization of root surface caries. Gerodontology 5, 25-31.

Newbrun E (1986) Prevention of root caries. Gerodontology 5, 33-41.

Nyvad B and Fejerskov O (1986) Active root surface caries converted into inactive caries as a response to oral hygiene. Scand J Dent Res 94, 281-284.

Nyvad B and Fejerskov O (1990) An ultrastructural study of bacterial invasion and tissue breakdown in human experimental root-surface caries. J Dent Res 69, 1118-1125.

Øgaard B, Seppä L and Rølla G (1994) Relationship between oral hygiene and approximal caries in 15-year-old Norwegians. Caries Res 28, 297-300.

Ohgushi K and Fusayama T (1975) Electron microscopic structures of the two layers of carious dentin. J Dent Res 54, 1019-1026.

Ravald N and Birkhed D (1992) Prediction of root caries in periodontally treated patients maintained with different fluoride programmes. Caries Res 26, 450-458.

Reiser K, McCormick RJ and Rucker RB (1992) Enzymatic and nonenzymatic cross-linking of collagen and elastin. FASEB J 6, 2439-2449.

Schüpbach P, Guggenheim B and Lutz F (1989) Human root caries: histopathology of initial lesions in cementum and dentin. J Oral Pathol Med 18, 146-156.

Schüpbach P, Osterwalder V and Guggenheim B (1995) Human root caries: microbiota in plaque covering sound, carious and arrested root surfaces. Caries Res 29, 382-395.

Schüpbach P, Osterwalder V and Guggenheim B (1996) Human root caries: microbiota of a limited number of root caries lesions. Caries Res 30, 52-64.

Scott PG and Leaver AG (1974) The degradation of human dentine collagen by trypsin. Connect Tissue Res 2, 299-307.

Sodek J and Mandell SM (1982) Collagen metabolism in rat incisor predentin in vivo: synthesis and maturation of type I, α_1(I) trimer, and type V collagens. Biochemistry 21, 2011-2015.

Takagi M, Hishikawa H, Hosokawa Y, Kagami A and Rahemtulla F (1990) Immunohistochemical localization of glycosaminoglycans and proteoglycans in predentin and dentin of rat incisors. J Histochem Cytochem 38, 319-324.

Thylstrup A and Fejerskov O (1994) Clinical and pathological features of dental caries. In: Textbook of clinical cariology (eds. Thylstrup A and Fejerskov O), 2nd ed., Chap. 6, pp. 111-157. Munksgaard, Copenhagen, Denmark.

Van der Rest M and Bruckner P (1993) Collagens: diversity at the molecular and the supramolecular levels. Curr Opin Struct Biol 3, 430-436.

Van Houte J, Lopman J and Kent R (1994) The predominant cultivable flora of sound and carious human root surfaces. J Dent Res 73, 1727-1734.

Van Strijp AJP, Van Steenbergen TJM and Ten Cate JM (1997) Bacterial colonization of mineralized and completely demineralized dentine in situ. Caries Res (in press).

Vater CA, Harris ED and Siegel RC (1979) Native cross-links in collagen fibrils induce resistance to human synovial collagenase. Biochem J 181, 639-645.

Vehkalahti M and Paunio I (1994) Association between root caries occurrence and periodontal state. Caries Res 28, 301-306.

Walters C and Eyre DR (1983) Collagen crosslinks in human dentin: increasing content of hydroxypyridinium residues with age. Calcif Tissue Int 35, 401-405.

Waltimo J (1996) Unusual forms of collagen in human dentin. Matrix Biol 15, 53-56.

Wu JJ and Eyre DR (1988) Fine powdering exposes the mineral-protected collagen of bone to protease digestion. Calcif Tissue Int 42, 243-247.

Yamauchi M, Chandler GS and Katz EP (1992) Collagen cross-linking and mineralization. Excerpta Med Int Congr Ser 1002, 39-46.

Yamauchi M and Katz EP (1993) The post-translational chemistry and molecular packing of mineralizing tendon collagens. Connect Tissue Res 29, 81-98.

Chapter 2

THE INFLUENCE OF THE ORGANIC MATRIX ON DEMINERALIZATION OF BOVINE ROOT DENTIN IN VITRO*

GA Kleter, JJM Damen, V Everts, J Niehof and JM ten Cate

Abstract. - The effect of matrix degradation on the rate of demineralization of dentin lesions was investigated. It was hypothesized that the demineralized matrix would inhibit the demineralization of the underlying mineralized dentin. Bovine root dentin specimens were alternately demineralized and incubated with either a bacterial collagenase or buffer (control). The demineralization was carried out under various conditions: Acetic acid solutions were used to form incipient and advanced erosive lesions, and lactic acid solutions containing a bisphosphonate were used to form incipient subsurface lesions. Under all conditions, the demineralization was found to be accelerated when the matrix was degraded by collagenase. This increase was more pronounced in advanced erosive lesions than in incipient lesions. Microscopic examination of collagenase-treated specimens revealed that the matrix of erosive lesions contained several layers of differently affected matrix, whereas the matrix of subsurface lesions appeared to be equally affected throughout the lesion. In conclusion, the matrix degradation was different in erosive and subsurface lesions but promoted the demineralization in both types of lesions.

INTRODUCTION

Root caries can occur when tooth roots are exposed to the oral environment, for example after periodontal surgery or gingival recession. Two stages are distinguished microscopically. First, the dentin mineral is dissolved and bacteria penetrate the tubules. Second, the demineralized dentin matrix is degraded, and bacteria infiltrate the intertubular area (Frank et al., 1989; Frank, 1990; Schüpbach et al., 1989). This sequence of events may indicate that the degradation of the dentin matrix occurs after it has become accessible by the removal of mineral. In an in vitro study, Klont and Ten Cate (1991) confirmed that the dentin matrix cannot be degraded unless it is demineralized.

The excretion of proteolytic enzymes by plaque microorganisms (Suido et al., 1986) probably accounts for the proteolytic activity observed in carious dentin (Larmas et al.,1968; Larmas, 1972). Proteases may also derive from the crevicular fluid (Cimasoni et al., 1977), when the root

* Journal of Dental Research 73 (1994) 1523-1529

lesion is in contact with the sulcus. In addition, Dumas et al. (1985) purified a collagenase from human teeth, which is activated upon acidic challenge (Dayan et al., 1983). The root lesion may therefore contain proteases from different sources.

Clarkson et al. (1986) conclude that proteolytic enzymes contribute to root lesion formation. Accordingly, Katz et al. (1987) found root cavitation with loss of matrix to occur in mild acidic solutions only in the presence of proteases. It is conceivable that the degradation of the matrix promotes the formation of a root lesion in two ways. First, the matrix forms a barrier to ionic diffusion, which is removed by degradation. Second, the degradation of the matrix yields nutrients, which may sustain the growth of cariogenic bacteria (Hojo et al., 1991).

The aim of this study was to compare the rates of calcium loss from dentin lesions where the amount of demineralized matrix increased, with those from lesions where the demineralized matrix was degraded by collagenase. The root caries process initially affects the cementum layer on the root surface. This layer may vary locally with regard to its presence, thickness, and cellular nature. In addition, the root surface is irregularly shaped. We therefore used specimens with their natural surfaces removed, in order to create comparable starting conditions. Bacterial collagenase from *Clostridium histolyticum* was used for the enzymatic digestion of demineralized dentin. For the demineralization of dentin, we used acetic acid solutions and lactic acid-bisphosphonate solutions in order to produce erosive and subsurface lesions respectively, since both types of lesions occur in root surface caries (Schüpbach et al., 1989). Bisphosphonates interact with mineral surfaces, thereby protecting these surfaces against dissolution (Thylstrup et al., 1983; Holmen et al., 1985). A bisphosphonate can thus be used to produce subsurface lesions in dentin (Featherstone et al., 1987; Klont and Ten Cate, 1991).

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade, unless mentioned otherwise.

Lactic acid was dissolved and gently boiled for 30 minutes prior to use so that any polymerized acid would be disrupted.

The following solutions were used for the experiments:

- HAc 0.1 M acetic acid, 5 mM NaN_3.
- HLac-MHDP 0.1 M lactic acid, 0.2 mM methane hydroxy diphosphonate, 5 mM NaN_3.
- Buffer 50 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), 0.25 mM $CaCl_2$, 0.2 M NaCl, 5 mM NaN_3, pH 7.8. The addition of calcium is necessary for collagenase to exert its activity.
- Collagenase 400 U / ml collagenase from *Clostridium histolyticum* (highly purified, type VII, Sigma, St. Louis, USA) in buffer. According to the manufacturer's specifications, this enzyme preparation contains minimal activities of other proteases, e.g., clostripain.

Preparation of root specimens. Adult bovine mandibles were obtained from a local slaughterhouse, one or two days after slaughter. The jaws were kept at 4°C and processed within three days. The incisors were extracted and the roots were separated. Most of the adhering soft tissue was removed and the remaining soft and pulpal tissues were destroyed in a 10% sodium hypochlorite solution (NaOCl, technical grade, Merck, Darmstadt, Germany) for two hours. Round dentin specimens (6 mm diameter) were prepared by drilling a hollow tube (ID 6 mm) through the incisor roots. The specimens were embedded in Vertex polymer (Dentimex, Zeist, The Netherlands) and polished on wet 240-grit sandpaper to remove all Vertex covering the dentin surfaces. The specimens were kept at 4°C in distilled water (approximately 1 mM NaN_3) until further use.

Formation of incipient lesions. Specimens were exposed alternately to acid and collagenase (fig. 1). Erosive lesions were formed by demineralization in HAc (pH 5.0 and pH 5.5), subsurface lesions by demineralization in HLac-MHDP (pH 4.5 and pH 5.0). All specimens were incubated separately in 1.0 ml acidic solutions for six hours and 1.0 ml collagenase or buffer for 18 hours daily. Under these conditions, the amount of degradable organic matrix is proportional to mineral loss (Klont and Ten Cate, 1991). Between the incubations, the specimens were rinsed briefly with distilled water and dried with paper. Each acid / collagenase and acid / buffer group contained five specimens. The experimental period was ten days. All incubations were carried out at 37°C without stirring.

Preliminary experiments showed that the 18-hour period of collagenase incubation was sufficient for a maximal collagen degradation in the lesions. Samples were taken from all solutions after the incubations to determine the release of calcium from the specimens. For the determi-

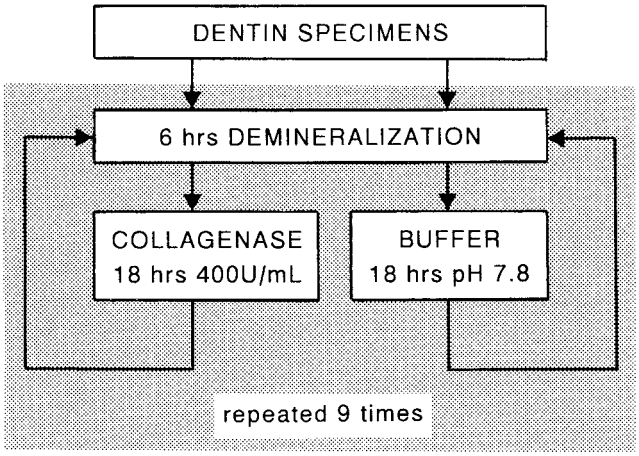


Figure 1
Formation of incipient lesions in root dentin. For further details, see "Materials and methods".

nation of collagen degradation, samples of every two or four consecutive incubations were pooled and assayed for hydroxyproline. The starting value of collagen degradation was determined in separate specimens. The starting value for subsurface lesions was determined after 15 minutes of incubation in 1.0 ml HLac-MHDP, pH 5.0, in order to account for possible effects of the bisphosphonate on the degradability of the collagenous matrix.

Formation of advanced lesions. In order to establish the effect of a demineralized matrix that was thicker than that obtained in the previous experiments, we made advanced erosive lesions by incubating specimens in HAc at pH 5.0 and pH 5.5 for 12 days (fig. 2). The demineralization solutions (20 ml HAc per specimen) were refreshed after four and eight days. Part of the lesions were subsequently incubated with 1.0 ml collagenase for six days, the other part with buffer; solutions were refreshed after three days. The specimens were then divided into three experimental groups. Advanced lesions with a non-degraded demineralized matrix were subjected to daily alternating exposures to 1.0 ml HAc (six hours) and 1.0 ml buffer (18 hours) for ten days; advanced lesions where the demineralized matrix was degraded, were subjected to a daily acid / collagenase treatment. A third group of advanced lesions, where the demineralized matrix was degraded, was subjected to a daily acid / buffer treatment, which allowed the demineralized matrix to increase again.

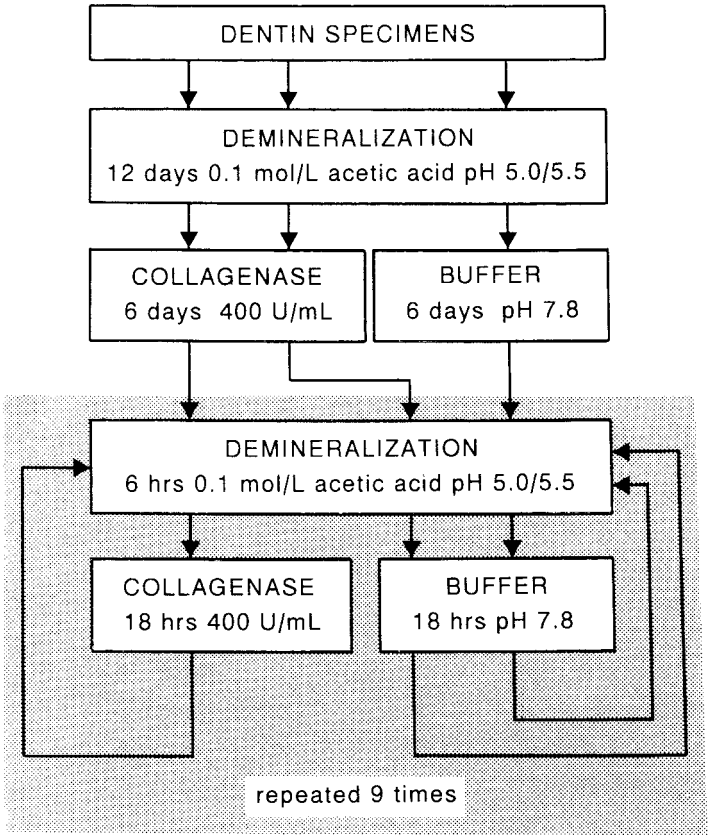


Figure 2
Formation of advanced erosive lesions in root dentin. For further details, see "Materials and methods".

Each group contained five specimens. All incubations were carried out at 37°C without stirring.

Samples were taken from each solution after incubation for calcium determination. Samples of the collagenase incubations were assayed for hydroxyproline.

Determination of calcium. The calcium content of the solutions was determined by atomic absorption spectrometry. To 100-µl samples, 3 ml of 1.56% $La(NO_3)_2$ in 50 mM HCl was added. The atomic absorption was measured on a Perkin Elmer 372 atomic absorption spectrophotometer at 423 nm.

Determination of hydroxyproline. In order to determine the amount of degraded collagen, we measured the hydroxyproline content of the incubation solutions. Samples of the incubation solutions were freeze-dried in ampuls and hydrolyzed in 500 µl of double-distilled 6 N HCl at 110°C for 16 hours. Samples of the hydrolyzate were dried in microvessels in vacuum over KOH and assayed for hydroxyproline according to Jamall et al. (1981). The absorbance at 558 nm was measured in 1-ml glass cuvets on a Perkin Elmer 550 S UV-VIS spectrophotometer against distilled water as reference. We obtained the amount of collagen (µg) by multiplying the hydroxyproline values (µg) by 7.98, as calculated from the composition of bovine dentin collagen (Volpin and Veis, 1973).

Microscopic analysis. From each experimental group of specimens with incipient lesions, two specimens were selected randomly for microscopic analysis. Two adjacent slices (500 µm thickness) were prepared from each specimen with a diamond wire sectioning machine (model 3242, Well, Le Locle, Switzerland). The slices were fixed in 1.0 ml 4% paraformaldehyde, 1% glutardialdehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for one week. One slice of each pair was subsequently demineralized in 2.0 ml 0.1 M acetic acid, 2.5% glutardialdehyde, pH 4.0.

Specimens were post-fixed in 1% OsO_4 in sodium cacodylate buffer, dehydrated in ascending alcohol solutions, and embedded in Epon LX 112. Semi-thin sections (1.0 µm thickness) were cut with a diamond knife and stained with Richardson's dye (contains methylene blue) or PAS-reagent. Micrographs were made with an Olympus New Vanox microscope.

Statistics. The levels of significance between two sets of unpaired results were determined according to Student's t test.

RESULTS

Effect of matrix degradation on demineralization during formation of incipient lesions

Erosive lesions. We determined the daily calcium loss from the specimens by combining the releases of calcium in consecutive incubations with acid and either collagenase or buffer. We calculated the cumulative calcium loss for each specimen by combining the daily calcium losses.

The calcium loss from specimens incubated with collagenase became significantly higher than that from control specimens on day 1 and day 9, when they were demineralized at pH 5.0 and pH 5.5, respectively (fig.3).

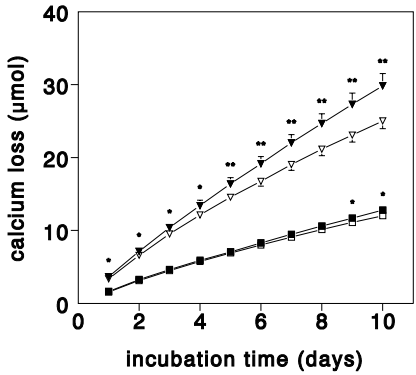


Figure 3
*Effect of matrix degradation on the rate of demineralization of incipient erosive lesions in root dentin. Specimens were subjected to daily alternating incubations with HAc pH 5.0 (▽,▼) or pH 5.5 (■,□) and either collagenase (▼,■) or buffer (▽,□) (n=5). Values represent mean ± SD. Levels of significance were calculated according to Student's t test. *P<0,05, **P<0,01 vs. buffer-treatment.*

At the end of the ten-day incubation period, the cumulative calcium loss from collagenase-treated specimens proved to be 19% (pH 5.0) and 6% (pH 5.5) higher than from buffer-treated specimens. The marginal changes in calcium concentration during collagenase- or buffer treatment were included in these figures.

The amounts of collagen degraded in specimens demineralized at pH 5.0 and pH 5.5 are shown in figure 4. A considerable amount of collagen was degraded in additional specimens prior to demineralization (t=0). This probably represents smear layer collagen that had formed during polishing. In addition, dentin tubules might have been opened by polishing, thereby exposing non-mineralized collagen. Although the col-

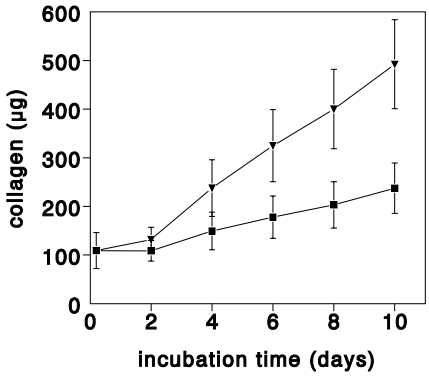


Figure 4
Degradation of collagen during the formation of incipient erosive lesions in root dentin. Specimens were alternately demineralized in HAc pH 5.0 (▼) or pH 5.5 (■) and incubated with collagenase (n=5). Values represent mean ± SD.

lagen degradation was higher in specimens demineralized at pH 5.0 than in specimens demineralized at pH 5.5, the ratios of degraded collagen to calcium loss over ten days were not significantly different: 12.8 ± 2.5 µg / µmol and 10.1 ± 4.0 µg / µmol, respectively. Preliminary experiments had shown that only a low amount of collagen was released during incubation with demineralization solution and buffer.

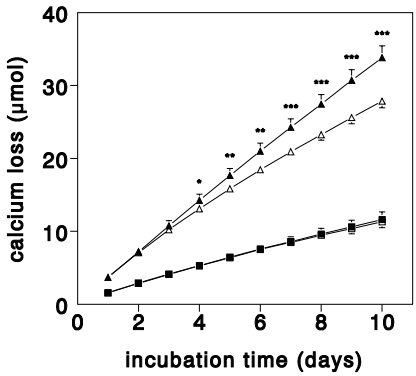


Figure 5
*Effect of matrix degradation on the rate of demineralization of incipient subsurface lesions in root dentin. Specimens were subjected to daily alternating incubations with HLac-MHDP pH 4.5 (△,▲) or pH 5.0 (■,□) and either collagenase (▲,■) or buffer (△,□) (n=5). Values represent mean ± SD. Levels of significance were calculated according to Student's t test. *P<0.05, **P<0.01, ***P<0.001 vs. buffer-treatment.*

Subsurface lesions. When specimens demineralized at pH 4.5 were treated with collagenase, this resulted in a higher calcium loss than the buffer treatment (+21% after ten days), an effect which was not found in the case of specimens demineralized at pH 5.0 (fig. 5).

In contrast to erosive lesions formed at pH 5.0 and pH 5.5, not only the amount of collagen that was degraded (fig. 6) but also the ratio of degraded collagen to calcium loss over ten days were much higher in lesions formed at pH 4.5 (8.7 ± 1.2 µg/µmol) than in lesions formed at pH 5.0 (3.8 ± 2.7 µg/µmol).

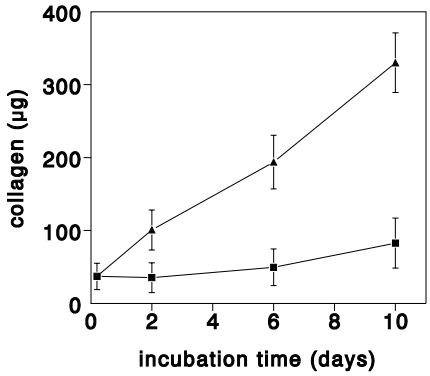


Figure 6
Degradation of collagen during the formation of incipient subsurface lesions in root dentin. Specimens were alternately demineralized in HLac-MHDP pH 4.5 (▲) or pH 5.0 (■) and incubated with collagenase (n=5). Values represent mean ± SD.

Effect of matrix degradation on demineralization of advanced lesions

The preparation of deep lesions in HAc at pH 5.0 and pH 5.5 for 12 days resulted in calcium losses of 81.0 ± 6.7 µmol and 48.8 ± 2.3 µmol, respectively. By the subsequent treatment with collagenase for six days, 2.53 ± 0.21 mg and 1.27 ± 0.15 mg collagen, respectively, was degraded.

When the advanced lesions where the matrix was degraded were subsequently subjected to the alternating HAc/collagenase treatment, the demineralization was considerably more rapid than during HAc/buffer treatment of advanced lesions with a demineralized matrix still present: +82% at pH 5.0 (fig. 7) and +52% at pH 5.5 (fig. 8).

The rate of demineralization of advanced lesions where the matrix was degraded, was slightly higher during the subsequent daily HAc/collagenase treatment than during the HAc/buffer treatment at pH 5.0 (fig. 7), but there was no difference at pH 5.5 (fig. 8).

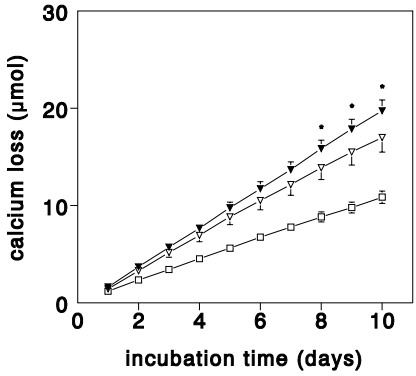


Figure 7
*Effect of matrix-degradation on the continued demineralization of advanced erosive lesions. Specimens were demineralized in HAc pH 5.0 for 3 x 4 days and incubated with collagenase (▼,▽) or buffer (□) during 2 x 3 days. Thereafter, specimens were subjected to daily alternating incubations with HAc pH 5.0 and either collagenase (▼) or buffer (▽,□) (n=5). Values represent mean ± SD. Levels of significance were calculated according to Student's t test. ▽ vs. □: P<0.05 (day 1) and P<0.001 (day 2-10); ▼ vs. □: P<0.001; and ▽ vs.▼: *P<0.05.*

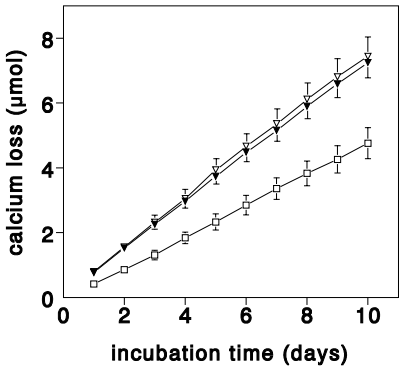


Figure 8
Same as in figure 7, except that demineralization was carried out at pH 5.5. ▽ vs. □: P<0.001; ▼ vs. □: P<0.001; ▽ vs. ▼: not significant.

The ratios of degraded collagen to calcium loss for specimens demineralized at pH 5.0 and pH 5.5 and treated with collagenase throughout the experiment were 27.7 ± 1.4 µg/µmol and 24.5 ± 2.4 µg/µmol respectively. These values were close to the ratio found by Klont and Ten Cate (1991) for completely demineralized dentin (23.3 µg/µmol).

Microscopy

Both Richardson's stain and PAS yielded similar patterns of staining intensity. PAS is somewhat more selective for proteoglycans, while a variety of matrix macromolecules is stained by Richardson's stain.

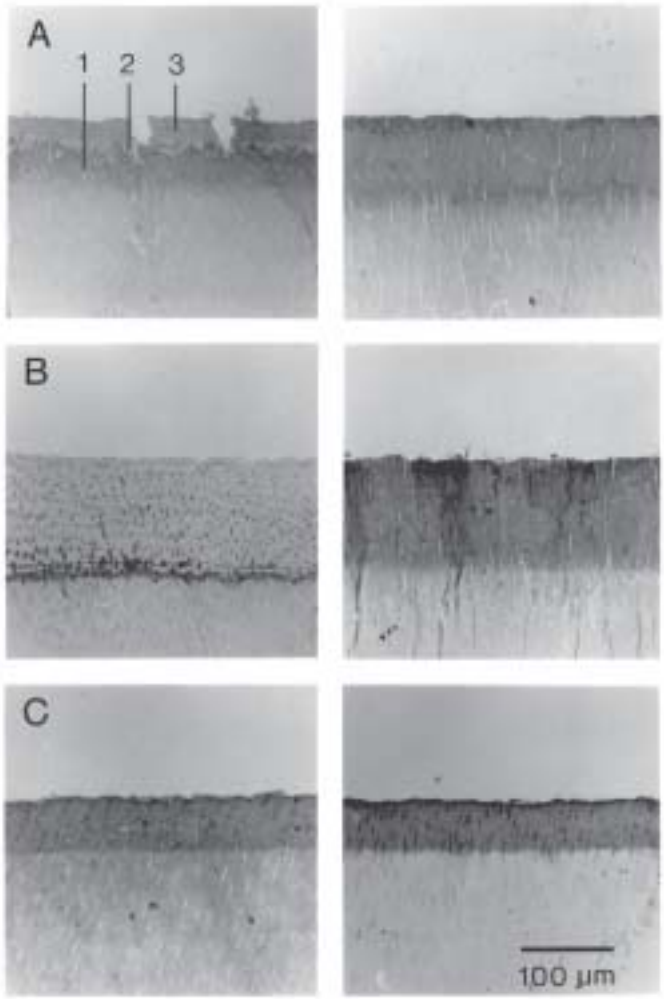


Figure 9
Light micrographs of incipient lesions, which were formed by alternating incubations with HAc pH 5.0 (A), HLac-MHDP pH 4.5 (B), and HLac-MHDP pH 5.0 (C) and either collagenase (left) or buffer (right). 9A (left): 1 = deepest layer, 2 = intermediate layer, 3 = top layer. Stain: Richardson's.

In lesions not treated with collagenase, the matrix appeared to be unaffected, since no differences were observed between the matrix in the lesions and the underlying dentin, when the latter had been demineralized during the preparation of the specimens for microscopy.

After incubation with collagenase, various changes were seen. In incipient erosive lesions formed in HAc at pH 5.0, three differently stained layers were observed (fig. 9A). When the sections were taken from slices demineralized after fixation, the layer on the bottom of the lesion (1) could not be distinguished from the underlying mineralized dentin and was apparently unaffected. It was covered by a narrow and faintly stained layer of irregular thickness (2). Tubules surrounded by more intensely stained material were present in this area. The top layer (3) consisted of intensely stained material and had a uniform appearance, with a few tubules still discernible. Erosive lesions formed in HAc at pH 5.5 resembled those formed in HAc at pH 5.0, except that the top layer was absent. The depth of the lesions could be measured accurately only in buffer-treated lesions and was approximately 90 µm (pH 5.0) and 50 µm (pH 5.5).

In incipient subsurface lesions alternately formed at pH 4.5 and treated with collagenase, the whole matrix appeared evenly affected (fig. 9B). Most of it was faintly stained, except for the tubules, which could easily be distinguished. No differences were found between buffer-treated subsurface lesions and those treated with collagenase, when both were formed at pH 5.0 (fig. 9C). The depths of the buffer-treated lesions formed at pH 4.5 and pH 5.0 were approximately 120 µm and 50 µm, respectively.

Discussion

In a previous investigation, Klont and Ten Cate (1991) showed that dentin must be demineralized before its matrix can be degraded by proteases. In the present study, it was demonstrated that proteolytic degradation of the demineralized matrix enhanced the susceptibility of dentin lesions to acid-dependent demineralization.

In both incipient erosive and subsurface lesions, the demineralization rate was found to decrease slowly as the amount of demineralized matrix increased. In advanced erosive lesions, this effect was more pronounced: The demineralization was reduced by 45% and 34%, respectively, when demineralized at pH 5.0 and pH 5.5. The lower release of calcium during demineralization of buffer-treated specimens may be explained by assuming that calcium ions were entrapped by the demineralized matrix. However, this was not the case, since digestion of the demineralized

matrix by collagenase did not result in a significant release of calcium. The demineralized matrix very likely hampers ionic diffusion into and out of the demineralizing area. A similar impaired diffusion of lactic acid and sodium lactate through a polyacrylamide gel after the incorporation of protein was demonstrated by Chu et al. (1992).

Klont and Ten Cate (1991) determined the ratio of collagen to calcium for dentin, which is consistent with the ratio of degraded collagen to calcium loss in advanced erosive lesions, while this ratio was lower in incipient lesions. In the initial phase of lesion formation, a relatively large part of the collagen is apparently insusceptible to the action of collagenase.

The three layers of organic matrix, which could be distinguished at the microscopic level in collagenase-treated incipient erosive lesions, probably represented different phases of matrix degradation. In the incipient subsurface lesions formed at pH 4.5, the collagenase treatment caused pronounced loss of intertubular stainable material throughout the lesion. The presence of a mineralized surface layer apparently did not prevent collagenase from penetrating the underlying demineralized matrix, most likely as a result of diffusion through local porosities or tubules. It may be speculated that the difference in microscopic patterns between these and erosive lesions is caused by this surface layer: By keeping the degraded matrix 'upright', it may allow collagenase to pass more easily through these than through erosive lesions, in which remnants of the degraded matrix may aggregate, thereby blocking collagenase movement.

The collagenase treatment of specimens demineralized in HLac-MHDP at pH 5.0 did not cause visible differences between the matrix of collagenase-treated lesions and that of buffer-treated lesions. This is consistent with the fact that the ratio of degraded collagen to calcium loss was lower for collagenase-treated lesions demineralized at pH 5.0 than for lesions demineralized at pH 4.5.

In both types of lesion, the peritubular matrix appeared to be more resistant to proteolytic activity than the intertubular matrix, which may be due to a compositional difference between the matrices. In this respect, it is interesting to note that Takagi et al. (1990) found the calcification of dentin to be accompanied by the degradation of proteoglycans throughout the intertubular matrix, but not in the peritubular matrix. However, there is no evidence that there is less degradation of the peritubular than the intertubular matrix, when demineralized dentin specimens are exposed to the oral environment (Van Strijp et al., 1992). This discrepancy can be explained by the assumption that a wide variety of enzymes participates in the degradation of the dentin matrix in vivo.

The present in vitro data indicate that the presence of the organic

matrix inhibits dentin demineralization, especially in advanced lesions. Therefore, the degradation of the organic matrix in vivo probably promotes the development of lesions during root surface caries.

Acknowledgement. The authors wish to thank Mr. A.J. Lammens for his technical assistence, and Ms. B. Fasting and Mr. J. Verouden for their comments on the manuscript.

REFERENCES

Chu CL, Spiess WEL and Wolf W (1992) Diffusion of lactic acid and Na-lactate in a protein matrix. Food Sci Technol 25, 476-481.

Cimasoni G, Ishikawa I and Jaccard F (1977) Enzyme activity in the gingival crevice. In: The borderland between caries and periodontal disease (ed. Lehner T), pp. 13-41. Academic, London UK.

Clarkson BH, Hall DL, Heilman JR and Wefel JS (1986) Effect of proteolytic enzymes on caries lesion formation in vitro. J Oral Pathol 15, 423-429.

Dayan D, Binderman I and Mechanic GL (1983) A preliminary study of activation of collagenase in carious human dentine matrix. Arch Oral Biol 28, 185-187.

Dumas J, Hurion N, Weill R and Keil B (1985) Collagenase in mineralized tissues of human teeth. FEBS Lett 187, 51-55.

Featherstone JDB, McIntyre JM and Fu J (1987) Physico-chemical aspects of root caries progression. In: Dentine and dentine reactions in the oral cavity (eds. Thylstrup A, Leach SA and Qvist V), pp. 127-137. IRL Press, Oxford UK.

Frank RM (1990) Structural events in the caries process in enamel, cementum and dentin. J Dent Res 69, 559-566.

Frank RM, Steuer P and Hemmerle J (1989) Ultrastructural study on human root caries. Caries Res 23, 209-217.

Hojo S, Takahashi N and Yamada T (1991) Acid profile in carious dentin. J Dent Res 70, 182-186.

Holmen L, Thylstrup A, Featherstone JDB, Fredebo L and Shariati M (1985) A scanning electron microscopic study of surface changes during development of artificial caries. Caries Res 19, 11-21.

Jamall IS, Finelli VN and Quee Hee SS (1981) A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. Anal Biochem 112, 70-75.

Katz S, Park KK and Palenik CJ (1987) In-vitro root surface caries studies. J Oral Med 42, 40-48.

Klont B and Ten Cate JM (1991) Susceptibility of the collagenous matrix from bovine incisor roots to proteolysis after in vitro lesion formation. Caries Res 25, 46-50.

Larmas M (1972) Observations on endopeptidases in human carious dentin. Scand J Dent Res 80, 520-523.

Larmas M, Mäkinen KK and Scheinin A (1968) Histochemical studies on the aryl-aminopeptidase activity in human carious dentine. Acta Odontol Scand 26, 127-136.

Schüpbach P, Guggenheim B and Lutz F (1989) Human root caries: histopathology of initial lesions in cementum and dentin. J Oral Pathol Med 18, 146-156.

Suido H, Nakamura M, Mashimo PA, Zambon JJ and Genco RJ (1986) Arylaminopeptidase activities of oral bacteria. J Dent Res 65, 1335-1340.

Takagi M, Hishikawa H, Hosokawa Y, Kagami A and Rahemtulla F (1990) Immunohistochemical localization of glycosaminoglycans and proteoglycans in predentin and dentin of rat incisors. J Histochem Cytochem 38, 319-324.

Thylstrup A, Featherstone JDB and Fredebo L (1983) Surface morphology and dynamics of early enamel caries development. In: Demineralisation and re-mineralisation of the teeth (eds. Leach SA and Edgar WM) pp. 165-184. IRL Press, Oxford UK.

Van Strijp AJP, Klont B and Ten Cate JM (1992) Solubilization of dentin matrix collagen in situ. J Dent Res 71, 1498-1502.

Volpin D and Veis A (1973) Cyanogen bromide peptides from insoluble skin and dentin bovine collagens. Biochemistry 12, 1452-1464.

Chapter 3

DISCOLORATION OF DENTAL CARIES LESIONS: A REVIEW*

Abstract. - The mechanism by which tooth substance darkens during caries has not been resolved yet. Candidate pigments are divided in two categories: either products from local reactions or exogenous pigments. The first category comprises the Maillard reaction and enzymatic browning, the second bacterial pigments, heme, iron, and food pigments. It is concluded that the Maillard reaction appears the most likely cause for the discoloration.

Dental caries is generally acknowledged to be a process during which bacterial acids destroy hard dental tissues (Kleinberg, 1982). Lesions are characterized by conspicuous discoloration, which becomes more enhanced after the carious attack has ceased. In the course of the process in enamel, an opaque "white spot" lesion may become arrested as a "brown spot" (Ripa, 1977). In case of root surface caries, a slightly brown incipient lesion becomes dark and hard on probing after caries arrestment (Banting, 1991; Fejerskov and Nyvad, 1986). One recent report, however, describes soft black active root lesions (Lynch and Beighton, 1994).

It is tempting to speculate on the nature of the pigment formed in the caries process and on its relationship with caries arrestment. Knowledge of the cause of caries arrestment may help provide ways to stop caries in a preliminary phase. However, many causes could account for the discoloration in caries, often explaining the different opinions dentists have on this matter. Therefore, a review of the chemical backgrounds of the color changes was felt necessary.

Two different categories of pigments may be involved in lesion discoloration: pigments resulting from chemical reactions of the organic contents of the lesion, and exogenous pigments from bacteria or food, which penetrate the lesion and bind to lesion constituents.

CHEMICAL REACTIONS IN THE LESION

Maillard reaction. The Maillard reaction is also known as non-enzymatic browning, non-enzymatic glycosylation, and glycation. It comprises the spontaneous reaction between carbonyl and amino compounds, such as sugars and proteins, respectively. It is especially well-known in the

chemistry of heated foods. In addition, the physiological Maillard reaction in humans is associated with the complications of diabetes, atherosclerosis, and aging. For review articles on this topic, the interested reader is referred to a recent book (Labuza et al., 1994).

The initial products of the reactions between sugars and proteins may enter a cascade of reactions yielding fluorescence, browning, and polymerization of proteins ("cross-linking"). The brown pigments, so-called melanoidins, are polymers whose composition has not yet been established completely. Melanoidins bind calcium and may thus interfere with de- and remineralization in caries.

The Maillard reaction in teeth in vitro was studied especially during the 50s and 60s by Dreizen and coworkers and by Armstrong, and is reviewed by Armstrong (1964). Pigmentation of demineralized teeth, resistance of browned demineralized dentin to proteolysis, and acid-precipitated pigments were produced artificially in vitro and showed strong resemblance to in vivo observations. In addition, carious dentin showed an increase in bound carbohydrate as determined with color reactions. With respect to the carbonyl compounds capable of reacting with dentin, simple carbohydrates, hexosamines, and several carbonyl metabolites (dihydroxyacetone, glyceraldehyde, and methylglyoxal) have been tested. Nevertheless, there have been few attempts to demonstrate specific glycosylation products, such as a glycosylated peptide (Armstrong, 1968) and hexitollysine (Kuboki et al., 1977).

Armstrong attributed the increased resistance of dentin matrix to proteolysis to the blockage of susceptible sites by covalently bound carbohydrate. Later it became clear that the Maillard reaction induces the formation of covalent bonds (cross-links) between protein molecules, accounting for such resistance as well. The presence of non-degradable matrix proteins inhibits mineral dissolution (Chapter 2). In addition, both brown pigments and cross-linked proteins inhibit the production of extracellular polysaccharides by cariogenic streptococci (Kobayashi et al., 1990).

Interestingly, the Maillard reaction has also been implicated as causing teeth discoloration in patients receiving chlorhexidine. The demonstration of a reaction intermediate, furfural (Nordbö et al., 1977), and decreased browning by a Maillard reaction inhibitor (Nathoo and Gaffar, 1995) serve as proof. This has been mitigated, however, by others who demonstrated either iron-sulphide staining or chlorhexidine-mediated binding of food pigments to tooth surfaces (Addy and Moran, 1995).

Unfortunately, the in vitro studies mentioned-above sometimes employed rather unnatural reactant concentrations and reaction conditions for simulation of the Maillard reaction. Little attention was paid to the likeliness of the reaction under the circumstances prevailing in the caries lesion in vivo. To provide a better understanding of the different

molecular species that may be involved in browning reactions during caries, they are summarized below.

(i) *simple carbohydrates.* Free reducing sugars are present in plaque. Their reaction with amino compounds is inhibited in the mildly acidic environment of a caries lesion. The acid environment rich in phosphates and acids, however, favours formation of furfurals (Nordbö et al., 1979) from reducing sugars. Furfurals are reactive intermediates of the Maillard reaction, which react with proteins. The demineralized matrix of enamel and dentin is browned intensely by furfurals (Armstrong, 1964; Dreizen et al., 1964; Engel, 1968).

(ii) *microbial metabolites.* Both extracellular and intracellular microbial carbonyl compounds are likely to be present in a caries lesion. Generally, these short (C_2-C_4) metabolites are more reactive than simple carbohydrates (C_6), especially at the pH values (4-6) of caries lesions.
Lysis of bacteria in caries lesions (Schüpbach et al., 1992) will release intracellular metabolites. Glycolysis products such as glyceraldehyde and dihydroxyacetone brown teeth in vitro (Armstrong, 1964; Dreizen et al., 1964; Engel, 1968).
Acetaldehyde and acetoin can be excreted in substantial amounts by lactic acid bacteria, in addition to diacetyl and methylglyoxal. For example, acetoin excretion has been studied in *Streptococcus mutans* (Hillman et al., 1987). Acetaldehyde (Nordbö, 1971) and methylglyoxal (Armstrong, 1964) stain teeth in vitro.
So far, only one report (Engel, 1971) has identified (by infrared spectrometry) a discoloration in enamel as an analogon of the glyceraldehyde / glycine pigment.

(iii) *lipid oxidation products.* Oxidation of polyunsaturated fatty acid residues yields fragments with reactive epoxy, peroxy, and carbonyl groups. The reaction between such compounds and proteins initiates browning, fluorescence, and cross-linking. Both enzymatic and non-enzymatic lipid oxidation occurs in the oral environment, especially in gingival crevicular fluid during periodontitis. Lipid oxidation in carious dentin is mentioned by Dirksen (1963), but no detailed account has been given.

Enzymatic browning. Phenol-oxidizing enzymes (such as tyrosinase and peroxidase) oxidize tyrosine residues into reactive quinone derivatives, which will condense into colored polymers (melanins). Melanins are rich in carboxyl groups and therefore have high affinity for divalent metal ions such as calcium.

Peroxidases from saliva, crevicular fluid, bacteria, and fungi may contribute to this reaction in caries lesions. Although deeper layers of the carious microflora are assumed to be anaerobic, the oxygen required for the reaction may reach the deeper parts of the plaque via oxygen channels (Marquis, 1995). Lactobacilli, however, cause browning of dentin in the absence of tyrosinase (Dreizen et al., 1957).

Histochemical evidence for melanins in caries lesions has been presented, based on silver staining and bleaching by hydrogen peroxide. These reports, however, are contradictory as far as the pigment location is concerned: circumventing the lesion (Opdyke, 1962), diffuse throughout the lesion (Ermin, 1968), and superficial (Meyer and Baume, 1966).

Humic substances. Analogous to the reactions described above, humic substances (the polymeric pigments from soil (humus) and marine sediments) can be formed by both enzymatic and non-enzymatic browning. High concentrations of free calcium and phosphate ions and supersaturation with respect to hydroxyapatite can sustain in soil, because adsorption of humic acids to mineral surfaces inhibits crystal growth (Inskeep and Silvertooth, 1988). A similar adsorption to tooth mineral in a caries lesion can be anticipated for polycarboxylic polymers from either the Maillard reaction or enzymatic browning.

EXOGENOUS PIGMENTS

Bacterial pigments. Some bacteria commonly found in caries lesions are known to produce pigments. For example, the black staining of plaque is related with *Actinomyces* (Slots, 1974), but its chemical nature remains unknown. Black pigmented *Prevotella* produces both iron sulphide and heme pigments (Shah et al., 1979). In addition, *Propionibacterium* forms porphyrins (Lee et al., 1978). Bacterial iron-binding peptides, which can contribute to discoloration, increase in the saliva of subjects with a high caries frequency (Nordh, 1969).

From carious enamel, a brown pigment-producing *Actinomyces* has been isolated (Hurst et al., 1948). Black caries lesions contained higher numbers of *Actinomyces*, *Lactobacillus*, and *Veilonella* (but not black pigmented *Prevotella*) than unstained lesions (Boue et al., 1987).

Heme and iron. Aside from bacterial heme, the host him / herself may contribute to heme- and iron-derived pigmentations. Heme and iron compounds may originate from either the pulp or the oral cavity. Pulp-derived discolorations are known, for example, from traumatic teeth (Stanley et al., 1978). The pulp underlying caries lesions may become

irritated or even pulpitic (Seltzer and Bender, 1965) implicating increased vascular permeability and eventually hemolysis. Hemoglobin infiltrates the dentinal tubules and releases heme, which is degraded to bile pigments (bilirubin, biliverdin). In addition, the iron liberated from heme is converted to hemosiderin or to black precipitates with sulphur compounds (Stangel et al., 1996). Heme and iron may additionally be derived from heme proteins in saliva and gingival crevicular fluid, but the salivary iron concentration is rather low.

In arrested enamel lesions, increased contents of iron (Torell, 1957a) and iron phosphate crystals (Torell, 1957b) have been observed. In pigmented dentin from carious teeth, however, no increase of iron or heavy metals has been found (Malone et al., 1966).

Food pigments. Well-described are tooth discolorations associated with the consumption of coffee, tea, wine, and betel nuts. This discoloration by food and beverages has been mimicked in vitro with caries lesions (Kidd et al., 1990) and sound teeth (Chan et al., 1981).

The accumulation of food pigments may promote caries arrestment. The binding of tea tannins renders dentin collagen resistant to proteolytic degradation (Armstrong, 1958). Tannins from tea inhibit streptococcal glycosyltransferase and reduce caries in animal experiments (Sakanaka et al., 1992). In addition, an acidic polymeric pigment from dark beer inhibits streptococcal synthesis of extracellular polysaccharides (Murata et al., 1995).

Binding of dyes to demineralized dentin has been used to assess the condition of carious dentin, as an indicator of decay levels during cavity preparation (Kuboki et al., 1983)

CONCLUSIONS

Considering the scarce evidence for most of the explanations reviewed both here and previously (Van Reenen, 1955), no unequivocal conclusion on the cause of carious discoloration seems possible. Apparently, discoloration precedes the infiltrating microorganisms in carious dentin (Fusuyama et al., 1966). This eliminates many of the above-mentioned possible reactions for this stage of caries. Most likely, molecules infiltrating in advance of the bacteria are responsible for the browning reaction. Since the advancing front of the dentin lesion is both anaerobic and acidic, and because collagen (the main organic constituent of dentin) is poor in aromatic amino acids, oxidative enzymatic browning does not seem a likely candidate. On the other hand, short carbonyl metabolites will react readily with the organic matrix, even in an anaerobic acidic

environment. Furfurals can be formed from hexoses, pentoses, or ascorbic acid in this environment and may contribute to a local browning reaction.

It is noteworthy that several of the above-mentioned mediators of discoloration have been found to inhibit streptococcal proliferation. In addition, collagen cross-linking caused by enzymatic and non-enzymatic browning or by tannage with tannins might render dentin less susceptible to proteolytic degradation.

So far, the most convincing evidence for the discoloration of caries lesions has been provided for the Maillard reaction. Since few investigations have attempted to identify Maillard products straightforwardly in carious material, further research in this field should be undertaken. In addition, the influence of discolored demineralized matrix, resistant to degradation, on the accessibility of the underlying sound tissue for acids and infiltrating bacteria should be established.

References

Addy M and Moran J (1995) Mechanisms of stain formation on teeth, in particular associated with metal ions and antiseptics. Adv Dent Res 9, 450-456.

Armstrong WG (1958) Modification of the organic matrix of sound dentin to collagenase-resistant forms. J Dent Res 37, 1016-1034.

Armstrong WG (1964) Modifications of the properties and compositon of the dentin matrix caused by dental caries. Adv Oral Biol 1, 309-332.

Armstrong WG (1968) A method for the simultaneous separation and assays of peptides and attached carbohydrate and fluorescent components. Automat Anal Chem (Technicon Symp 3rd, 1967) 1, 295-299.

Banting DW (1991) Management of dental caries in the older patient. In: Geriatric dentistry (eds. Papas AS, Niessen LC and Chauncey HH), Chap. 9, pp. 141-167. Mosby Year Book, St. Louis MO, USA.

Boue D, Armau E and Tiraby G (1987) A bacteriological study of rampant caries in children. J Dent Res 66, 23-28.

Chan KC, Hormati AA and Kerber PE (1981) Staining calcified dental tissues with food. J Prosthet Dent 46, 175-178.

Dirksen TR (1963) Lipid components of sound and carious dentin. J Dent Res 42, 128-132.

Dreizen S, Gilley EJ, Mosny JJ and Spies TD (1957) Experimental observations on melanoidin formation in human carious teeth. J Dent Res 36, 233-236.

Dreizen S, Spirakis CN and Stone RE (1964) In vitro studies of the chromogenic reactions between selected carbohydrate derivatives and the amino acids common

to human enamel and dentine. Arch Oral Biol 9, 733-737.

Engel W (1968) Chemische Untersuchungen zur Verfaerbung des Zahnschmelzes beim Kariesprozess. Bull Group Int Rech Sci Stomatol 11, 365-380 and 431-444.

Engel W (1971) Zum biochemischen Ablauf der Schmelzverfaerbung beim Kariesprozess unter dem Einfluss verschiedener Fluorkonzentrationen. Bull Group Int Rech Sci Stomatol 14, 243-258.

Ermin R (1968) Lichtmikroskopische Histologie und Histochemie der karioesen Laesionen. Bull Group Int Rech Sci Stomatol 11, 345-363 and 431-444.

Fejerskov O and Nyvad B (1986) Pathology and treatment of dental caries in the aging individual. In: Geriatric dentistry (eds. Holm-Pedersen P and Löe H), Chap. 19, pp. 238-262. Munksgaard, Copenhagen, Denmark.

Fusuyama T, Okuse K and Hosoda H (1966) Relationship between hardness, discoloration, and microbial invasion in carious dentin. J Dent Res 45, 1033-1046.

Hillman JD, Andrews SW and Dzuback AL (1987) Acetoin production by wild-type strains and a lactate dehydrogenase-deficient mutant of Streptococcus mutans. Infect Immun 55, 1399-1402.

Hurst V, Frisbie HE, Nuckolls J and Marshall MS (1948) In vitro studies of caries of the enamel in the Syrian hamster. Science 107, 42-44.

Inskeep WP and Silvertooth JC (1988) Inhibition of hydroxyapatite precipitation in the presence of fulvic, humic, and tannic acids. Soil Sci Soc Am J 52, 941-946.

Kidd EAM, Joyston-Bechal S and Smith MM (1990) Staining of residual caries under freshly-packed amalgam restorations exposed to tea/chlorhexidine in vitro. Int Dent J 40, 219-224.

Kleinberg I (1982) Dental caries. In: Oral microbiology (ed. Nolte WA), 4th. ed., Chap. 24, pp. 605-624. Mosby, St. Louis MO, USA.

Kobayashi S, Koga K, Hayashida O, Nakano Y and Hasegawa Y (1990) Specific inhibition of insoluble glucan synthase (GTF-I) by Maillard reaction products from casein and albumins. Agric Biol Chem 54, 1417-1424.

Kuboki Y, Liu CF and Fusuyama T (1983) Mechanism of differential staining in carious dentin. J Dent Res 62, 713-714.

Kuboki Y, Ohgushi K and Fusuyama T (1977) Collagen biochemistry of the two layers of carious dentin. J Dent Res 56, 1233-1237.

Labuza TP, Reineccius GA, Monnier V, O'Brien J and Baynes J (1994) Maillard reactions in chemistry, food, and health. Spec Publ - R Soc Chem 151.

Lee WLS, Shalita AR and Poh-Fitzpatrick MB (1978) Comparative studies of porphyrin production in Propionibacterium acnes and Propionibacterium granulosum. J Bacteriol 133, 811-815.

Lynch E and Beighton D (1994) A comparison of primary root caries lesions classified according to colour. Caries Res 28, 233-239.

Malone WF, Bell C and Massler M (1966) Physicochemical characteristics of active and arrested carious lesions of dentin. J Dent Res 45, 16-26.

Marquis RE (1995) Oxygen metabolism, oxidative stress and acid-base physiology of dental plaque biofilms. J Ind Microbiol 15, 198-207.

Meyer J and Baume LJ (1966) Pathologie de la mélanodontie infantile, de l'odontoclasie et de la carie circulaire. Schw Monatsschr Zahnheilk 76, 48-91.

Murata M, Nakajima Y and Homma S (1995) Inhibition of cariogenic glucan synthesis by dark beer. Lebensm Wiss Technol 28, 201-207.

Nathoo SA and Gaffar S (1995) Studies on dental stains induced by antibacterial agents and rational approaches for bleaching dental stains. Adv Dent Res 9, 462-470.

Nordbö H (1971) Discoloration of human teeth by a combination of chlorhexidine and aldehydes or ketones in vitro. Scand J Dent Res 79, 356-361.

Nordbö H, Sörensen R and Sönju T (1977) Furfurals in chlorhexidine-discolored pellicle. Scand J Dent Res 85, 606-609.

Nordbö H, Eriksen HM and Rölla G (1979) Affinity of hydroxyapatite for furfural and a brown pigment formed by furfural and glycine. Scand J Dent Res 87, 395-397.

Nordh F (1969) Siderochromes in saliva in persons with high and low caries frequency. Acta Odontol Scand 27, 493-504.

Opdyke DLJ (1962) The histochemistry of dental decay. Arch Oral Biol 7, 207-219.

Ripa LW (1977) The nature of the carious lesion. In: Oral hygiene in oral health (eds. Goldberg HJV and Ripa LW), Chap. 7, pp. 233-255. Thomas, Springfield IL, USA.

Sakanaka S, Shimura N, Aizawa M, Kim M and Yamamoto T (1992) Preventive effect of green tea polyphenols against dental caries in conventional rats. Biosci Biotech Biochem 56, 592-594.

Schüpbach P, Lutz F and Guggenheim B (1992) Human root caries: histopathology of arrested lesions. Caries Res 26, 153-164.

Seltzer S and Bender IB (1965) The dental pulp. Biological considerations in dental procedures, pp. 117-119. Lippincott, Philadelphia PA, USA.

Shah HN, Bonnett R, Mateen B and Williams RAD (1979) The porphyrin pigmentation of subspecies of Bacteroides melaninogenicus. Biochem J 180, 45-50.

Slots J (1974) The microflora of black stain on human primary teeth. Scand J Dent Res 84, 484-490.

Stangel I, Valdes E and Xu J (1996) Absorption of iron by dentin: its role in discoloration. J Biomed Mat Res 31, 287-292.

Stanley HR, Weisman MI, Michanowicz AE and Bellizzi R (1978) Ischemic infarction of the pulp: sequential degenerative changes of the pulp after traumatic injury. J Endodont 4, 325-335.

Torell P (1957a) Determination of iron in dental enamel. Odont Tidskr 65, 20-23.

Torell P (1957b) The substance of arrested caries in relation to intact enamel. Odont Tidskr 65, 28-35.

Van Reenen JF (1955) The yellow-brown pigmentation of dental caries. A survey of the literature. J Dent Assoc S Afr 10, 262-267.

Chapter 4

THE MAILLARD REACTION IN DEMINERALIZED DENTIN IN VITRO*

GA Kleter, JJM Damen, MJ Buijs and JM ten Cate

Abstract. - The Maillard reaction between carbohydrate and protein has been proposed as a cause of the browning of carious lesions. The aim of the present investigation was to determine the occurrence of this reaction in bovine dentin collagen in vitro and to establish the effect of the reaction on the proteolytic degradation of bovine dentin collagen in vitro. Slices of demineralized bovine dentin were incubated with 0.2 M glucose or buffer for ten weeks at 37°C. The formation of initial (furosine) and advanced (pentosidine) products of the Maillard reaction in dentin exposed to glucose was confirmed by HPLC. After reduction with $NaBH_4$ to prevent intermediate Maillard products from further reaction, slices were either degraded with collagenase for fluorescence measurement or incubated with trypsin or pepsin to assess enzymatic degradation. Fluorescence characteristic for the Maillard reaction increased in glucose-exposed slices. Degradation of collagen by pepsin, but not by trypsin, was greatly depressed following glucose pretreatment. This may indicate an altered sensitivity to proteolytic degradation; the Maillard reaction thus has a potential role in caries arrestment.

INTRODUCTION

Brown staining of a lesion is a well known phenomenon in dental caries. Even further, the severity of the discoloration has been related to the stage and activity of the disease. Although active black lesions have recently been described (Lynch and Beighton, 1994), caries is generally considered still active in a soft or leathery brownish lesion, but arrested in a hard black lesion (Banting, 1991; Fejerskov and Nyvad, 1986). One explanation for the browning involves the Maillard reaction (Armstrong, 1964; Dreizen et al., 1964). The mechanisms of this reaction between carbohydrate and protein are under extensive investigation because of its contribution to the browning of heated foods and its involvement in diabetic pathology (Van Boekel, 1991).

Collagen is the main protein of the organic matrix in dentin and, once demineralized, it is accessible to proteolytic enzymes (Klont et al., 1991). A number of observations, however, indicate that carious dentin becomes resistant towards enzymatic protein degradation (Young and Massler, 1963). The collagenase-resistant part is rich in carbohydrate

* European Journal of Oral Sciences 105 (1997) 278-284

(Armstrong, 1960a), implying a role for the Maillard reaction in the arrestment of caries.

Several markers for the Maillard reaction have been described in the literature. For example, the product initially formed between glucose and lysine is partly transformed into furosine (Heyns et al., 1968) on acid hydrolysis. Conversely, the fluorescent amino acid pentosidine (Sell and Monnier, 1989) is an advanced glycation endproduct (AGE) and may form covalent bonds between proteins (cross-linking). Furthermore, the Maillard reaction leads to an increase in characteristic fluorescence (excitation 370 nm, emission 440 nm) (Monnier et al., 1984; Pongor et al., 1984).

Apart from the anomalous cross-linking resulting from the Maillard reaction, the relative proportions of physiological cross-links present in normal dentin collagen may undergo changes during caries. Labile difunctional cross-links diminish, probably because they dissociate by the action of acid (Kuboki et al., 1977). Another possibility is the formation of mature products from difunctional cross-links as reported in vitro (Davis et al., 1975; Deshmukh et al., 1971; Robins and Bailey, 1977), such as pyridinoline cross-links (Eyre, 1981; Uchiyama et al., 1981), which are naturally present in bovine dentin (Eyre and Oguchi, 1980; Linde and Robins, 1988; Walters and Eyre, 1983; Yamauchi et al., 1992).

The aim of this study was to find proof that the Maillard reaction can take place in demineralized dentin. Markers for the initial and advanced Maillard reaction as well as physiological cross-links were investigated after incubation of bovine dentin with glucose. Changes in susceptibility to protein-degrading enzymes and in fluorescence specific for the reaction were additionally measured.

Materials and Methods

Materials. All reagents were analytical grade. Solutions were prepared in demineralized water. Central incisors were extracted from lower jaws of four-year old cows obtained from a local slaughterhouse. Pentosidine was a kind gift from Prof. V. Monnier, Case Western Reserve University, Cleveland OH, USA. Furosine was from Neosystem, Strasbourg, France, and hydroxylysylpyridinoline and lysylpyridinoline from Metra Biosystems, Palo Alto CA, USA.

Dentin slices. Enamel-dentin specimens were prepared by drilling a hollow cylinder through the crowns of the incisors. Dentin slices (diameter 6 mm, thickness 0.50 mm) were made as transversal sections with a water-cooled diamond wire sawing machine (Well, model 3142; W. Ebner,

Mannheim, Germany). The slices were kept refrigerated in 5 mM sodium azide. Demineralization was done in 0.5 M EDTA, pH 7.4, at 4°C, while the release of calcium was measured by atomic absorption spectrometry. Mean collagen mass of the experimental slices was 3.33 ± 0.36 mg or 11.1 ± 1.2 nmol.

Experimental. An overview of the experimental procedures is given in figure 1.

Glucose incubation. Individual slices were incubated in 4.0 ml of 50 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, in distilled water with or without 0.2 M glucose at 37°C in 5-ml culture tubes with Teflon-sided screw caps for ten weeks. A droplet of toluene was added to prevent bacterial growth.

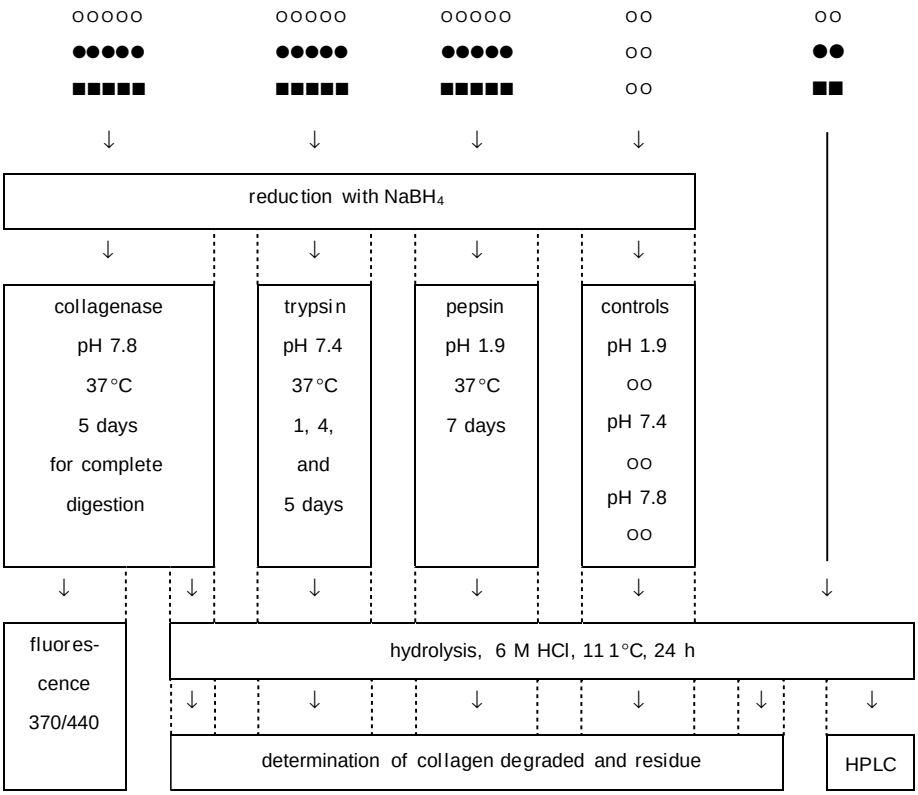


Figure 1
Overview of the experiment. O*: non-exposed dentin slice;* ●*: dentin slice exposed to phosphate buffer pH 7.4;* ■*: dentin slice exposed to glucose.*

Reduction. To prevent the formation of advanced Maillard products from initial products during incubations following the reaction with glucose, $NaBH_4$ reduction of the initial products was carried out, except for slices to be analyzed by HPLC after hydrolysis. Slices were reduced in 0.50 ml 0.9% NaCl in 50 mM NaH_2PO_4 / Na_2HPO_4, pH 7.4, by addition of 50 µl $NaBH_4$ in 0.01 M NaOH (5 mg / ml). After mixing for one hour, 100 µl acetic acid / water (1:1, by vol.) was added and mixed for 20 minutes to inactivate borohydride. Slices were washed in the appropiate buffer solutions prior to incubations with collagenase, trypsin, and pepsin.

Protease incubation. Reduced slices were immersed in the following solutions with a drop of toluene in 5-ml culture tubes with Teflon-sided screw caps at 37°C:

- *collagenase* (C-0773, Sigma Chem. Co., St. Louis MO, USA) from *Clostridium histolyticum*, highly purified, 400 U / ml (collagen digestion) in 50 mM HEPES, 0.2 M NaCl, 10 mM $CaCl_2$, pH 7.8, 2.0 ml / sample, 72 ± 5 U / nmol collagen, incubation for 5 days to obtain complete digests,
- *trypsin* (Sigma T-8642) from bovine pancreas, type XIII, TPCK treated, 11500 BAEE U / mg, 0.50 mg / ml in 50 mM HEPES, 10 mM $CaCl_2$, pH 7.4, 1.0 ml / sample, $(0.51 \pm 0.07)\cdot 10^3$ U / nmol collagen, incubation for 1, 4, and 5 days,
- *pepsin* (Sigma P-6887) from pig stomach mucosa, 3200 U / mg (hemoglobin digestion), 0.48 mg / ml in 0.01 M HCl, 1.0 ml / sample, $(0.14 \pm 0.02)\cdot 10^3$ U / nmol collagen, incubation for 7 days.

The incubation conditions for trypsin and pepsin were chosen after preliminary tests showed that measurable amounts of collagen were released into the incubation solutions. In trypsin incubations, degradation is fastest on day 1 and decreasing in velocity thereafter. Slices digested extensively by pepsin were fragile. Intermittent changes of pepsin solutions were therefore avoided.

Fluorescence. Collagenase digests of dentin collagen were sixty-fold diluted in 50 mM HEPES, pH 7.4. Measurements were made in 3-ml quartz cuvettes with a Waters 470 fluorescence detector with a JASCO cuvette accessory, connected to a strip chart recorder. Emission spectra (380-700 nm) were measured at 370 nm excitation. For measurements at fixed excitation- and emission wavelengths 370 / 440 nm (excitation and emission bandwidth 18 nm), fluorescence was read four minutes after cuvet insertion, corrected for contribution by the collagenase containing buffer (≤ 57%), and calculated as permillage of the fluorescence of 3.0 µM tyrosine in 50 mM HEPES, pH 7.4, at 275 / 303 nm. The results were normalized to a collagen concentration of 20 µg / ml for comparison.

Hydrolysis. To 0.50 ml of a protease solution or a dentin slice in 0.50 ml water was added 0.50 ml 12 M HCl. Nitrogen was blown over the solutions and the tubes were closed and heated at 111°C for 24 hours in a hot air oven. Aliquots were dried in triplicate in vacuo over NaOH for collagen determination and HPLC analysis.

Collagen determination. In order to calculate the amount of collagen in a sample, hydroxyproline was measured in acid hydrolyzates (Jamall et al., 1981). After oxidation, hydroxyproline reacts with p-dimethylaminobenzaldehyde to give a red colour, which is measured by spectrophotometry. For bovine dentin, collagen mass was estimated as 8.0 × the hydroxyproline value as calculated from previous data (Volpin and Veis, 1973). To calculate the molar quantities of collagen, a molecular mass of 300 kDa was used.

HPLC. The system for high performance liquid chromatography consisted of two pumps for gradient formation, a manual injector, a column heater (75°C) with a 120 × 4.6 mm Merck (Darmstadt, Germany) Polyspher AA NA column (strong cation exchange), a pump for reagent delivery, a mixing tee with reaction coil, and a fluorescence detector (Waters, Milford MA, USA).

Samples were dissolved in 67 mM citric acid solution, pH 2 (HNO_3), with 0.3 mM thymol and one of the internal standards: 4.0 µM pyridoxine (fluorescent cross-links) or 24 µM homoarginine (furosine), filtered, and 100 µl was injected. Eluent A: 67 mM citric acid, 0.3 mM thymol, pH 3.0 (HNO_3) for analysis of pyridinolines and pentosidine, but pH 5.0 for furosine analysis. Eluent B: 247 mM sodium nitrate, 24 mM boric acid, pH 10.2 (NaOH). Acidifying reagent (fluorescent cross-links): 67 mM citric acid, pH 2 (HNO_3). OPA-reagent (furosine): 5 mM o-phtalic dialdehyde (OPA) dissolved in ethanol, 20 mM N-acetylcysteine, and 7 mM sodium N-lauroylsarcosinate in 0.5 M $K_2B_4O_7$, pH 10. OPA-reagent was stored overnight prior to use and shielded from light. Gradient composition and detection methods are given in table 1.

Table 1
Gradients employed for the HPLC analysis of Maillard products and physiological cross-links.

analyte	hydroxylysylpyridinoline lysylpyridinoline pentosidine		furosine	
gradient[a]	pH 3.0 - 10.2		pH 5.0 - 10.2	
	%B	min	%B	min
	0	0	0	0
	50	10	40	10
	70	30	100	90
	82	60	100	110
	92	110	0	115
	100	115		
	100	135		
	0	140		
reagent[a]	67 mM citric acid, pH 2		OPA-reagent, pH 10	
detection[b]	297 / 395[c]; 335 / 385[d]		330 / 440	
internal standard	0.40 nmol pyridoxine		2.4 nmol homoarginine	

[a] buffers A, B, and reagents: see "Materials and methods", A+B 0.20 ml / min, reagent 0.20 ml / min; [b] fluorescence, $\lambda_{ex} / \lambda_{em}$ (nm); [c] for detection of hydroxylysylpyridinoline, lysylpyridinoline, and pyridoxine; [d] for detection of pentosidine.

RESULTS

The slices turned pale yellow by exposure to glucose. Slices incubated in buffer showed no discoloration.

Fluorescence. Upon excitation at 370 nm, the emission spectrum from the digests of glucose-exposed dentin slices showed a broad peak (maximum 420 nm), which was strongly increased compared with the background peak in buffer-exposed dentin. This background peak was no contamination of buffer salts, since it was also present in demineralized water. The broad peak at 420 nm also overlapped a broad shoulder peak at 480 nm that occurred in the buffer-exposed dentin (fig. 2). The 370 / 440 nm fluorescence of glucose-exposed dentin slices was significantly increased compared with controls (table 2).

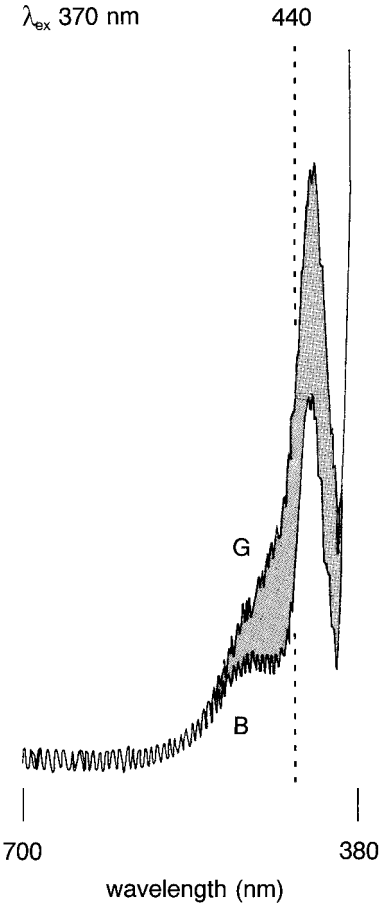


Figure 2
Emission spectra of buffer-exposed (B) and glucose-exposed (G) dentin collagenolysates. 200 pmol of digested collagen in 3 ml buffer pH 7.4, scanspeed 3.6 nm/s.

Table 2
Fluorescence of collagenase digests (200 pmol collagen / 3 ml) of dentin slices (Ø 6 mm, thickness 0.5 mm) incubated with glucose and buffer during ten weeks at 37°C, and non-incubated controls (n=5), λ_{ex} 370 nm, λ_{em} 440 nm, pH 7.4, expressed as 1 / 1000 of fluorescence of 3.0 µM tyrosine, pH 7.4, λ_{ex} 275 nm, λ_{em} 303 nm.

incubation	fluorescence
glucose	12.1 ± 1.5
buffer	3.0 ± 0.1[a]
control	3.1 ± 0.5[a]

[a] P<0.001 vs. glucose.

HPLC. Furosine and pentosidine, indicators of the initial and advanced stages of the Maillard reaction, respectively, increased in glucose-exposed slices (figs. 3,4; table 3). Hydroxylysylpyridinoline apparently did not form in the demineralized dentin at 37°C, as it did not increase in dentin exposed at 37°C (table 3).

Table 3
Furosine and fluorescent cross-links (mol amino acid / mol collagen, except pentosidine: mmol / mol) determined by HPLC in hydrolyzates of dentin slices exposed to glucose and buffer, pH 7.4, and non-exposed controls (n=2).

incubation	amino acid			
	furosine	hydroxylysylpyridinoline	lysylpyridinoline	pentosidine
glucose	2.1; 2.1	0.26; 0.29	ND	14.4; 15.3
buffer	ND	0.32; 0.27	ND	ND
control	ND	0.36; 0.28	ND	ND

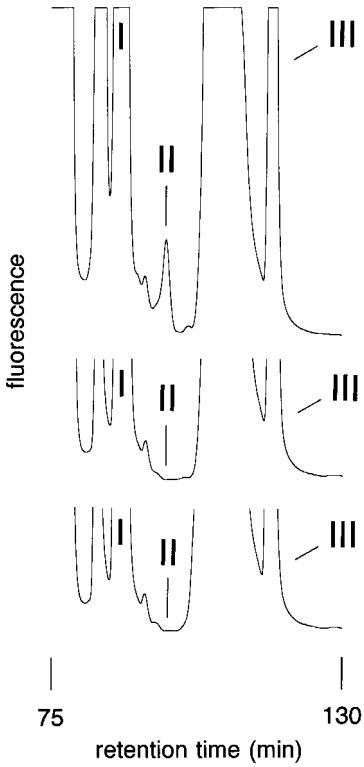
ND: non-detectable.

Collagen degradation. Trypsin-dependent loss of collagen was lower in glucose-treated dentin after ten days, but not significantly. A noticeable difference was seen for pepsin with 58% less degradation after incubation with glucose (table 4). A ghost of the dentin slice was left after collagenase digestion of one slice previously exposed to buffer and after digestion of all control slices. Such ghosts contained no detectable collagen.

Table 4
Proteolytic degradation of collagen in demineralized dentin slices after incubation with glucose or buffer, or non-incubated controls (n=5).

	% degradation (mean ± SD)			
	trypsin			pepsin
pretreatment	1 day	5 days	10 days	7 days
glucose	14.1 ± 3.4	28.2 ± 7.6	37.7 ± 9.9	36.6 ± 8.5
buffer	24.2 ± 11.1	40.3 ± 15.9	49.6 ± 17.3	90.1 ± 1.1[b]
control	20.9 ± 4.4[a]	37.2 ± 7.1	47.3 ± 7.8	86.5 ± 6.0[b]

[a] $P<0.05$, [b] $P<0.001$ vs. glucose pretreatment.



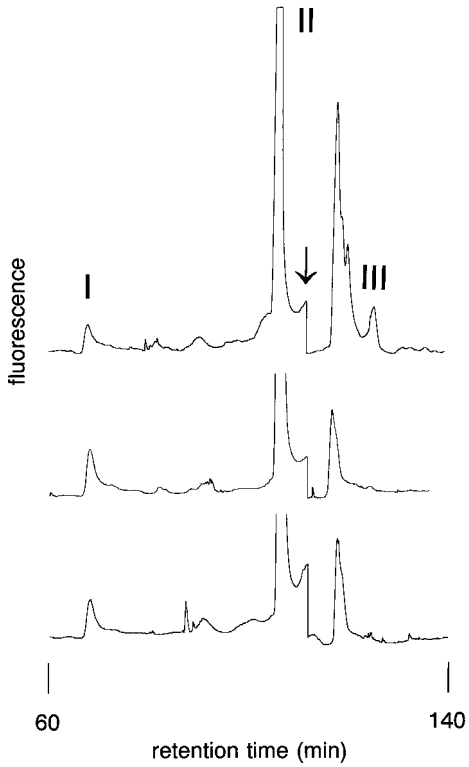


Figure 3

HPLC analysis of furosine (peak II) in hydrolyzates of non-exposed- (bottom), buffer-exposed (middle), and glucose-exposed (top) dentin samples. Dentin was not reduced prior to hydrolysis. Only the relevant parts of the chromatograms are shown. Amino acids are visualized after post-column labelling with a fluorescent dye. I lysine, II furosine, III homoarginine (internal standard). Column: Merck Polyspher AA-NA 120 × 4.6 mm; flow 0.2 ml/min; gradient pH 5.0 - 10.2; post-column reagent 0.2 ml/min; fluorescence λ_{ex} 330 nm, λ_{em} 440 nm; 100-µl injections in buffer pH 2.

Figure 4

As figure 3, except that intrinsically fluorescent cross-links were separated by another gradient and detected by their own fluorescence. I hydroxylysylpyridinoline, II pyridoxine (internal standard), III pentosidine, ↓ shift in detector settings. Lysylpyridinoline at 74 minutes was not observed. Gradient pH 3.0 -10.2; post-column reagent (pH 2) added for enhancement of pentosidine fluorescence; fluorescence detection λ_{ex} /λ_{em} (nm): 297/395 for hydroxylysylpyridinoline and pyridoxine, 335/385 for pentosidine.

Discussion

The aim of this study was to verify previous conclusions that dentin collagen resists enzymatic attack after reaction with glucose. The results show that, following the Maillard reaction, dentin collagen indeed is less degraded by pepsin, but not by trypsin.

After ten weeks of incubation with glucose at 37°C, notable increases were observed in markers for the initial (furosine) and advanced (pentosidine) Maillard reaction, and the characteristic 370/440 nm fluorescence (figs. 2-4; tables 2, 3). The fluorescence emission peak was composed of a sharp (420 nm) and a broad peak (480 nm), of which the first increased in height after glucose exposure. It could not be determined from the emission spectrum if the increase was caused by either a higher peak at 420 nm or an additional peak at 440 nm. The level of pentosidine was approximately 0.01 mol/mol collagen, in the same range as previously observed in rat tail tendon collagen in vitro (Bailey et al., 1995). The same concentration of pentosidine was found in skin collagen of aged (12 years) cattle (Monnier and Sell, 1994). Although the reported increase of pentosidine alone probably has little effect on the properties of collagen, its formation is accompanied by a considerable increase of non-fluorescent cross-links (Bailey et al., 1995). This is very likely also the case in our system, since the level of pentosidine cannot account for the effect of the reaction with glucose on degradation by pepsin (see below).

Trypsin and pepsin were used to test the degradabilities of dentin collagen after the various treatments. Trypsin is a serine protease, active at neutral pH, whereas pepsin is a carboxylic protease, active in an acid environment. Carious dentin shows proteolytic activity at acid, neutral, and alkaline pH values (Larmas, 1972). The production of proteases has been demonstrated in oral pathogens, for example a trypsin-like protease in *Porhyromonas gingivalis* (Minhas and Greenman, 1989) and a carboxylic protease in *Candida albicans* (Rüchel, 1981). In addition, root caries lesions may be in contact with the gingival crevicular fluid, which contains host enzymes such as the serine protease cathepsin G (Tervahartiala et al., 1996) and the carboxylic protease cathepsin D (Cimasoni et al., 1977). Dentin collagen differs from other collagens in showing susceptibility towards trypsin and resistance against pepsin (Scott and Leaver, 1974; Carmichael et al., 1977). The degradation of dentin collagen by pepsin observed here is likely caused by acid denaturation (pH 2) of collagen during the incubation at 37°C, similar to the complete degradation of dentin collagen by trypsin after heat denaturation (Kuboki et al., 1981). It is unlikely that the pH in plaque or in a caries lesion will approach that of our pepsin incubations. Nevertheless, the experimental conditions allow a shorter period for simulation of

denaturation of dentin collagen and degradation by acid proteases during prolonged acid exposure in a caries lesion. The trypsin and pepsin incubations thus represent two extremes, which may occur during caries development in dentin.

Both trypsin and pepsin act on the non-helical and denatured segments of collagen molecules. It seems therefore paradoxal that the reaction of collagen with glucose inhibits collagen degradation by pepsin, as observed previously with tendon collagen at 37°C (Fu et al., 1994; Tian et al., 1996), but not by trypsin. This can be explained, however, by assuming that cross-links introduced by glucosylation will protect denatured but not highly ordered collagen, for example by inhibiting diffusion through the denatured protein gel (Yapel et al., 1994).

Surprisingly, glucose-exposed dentin slices were completely dissolved by collagenase, in contrast with the complete resistance reported elsewhere (Armstrong, 1960b). This variance can however be explained by the relatively extreme conditions employed previously, such as high concentrations of glucose and dentin powder, and a lower relative humidity. Even more, the relative low humidity was found to be necessary for colour development, which is at variance with the yellow discoloration observed here after incubation with aqueous glucose.

In conclusion, the Maillard reaction renders the dentin collagen less susceptible to the action of pepsin, but not trypsin. This implies a role for the reaction in caries, since dentin collagen is likely denatured under long lasting acid conditions like in the incubations with pepsin, thereby exposing itself to protein-degrading enzymes. The exposed collagen therefore is the playing field for the competition between proteolytic degradation and protection by Maillard reaction dependent cross-linking. Analysis of discolored dentin of natural lesions will be required to establish the extent of the Maillard reaction.

Acknowledgement. The authors wish to thank for valuable contributions: N. Sakké (TNO, Leiden, NL) and A. Lammens.

References

Armstrong WG (1960a) Carbohydrate material in carious dentine - (I): preliminary investigations. Arch Oral Biol 2, 69-77.

Armstrong WG (1960b) Reaction in vitro of sound dentine with glucose, glucosamine and carbohydrate fermentation and degradation products. Arch Oral Biol 5, 179-189.

Armstrong WG (1964) Modifications of the properties and composition of the

dentin matrix caused by dental caries. Adv Oral Biol 1, 309-332.

Bailey AJ, Sims TJ, Avery NC and Halligan EP (1995) Non-enzymatic glycation of fibrous collagen: reaction products of glucose and ribose. Biochem J 305, 385-390.

Banting DW (1991) Management of dental caries in the older patient. In: Geriatric dentistry (eds. Papas AS, Niessen LC and Chauncey HH), Chap. 9, pp. 141-167. Mosby Year Book, St. Louis MO, USA.

Carmichael DJ, Dodd CM and Veis A (1977) The solubilization of bone and dentin collagens by pepsin; effect of cross-linkages and non-collagen components. Biochim Biophys Acta 491, 177-192.

Cimasoni G, Ishikawa I and Jaccard F (1977) Enzyme activity in the gingival crevice. In: The borderland between caries and periodontal disease (ed. Lehner T), pp. 13-41. Academic, London UK.

Davis NR, Risen OM and Pringle GA (1975) Stable, nonreducible cross-links of mature collagen. Biochemistry 14, 2031-2036.

Deshmukh A, Deshmukh K and Nimni ME (1971) Synthesis of aldehydes and their interactions during the in vitro aging of collagen. Biochemistry 10, 2337-2342.

Dreizen S, Spirakis CN and Stone RE (1964) In vitro studies of the chromogenic reactions between carbohydrate derivatives and the amino acids common to human enamel and dentine. Arch Oral Biol 9, 733-737.

Eyre DR (1981) Crosslink maturation in bone collagen. Dev Biochem 22, 51-55.

Eyre DR and Oguchi H (1980) The hydroxypyridinium crosslinks of skeletal collagens: their measurement, properties and a proposed pathway of formation. Biochem Biophys Res Comm 92, 403-410.

Fejerskov O and Nyvad B (1986) Pathology and treatment of dental caries in the aging individual. In: Geriatric dentistry (eds. Holm-Pedersen P and Löe H), Chap. 19, pp. 238-262. Munksgaard, Copenhagen, Denmark.

Fu MX, Wells-Knecht KJ, Blackledge JA, Lyons TJ, Thorpe SR and Baynes JW (1994) Kinetics, mechanisms, and inhibition of late stages of the Maillard reaction. Diabetes 43, 676-683.

Heyns K, Heukeshoven J and Brose KH (1968) Degradation of fructose amino acids to N-(2-furoylmethyl)amino acids. Intermediates in the browning reaction. Angew Chem Int Ed 7, 628-629.

Jamall IS, Finelli VN and Que Hee SS (1981) A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. Anal Biochem 112, 70-75.

Klont B, Damen JJM and Ten Cate JM (1991) Degradation of bovine incisor root collagen in an in vitro caries model. Arch Oral Biol 36, 299-304.

Kuboki Y, Ohgushi K and Fusayama T (1977) Collagen biochemistry of the two layers of carious dentin. J Dent Res 56, 1233-1237.

Kuboki Y, Tsuzaki M, Sasaki S, Liu CF and Mechanic GL (1981) Location of intermolecular cross-links in bovine dentin collagen, solubilization with trypsin and

isolation of cross-link peptides containing dihydroxylysinonorleucine and pyridinoline. Biochem Biophys Res Comm 102, 119-126.

Larmas M (1972) Observations on endopeptidases in human carious dentin. Scand J Dent Res 80, 520-523.

Linde A and Robins SP (1988) Quantitative assessment of collagen crosslinks in dissected predentin and dentin. Collagen Rel Res 8, 443-450.

Lynch E and Beighton D (1994) A comparison of primary root caries lesions classified according to colour. Caries Res 28, 233-239.

Minhas T and Greenman J (1989) Production of cell-bound and vesicle-associated trypsin-like protease, alkaline phosphatase and N-acetyl--glucosaminidase by Bacteroides gingivalis strain W50. J Gen Microbiol 135, 557-564.

Monnier VM, Kohn RR and Cerami A (1984) Accelerated age-related browning of human collagen in diabetes mellitus. Proc Natl Acad Sci USA 81, 583-587.

Monnier VM and Sell DR (1994) The advanced Maillard reaction in aging and age-related diseases probed with pentosidine. Spec Publ - R Soc Chem 151, 235-243.

Pongor S, Ulrich PC, Bencsath FA and Cerami A (1984) Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. Proc Natl Acad Sci USA 81, 2684-2688.

Robins SP and Bailey AJ (1977) Some observations on the ageing in vitro of reprecipitated collagen fibres. Biochim Biophys Acta 492, 408-414.

Rüchel R (1981) Properties of a purified proteinase from the yeast Candida albicans. Biochim Biophys Acta 659, 99-113.

Scott PG and Leaver AG (1974) The degradation of human dentine collagen by trypsin. Connect Tissue Res 2, 299-307.

Sell DR and Monnier VM (1989) Structure elucidation of a senescence cross-link from human extracellular matrix; implication of pentoses in the aging process. J Biol Chem 264, 21597-21602.

Tervahartiala T, Konttinen YT, Ingman T, Häyrinen-Immonen R, Ding Y and Sorsa T (1996) Cathepsin G in gingival tissue and crevicular fluid in adult periodontitis. J Clin Period 23, 68-75.

Tian SF, Toda S, Higashimo H and Matsumara S (1996) Glycation decreases the stability of the triple-helical strands of fibrous collagen against proteolytic degradation by pepsin in a specific temperature range. J Biochem 120, 1153-1162.

Uchiyama A, Inoue T and Fujimoto D (1981) Synthesis of pyridinoline during in vitro aging of bone collagen. J Biochem 90, 1795-1798.

Van Boekel MAM (1991) The role of glycation in aging and diabetes mellitus. Mol Biol Rep 15, 57-64.

Volpin D and Veis A (1973) Cyanogen bromide peptides from insoluble skin and dentin bovine collagens. Biochemistry 12, 1452-1464.

Walters C and Eyre DR (1983) Collagen crosslinks in human dentin: increasing content of hydroxypyridinium residues with age. Calcif Tissue Int 35, 401-405.

Yamauchi M, Chandler GS and Katz EP (1992) Collagen cross-linking and mineralization. Int Congr Ser - Excerpta Med 1002, 39-46.

Yapel RA, Duda JL, Lin X and Von Meerwall ED (1994) Mutual and self-diffusion of water in gelatin: experimental measurement and predictive test of free-volume theory. Polymer 35, 2411-2416.

Young AM and Massler M (1963) Some physical chemical characteristics of carious dentine. Br Dent J 115, 406-412.

Chapter 5

MODIFICATION OF AMINO ACID RESIDUES IN CARIOUS DENTIN MATRIX*

GA Kleter, JJM Damen, MJ Buijs and JM ten Cate

Abstract. - The Maillard reaction between sugar and protein has been postulated as the cause for the browning and arrestment of carious lesions. This reaction has been implicated as cause for decreased degradability of collagen in vivo. Aim of the present study was to verify the occurrence of the reaction in vivo. Carious and sound dentin were taken from extracted human teeth and analyzed for fluorescence characteristic for the Maillard reaction and oxidation, and for Maillard products by HPLC. In addition, physiological cross-links were analyzed by HPLC. Oxidation- and Maillard reaction-related fluorescence increased in collagenase digests from carious dentin. Advanced Maillard products (carboxymethyllysine and pentosidine) increased, whereas furosine, a marker for the initial reaction, was not observed consistently. This implies no direct addition of sugars to protein, but rather the addition of smaller metabolites and glycoxidation products. In addition, the physiological cross-links hydroxylysinonorleucine and dihydroxylysinonorleucine decreased in carious dentin. Also for hydroxylysylpyridinoline a decrease was observed, but not consistently. In conclusion, the caries process modifies amino acids in dentin collagen, which can lead to increased resistance against proteolysis and ultimately to caries arrestment.

INTRODUCTION

Dentin discoloration is a common indicator of caries and more specifically the stage of the carious process. Thus a soft light-brown spot reflects an ongoing decay whereas a hard shiny black spot characterizes lesion arrestment.

The Maillard reaction between sugar and protein has been implicated as cause for this discoloration (Armstrong, 1964). The initial product between carbohydrate and a protein amino group can enter a cascade of reactions to ultimately form brownish polymers, cross-linked proteins, and advanced glycation end products (AGEs). Few products from this myriad have been characterized in the Maillard reaction under physiological conditions. Several AGEs, although colorless, have been used as markers for the advanced stages of the Maillard reaction throughout the literature. In dentin caries, the reaction has been implicated as the cause

for the increased resistance of dentin collagen against collagenolytic breakdown (Armstrong, 1964).

In this investigation we determined if two AGEs, pentosidine (Sell and Monnier, 1989) and carboxymethyllysine (Ahmed et al., 1986, Liardon et al., 1987), were present in carious dentin from extracted human teeth. In addition, furosine was measured, which forms during acid hydrolysis from the initial addition products of glucose to lysine (Heyns et al., 1968). It is a marker of the early stage of the Maillard reaction. Fluorescence related to the Maillard reaction and dityrosine were measured in collagen digests from carious dentin. Dityrosine is a fluorescent cross-linking amino acid formed by oxidation of proteins, such as collagen (LaBella et al., 1968). Physiological collagen cross-links were concomitantly studied since cross-links inhibit the enzymatic degradation of dentin collagen (Carmichael et al., 1977).

MATERIALS AND METHODS

Chemicals. All reagents were analytical grade unless specified differently. Bacterial high-purity collagenase was from Sigma (type VII). Pyridinoline cross-links were from Metra Biosystems (Palo Alto CA, USA), and furosine from Neosystem (Strasbourg, France). Carboxymethyllysine was kindly donated by Dr. P.A. Finot (Nestlé Research, Vevey, Switzerland), dityrosine by Dr. S.C. Fry (University of Edinburgh, United Kingdom), and pentosidine by Prof. V.M. Monnier (Case Western Reserve University, Cleveland OH, USA). Solutions were prepared in demineralized water.

Preparation of collagenolytic digests and hydrolyzates. Three batches (I-III) of extracted teeth with carious dentin were obtained from the Department of Oral Surgery of the Free University of Amsterdam. The project was approved by the regulating bodies of ACTA and the Department of Oral Surgery. Immediately after extraction, the teeth were transferred to plastic vials containing 5 mM sodium azide in demineralized water, and kept refrigerated. Specimens containing extensive caries lesions extending into the dentin of root and crown were selected. The batches I-III thus selected contained nine (age 20-73, median 25), seven (age 29-48, median 46), and four (age 29-43, median 29) teeth. Having been carefully dried under an airstream from a handpiece, the selected teeth were photographed under a stereomicroscope. Excavated carious dentin and a disc (Ø 6 mm, thickness 0.5 mm) of sound root dentin from each element were demineralized in 0.5 M EDTA, pH 7.4 (I, II) or 0.1 M acetic acid (III), at 4°C. The progress of demineralization was assessed as the amount of calcium released into the solution determined by atomic absorption

spectrometry. All carious dentin in our experiment was considered still active in caries, since it could be removed by excavator.

The organic material from specimens was chemically reduced to prevent the Maillard reaction from continuing during the subsequent collagenase-incubations and to stabilize labile difunctional physiological cross-links prior to hydrolysis. In addition, this reduction prevents the formation of carboxymethyllysine during hydrolysis of the initial Maillard product of lysine. Fully demineralized specimens from batches I and II were reduced in 0.50 ml 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4, with 0.25 mg $NaBH_4$ in 50 µl 0.01 M NaOH for one hour at room temperature. Borohydride was inactivated by addition of 100 µl HAc / H_2O (1:1 by vol.).

The specimens of batch I were digested by collagenase at 37°C for 7 days in culture tubes with Teflon-sided screw caps containing 2 ml 400 U / ml collagenase in 50 mM hydroxyethylpiperazine ethylsulfonic acid (HEPES), 0.2 M NaCl, 0.25 mM $CaCl_2$, pH 7.8. Toluene was added to prevent bacterial growth. Each digest was centrifuged and the supernatant was filtered (0.22 µm, Millipore Millex GV). 0.50 ml of the supernatants was mixed with an equal amount of concentrated hydrochloric acid (36.5-38% (Baker "Instra-Analyzed"), final concentration 6 M) in a culture tube with a Teflon-sided scew cap. Nitrogen was blown over the solutions before the tubes were closed and heated for 24 hours at 110°C in a hot air oven. Pellets were washed three times with water, mixed with 2.0 ml 6 M hydrochloric acid, and likewise hydrolyzed. After hydrolysis triplicate samples were dried in vacuo over NaOH for the determination of amino acid (as ninhydrin reactivity) - and collagen (as hydroxyproline) content.

For batch II the dissolution by collagenase was omitted and reduced specimens were hydrolyzed after addition of 0.50 ml demineralized water and 0.50 ml concentrated HCl (Baker "Instra-Analyzed"). This enabled the HPLC analysis of non-fluorescent cross-linking amino acids since acid hydrolysis of collagenase-containing solutions would yield interfering artifacts.

For the specimens of batch III, both the collagenase and borohydride treatments were omitted to enable analysis of furosine, which is formed by acid hydrolysis of the non-reduced glucose-lysine condensation product.

Fluorescence measurements. Collagenase digests (I) were diluted in the appropriate buffer in duplo to 60 µg collagen equivalents per 3.0 ml, corresponding to 200 pmol of collagen (MW 300 kDa). The following buffers were used:

- 50 mM H_3PO_4, pH 2.0, for fluorescence 328 / 378 (excitation / emission wavelength, nm),

- 50 mM HEPES, pH 7.4, for fluorescence 370/440,
- 50 mM $Na_2B_4O_7$, pH 10.0, for fluorescence 317/407.

The 328/378- and 370/440 fluorescences are characteristic for pentosidine and Maillard products, respectively (Dyer et al., 1991b) while the 317/407 fluorescence indicates dityrosine formation (Huggins et al., 1993).

Measurements were performed on a Waters 470 HPLC fluorescence detector equipped with a JASCO cuvette accessory and connected to a Perkin Elmer 561 strip chart recorder. Excitation and emission bandwidths were 18 nm. Emission spectra were measured for the three excitation wavelengths mentioned above and emission starting from 10 nm higher than excitation up to 700 nm. Fluorescence at fixed wavelengths was measured four minutes after cuvette insertion and expressed as permillage of the 275/303 fluorescence of 3.0 µM tyrosine in 50 mM HEPES, pH 7.4. Corrections were made for buffer- and blank collagenase fluorescence, and for signal attenuation.

Ninhydrin assay. Our method to determine reactivity towards ninhydrin was a modification of a method described previously (Moore and Stein, 1954; Moore, 1968). Briefly, dried samples were dissolved in 0.10 ml 0.1 M acetic acid and mixed with an equal volume of ninhydrin reagent (Sigma). After 15 minutes in a boiling water bath, samples were diluted with 0.80 ml ethanol/water (1:1 by vol.) and measured for absorbance at 550 nm on a flow-through spectrophotometer (Vitatron). Standards containing 0-0.6 mM leucine in 0.1 M HAc were included. Values were therefore calculated as leucine-equivalents.

Hydroxyproline assay. Hydroxyproline was measured largely according to Jamall et al. (1981). Dried samples were dissolved in 0.60 ml 50% (w/v) 2-propanol at least 30 minutes prior to further additions. To each sample 0.10 ml chloramine T reagent (5.6 mg/ml acetate-citrate buffer pH 6) and 0.50 ml Ehrlich's reagent ((0.9 g p-dimethylaminobenzaldehyde/ml 60% $HClO_4$) / 2-propanol = 3:11 by vol.) was added, with 10-20 minutes between the additions. After color development at 50°C for 1.5 hours, absorption was measured on a flow-through spectrophotometer at 550 nm. Appropriate standards containing hydroxyproline were included. For calculation of collagen content, it was assumed that collagen mass was 7.1 times the hydroxyproline mass, as calculated from the composition of human dentin collagen (Nkhumeleni et al., 1992). The molecular mass of collagen was assumed to be 300 kDa.

HPLC. The conditions applied for the analysis of hydrolyzates from different series are summarized in table 1.

Table 1
Conditions employed for HPLC analysis of dentin hydrolyzates

	batch I				batch II				batch III	
Analyses	CML		HP, LP, Pent		CML, (D)HLNL		HP, LP, Pent		Fur	
Injection	autosampler		autosampler		manual		manual		manual	
Injected volume	2.00 ml		2.00 ml		100 µl		100 µl		100 µl	
Collagen[a]	10 µg		50 µg		20 µg		50 µg		44 µg	
Internal standard	2.0 nmol hArg		0.40 nmol Pyd		2.4 nmol hArg		0.40 nmol Pyd		2.4 nmol hArg	
Gradient (A+B 0.20 ml/min)	%B	min	%B	min	%B	min	%B	min	%B	min
	0	0	0	0	0	0	0	0	0	0
	45	10	50	10	45	10	50	10	40	10
	55	48	70	30	52	48	70	30	100	90
	95	58	82	60	70	68	82	60	100	110
	100	73	92	110	82	88	92	110	0	115
	100	93	100	115	87	123	100	115		
	0	98	100	135	95	128	100	135		
			0	140	100	143	0	140		
					100	163				
					0	168				
Reaction coil[b]	1.9 m		0.7 m		1.9 m		0.7 m		1.9 m	
Reagent	OPA pH 10		CitrAc pH 2		OPA pH 10		CitrAc pH 2		OPA pH 10	
Detection, nm	330/440		297/395[c] and 335/385[d]		330/440		297/395[c] and 335/385[d]		330/440	

[a] collagen equivalents injected (mol. wt. 300 kDa); [b] ID = 0.75 mm; [c] for pyridinolines and pyridoxine; [d] for pentosidine; CML = carboxymethyllysine, (D)HLNL = (di)hydroxylysinonorleucine, Fur = furosine, HP = hydroxylysylpyridinoline, LP = lysylpyridinoline, Pent = pentosidine, hArg = homoarginine, Pyd = pyridoxine, A and B = eluents (see text), OPA pH 10 = reagent (see text), CitrAc pH 2 = acidifying reagent (see text); reagents delivered post-column at 0.2 ml/min.

The HPLC gradient system consisted of two pumps for solvent delivery, an injector, a 120 x 4.6 mm Polyspher AA-NA column (Merck, 75°C), a pump for reagent delivery, a mixing tee with reaction coil, and a fluorescence detector lined to an integrator (Waters).

Eluent buffers: A: 0.067 M sodium citrate, 0.33 mM thymol, pH 3.0, except pH 5.0 for furosine analysis; B: 0.25 M sodium nitrate, 0.024 M boric acid, pH 10.2 (pH adjusted with NaOH and HNO_3).

Reagents: OPA-reagent: 5 mM o-phtalic dialdehyde, 0.020 M N-acetylcysteine, 7 mM sodium lauroylsarcosinate, 0.5 M potassium borate, pH 10; acidifying reagent: 0.067 M sodium citrate, pH 2.0.

Sample dilution buffer: 0.067 M sodium citrate, 0.33 mM thymol, pH 2.0, plus the internal standard (see table 1): 0.40 nmol pyridoxine or 2.0-2.4 nmol homoarginine per injection.

In the analysis of carboxymethyllysine and non-fluorescent cross-links, the OPA-reagent reacts with amino acids eluting from the column into fluorescent compounds. Acidifying reagent was added yielding a final pH 2.6, to enhance pentosidine fluorescence. The injector, gradient, reagent, and reaction coil differed depending on the sample analyzed (table 1). Dried samples of acid protein hydrolyzates were dissolved in sample dilution buffer and filtered.

For calculation, it was assumed that 1 mol of hydroxylysinonorleucine, dihydroxylysinonorleucine, and carboxymethyllysine corresponded with 2, 2, and 1 mol of leucine equivalents, respectively, in the standard solutions as determined by the manual ninhydrin assay.

Results

Fluorescence measurements. Table 2 shows the average fluorescence values obtained for carious and sound dentin from extracted teeth at the three excitation / emission wavelengths.

Fluorescence increased significantly in carious samples compared with sound samples, especially at 370 / 440, indicative of the Maillard reaction. Figure 1 displays emission spectra of digests of sound and carious dentin from the same tooth.

Table 2
Fluorescence measured in collagenase digests of sound and carious dentin of extracted human teeth.

λ_{ex} / λ_{em} (nm)	fluorescence (arbitrary units)[a]		suspected fluorophore
	sound	carious[b]	
317 / 407	18 ± 3	35 ± 9	dityrosine
328 / 378	6 ± 2	16 ± 4	pentosidine
370 / 440	3 ± 1	17 ± 5	Maillard products

[a] mean values ± standard deviation (n=9); [b] $P<0.001$ for all fluorescences.

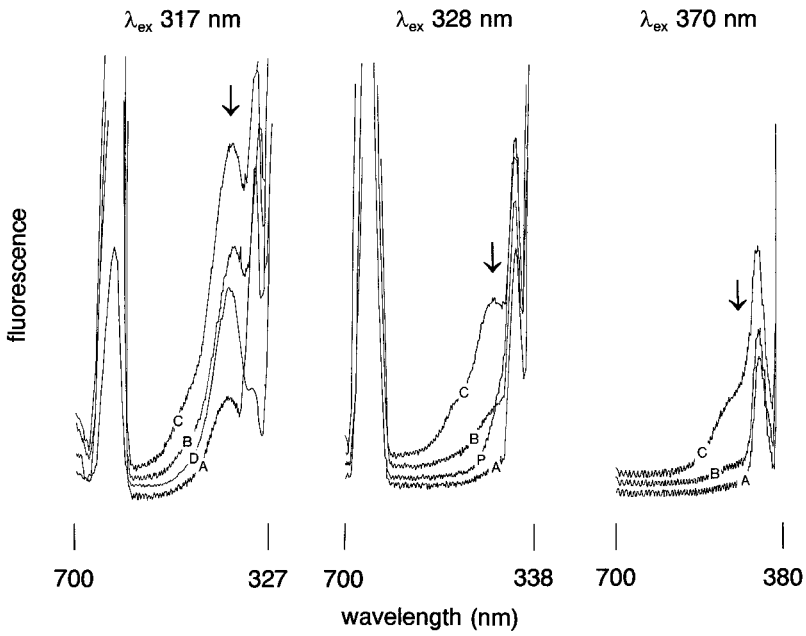


Figure 1
Emission spectra of blank (buffer, A), standards (D, 0.28 µM dityrosine at λ_{ex} 317 nm; P, 3.3 nM pentosidine at λ_{ex} 328 nm), digested sound dentin (B), and carious dentin (C) of a tooth from series I. Note the increase of fluorescence in carious dentin (↓).

HPLC analysis. Results of the analyses of physiological cross-links and Maillard products are summarized in table 3. Representative HPLC separations of the hydrolyzate of one dentin specimen from batch II are depicted in figures 2 and 3. In carious dentin from batch I, carboxymethyllysine was significantly elevated as was pentosidine in batches I and II. Since carboxymethyllysine was indistinguishable from a large flanking glucosamine peak in most carious specimens from batch II, no values are given in table 3. The physiological cross-links dihydroxylysinonorleucine and hydroxylysinonorleucine were decreased significantly in carious dentin (fig. 2). Hydroxylysylpyridinoline (fig. 3) decreased on average but significantly only in one group. For lysylpyridinoline no significant effect was observed (fig. 3). The latter, however, co-elutes with dityrosine having resembling fluorescence properties, which hampers the determination of either one.

Furosine was only observed in two carious samples out of four, with 0.22 and 0.24 mol furosine / mol collagen, representing a very low level of lysine glycosylation (fig. 4).

Table 3
Cross-link and Maillard product contents of sound and carious dentin of extracted teeth[a].

dentin	sound	carious
I, collagenase-digested specimens (n=9)		
fluorescent amino acids		
hydroxylysylpyridinoline	0.68 ± 0.12	0.41 ± 0.16[b]
lysylpyridinoline	0.07 ± 0.02	0.07 ± 0.03
pentosidine	2.6 ± 1.2	4.6 ± 1.1[c]
carboxymethyllysine	0.42 ± 0.06	0.96 ± 0.34[c]
II, non-digested specimens (n=7)		
fluorescent amino acids		
hydroxylysylpyridinoline	0.52 ± 0.07	0.45 ± 0.08
lysylpyridinoline	0.05 ± 0.02	0.05 ± 0.01
pentosidine	1.8 ± 0.7	3.3 ± 0.5[d]
non-fluorescent amino acids		
hydroxylysinonorleucine	0.16 ± 0.04	0.08 ± 0.03[c]
dihydroxylysinonorleucine	0.37 ± 0.05	0.16 ± 0.04[b]

[a] mol / mol collagen, except for pentosidine: mmol / mol collagen; mean values ± standard deviation; [b] $P<0.001$; [c] $P<0.01$; [d] $P<0.02$.

Figure 2
HPLC analysis of hydrolyzed sound (bottom) and carious (middle) dentin of a tooth from series II, and an amino acid standard (top).
Non-fluorescent amino acids were converted into fluorescence by a post-column reaction. Injection 67 pmol collagen of dentin hydrolyzates.
I carboxymethyllysine,
II hydroxylysylpyridinoline,
IV dityrosine,
VI dihydroxylysinonorleucine,
VII hydroxylysinonorleucine,
IX hydroxylysine,
X lysinoalanine,
III, V, and VIII unknown amino acids from bovine dentin.

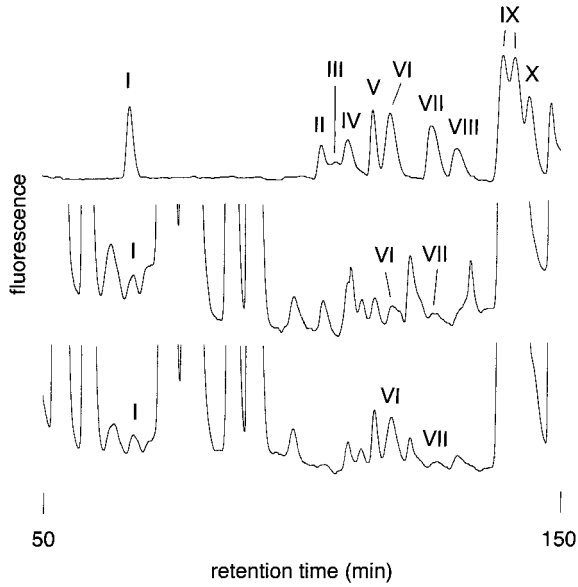
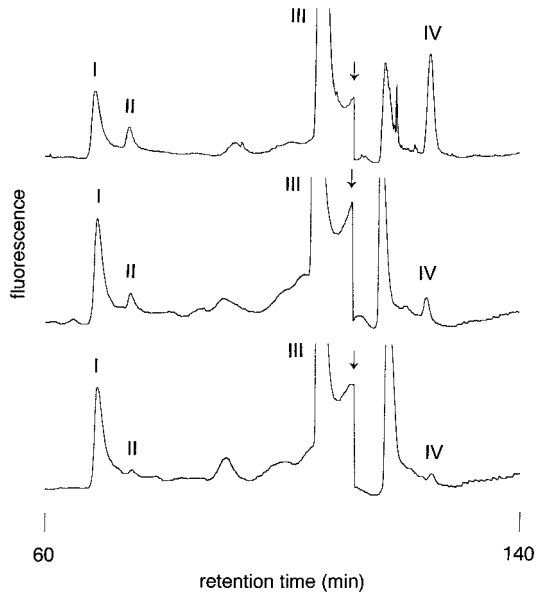


Figure 3
HPLC of fluorescent amino acids from same hydrolyzate of sound (bottom) and carious (middle) dentin as in figure 2, and of a standard mixture (top). Compounds were detected after post-column acidification. Injection 167 pmol collagen of dentin hydrolyzates.
I hydroxylysylpyridinoline,
II lysylpyridinoline,
III pyridoxine (internal standard),
IV pentosidine,
↓ shift in detector settings.



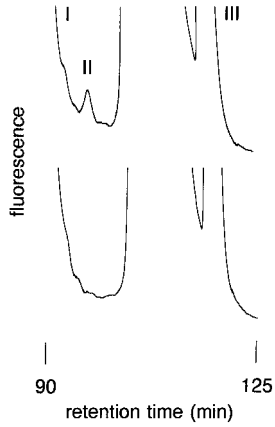


Figure 4
HPLC of non-reduced sound (bottom) and carious dentin (top) hydrolyzates from series III. Injection 148 pmol collagen. Detection as in figure 2.
I lysine, II furosine, III homoarginine (internal standard).

DISCUSSION

In the present investigation we sought to clarify the purported relation between dentin caries and matrix protein modifications such as the Maillard reaction and cross-linking.

The increase in either physiological-, oxidative stress-, and Maillard reaction-related peptide cross-linking is characterized by a concomitant increase in UV-fluorescence. The most pronounced increase in carious dentin was observed for the 370 / 440 fluorescence, generally associated with the Maillard reaction (table 2). The emission spectra (fig. 1) revealed the appearance of broad bands at 420, 410, and 410 nm concomitant with increases in 370 / 440, 328 / 378, and 317 / 407 fluorescence, respectively. The broad maxima correspond to those in previous reports (Dyer et al., 1991a; Huggins et al., 1993). The emission peak about 410 nm at 317 nm excitation would indicate the formation of dityrosine in carious dentin. The physiological pyridinoline cross-links resemble dityrosine in their fluorescence, but because no increase was found in the level of the major pyridinoline, hydroxylysylpyridinoline, by HPLC (table 3), the pyridinolines are not responsible for the higher fluorescence. The increase in the 328 / 378 fluorescence was associated with the merge of a new peak at 410 nm, which differed from that of pentosidine coalescing with the background peak at 370 nm (fig. 1) indicating a dityrosine-like fluorescence. Fluorescence alone cannot serve as proof of the Maillard reaction, however, since lipid peroxidation yields similar fluorescences as pentosidine- and Maillard products (Hidalgo and Zamora, 1993; Odetti et al., 1994).

For the analysis of Maillard reaction-related amino acids and cross-links in collagen, ion exchange chromatography by HPLC was conducted. This method is advantageous in requiring no prior clean-up of samples contrary to previously published methods, enabling a more reliable analyte quantification. Efforts were made to develop a gradient system, which allowed for the separation of the cross-links from the major amino acids, but we succeeded only in the reliable separation of dihydroxylysinonorleucine and hydroxylysinonorleucine. In addition, in hydrolyzates from specimens not pretreated with collagenase, bacterial contamination may preclude the analysis of certain amino acids. For example, the analysis of carboxymethyllysine in batch II was hampered by a large glucosamine peak, probably from bacterial polysaccharides.

The increase of carboxymethyllysine (batch I) and pentosidine (batches I and II) thus observed provided additional proof for the Maillard reaction in caries (table 3, figs. 2, 3). The pentosidine level ranged from about equal to a manifold of the level in sound dentin. The formation of pentosidine can only account for a fraction of the increase in 328/378 fluorescence, which is in accordance with a major contribution from a different fluorophore as stated above. Unfortunately, an increase of dityrosine as expected from the gain in 317/407 fluorescence (table 2) could not be substantiated unequivocally by HPLC analysis because dityrosine co-eluted with lysylpyridinoline. Even if we would consider dityrosine to originate the lysylpyridinoline peaks observed in HPLC of carious dentin, but not of sound dentin, only one quarter of the increase in 317/407 fluorescence would derive from dityrosine.

Surprisingly, only little furosine was detected in carious dentin (fig. 4). This implies that the advanced Maillard products must have formed through other reactions than the addition of sugars like glucose to lysine. This, however, would be in sharp contrast with the previously reported increase of dentin matrix glycosylation in caries (Kuboki et al., 1977; Higashi, 1979). Two explanations can be provided. First, sugars and related compounds, such as ascorbic acid, can be oxidized to shorter intermediates, which react with proteins to form carboxymethyllysine and pentosidine (Dunn et al., 1990; Slight et al., 1992; Wells-Knecht et al., 1995a,b). Second, short aldehyde metabolites from the glyoxylate cycle (Cioni et al., 1981) and glycolysis can be released by lysis of microbial cells caused, for example, by serum complement (Frank et al., 1987) or lysozyme (Pollock et al., 1987). Some of these aldehydes are capable of cross-linking collagen (Milch, 1963). Carboxymethyllysine could for instance be formed from glyoxal (Al-Abed and Bucala, 1995; Glomb and Monnier, 1995) and from glyoxylic acid (Acharya and Manning, 1983; King et al., 1977). Dihydroxyaceton and glyceraldehyde are potential pentosidine precursors (Dyer et al., 1991b).

Interestingly, sound human dentin contained a more or less constant level of the advanced Maillard products, pentosidine and carboxymethyllysine, irrespective of age (table 3). Although human dentin collagen is not turned over and the advanced products should thus increase during life, we suggest that the Maillard reaction occurs in predentin collagen, but is halted upon mineralization .

The decrease of the cross-link hydroxylysylpyridinoline (table 3, fig. 3) in carious specimens was unexpected since cross-linked regions of collagen fibrils are likely more resistant towards degradation (Kronick and Maleeff, 1990). Furthermore, incubation at physiological temperatures of demineralized collagens leads to pyridinoline cross-link formation (Eyre, 1981; Uchiyama et al., 1981). Deposition of new collagen by odontoblasts underneath the lesion front (Levine, 1972) and oxidation of the pyridinol moiety could both account for this divergence . The decrease of the difunctional cross-link dihydroxylysinonorleucine in carious dentin (table 3, fig. 2) thus cannot be simply explained by formation of hydroxylysylpyridinoline, but rather by dissociation of covalent bonds (Kuboki et al., 1977). The level of lysylpyridinoline was relatively low and no significant change was observed. The cross-link levels as a whole were relatively high compared with previous reports (Davis, 1973; Rivera and Yamauchi, 1993; Walters and Eyre, 1983).

Two opposing reactions thus likely occur in caries, namely the decrease in physiological cross-links and the formation of new cross-links in the advanced stages of the Maillard reaction. It is conceivable that the latter will prevail after a prolonged period.

In conclusion, the dentin matrix, like many tissue proteins, is subject to the Maillard reaction. For dentin, this reaction can only occur once the protein becomes exposed by demineralization during caries. The Maillard reaction of the organic matrix can influence the formation of dentin lesions in two ways. First, dentin collagen is likely to denature during long-term acid exposure during caries, thereby rendering previously resistant molecule segments susceptible to aspecific protein degrading enzymes. Degradation of dentin matrix promotes lesion demineralization (Chapter 2) and cavitation (Clarkson et al., 1986; Katz et al., 1987). The Maillard reaction with glucose in vitro renders demineralized dentin collagen more resistant to proteolytic breakdown (Chapter 4). Second, dentin demineralizes less after the reaction with the carbonyl compound glutardialdehyde (Boonstra et al., 1993). The Maillard reaction therefore likely inhibits lesion progression in discolored dentin.

Acknowledgement. The authors wish to thank M. Kuyck , Y. Klompmaker, and D.M. Stroink (Free University, Amsterdam) for supplying the extracted teeth and N. Verzijl (TNO, Leiden), A. Lammens, L.B. Peters, Dr. M.P.

Rudolphy, W. van der Borden, Dr. A.J.P. van Strijp, and Dr. K. Weerheijm for technical assistance. The work in this study was financially supported by the Netherlands Institute for Dental Sciences and the Academic Center for Dentistry Amsterdam.

REFERENCES

Acharya SA and Manning JM (1983) Reaction of glycolaldehyde with proteins: latent crosslinking potential of α-hydroxyaldehydes. Proc Natl Acad Sci USA 80, 3590-3594.

Ahmed MU, Thorpe SR and Baynes JW (1986) Identification of N-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. J Biol Chem 261, 4889-4894.

Al-Abed Y and Bucala R (1995) N-carboxymethyllysine formation by direct addition of glyoxal to lysine during the Maillard reaction. Bioorg Med Chem Lett 5, 2161-2162.

Armstrong WG (1964) Modification of the properties and composition of the dentin matrix caused by dental caries. Advances Oral Biol 1, 309-332.

Boonstra WD, De Vries J, Ten Bosch JJ, Ögaard B and Arends J (1993) Inhibition of bovine dentin demineralization by a glutardialdehyde pretreatment: an in vitro caries study. Scand J Dent Res 101, 72-77.

Carmichael DJ, Dodd CM and Veis A (1977) The solubilization of bone and dentin collagens by pepsin. Effect of cross-linkages and non-collagen components. Biochim Biophys Acta 491, 177-192.

Cioni M, Pinzauti G and Vanni P (1981) Comparative biochemistry of the glyoxalate cycle. Comp Biochem Physiol B 70B, 1-26.

Clarkson BH, Hall DL, Heilman JR and Wefel JS (1986) Effect of proteolytic enzymes on caries lesion formation in vitro. J Oral Pathol 15, 423-429.

Davis NR (1973) A method for the determination of lysine-derived collagen crosslinks. Biochem Biophys Res Comm 52, 877-883.

Dunn JA, Ahmed MU, Murtiashaw MH, Richardson JM, Walla MD, Thorpe SR and Baynes JW (1990) Reaction of ascorbate with lysine and protein under autoxidizing conditions: formation of N-(carboxymethyl)lysine by reaction between lysine and products of autoxidation of ascorbate. Biochemistry 29, 10964-10970.

Dyer DG, Blackledge JA, Katz BM, Hull CJ, Adkisson HD, Thorpe SR, Lyons TJ and Baynes JW (1991a) The Maillard reaction in vivo. Z Ernahrungswiss 30, 29-45.

Dyer DG, Blackledge JA, Thorpe SR and Baynes JW (1991b) Formation of pentosidine during nonenzymatic browning of proteins by glucose; identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo. J Biol Chem 266, 11654-11660.

Eyre DR (1981) Crosslink maturation in bone collagen. Dev Biochem 22, 51-55.

Frank MM, Joiner K and Hammer C (1987) The function of antibody and complement in the lysis of bacteria. Rev Infect Dis 9(Suppl 5), S537-S545.

Glomb MA and Monnier VM (1995) Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. J Biol Chem 270, 10017-10026.

Heyns K, Heukeshoven J and Brose KH (1968) Degradation of fructose amino acids to N-(2-furoylmethyl)amino acids. Intermediates in browning reactions. Angew Chem Int Ed 7, 628-629.

Hidalgo FJ and Zamora R (1993) Fluorescent pyrrole products from carbonyl-amine reactions. J Biol Chem 268, 16190-16197.

Higashi C (1979) Calcium binding and calcification induction of organic matrices of the two layers of carious dentin. Kokubyo Gakkai Zasshi 46, 245-255 (Jap).

Huggins TG, Wells-Knecht MC, Detorie NA, Baynes JW and Thorpe SR (1993) Formation of o-tyrosine and dityrosine in proteins during radiolytic and metal-catalyzed oxidation. J Biol Chem 268, 12341-12347.

Jamall IS, Finelli VN and Que Hee SS (1981) A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. Anal Biochem 112, 70-75.

Katz S, Park KK and Palenik CJ (1987) In-vitro root surface caries studies. J Oral Med 42, 40-48.

King TP, Kochoumian L and Lichtenstein LM (1977) Preparation and immunochemical properties of methoxypolyethylene glycol-coupled and N-carboxymethylated derivatives of ragweed pollen allergen, antigen E. Arch Biochem Biophys 178, 442-450.

Kronick P and Maleeff B (1990) Uneven natural cross-linking in hide fibrils. J Am Leather Chem Ass 85, 122-127.

Kuboki Y, Ohgushi K and Fusuyama T (1977) Collagen biochemistry of the two layers of carious dentin. J Dent Res 56, 1233-1237.

LaBella F, Waykole P and Queen G (1968) Formation of insoluble gels and dityrosine by the action of peroxidase on soluble collagens. Biochem Biophys Res Comm 30, 333-338.

Levine RS (1972) The distribution of hydroxyproline in the dentin of carious human teeth. Arch Oral Biol 17, 127-135.

Liardon R, De Weck-Gaudard D, Philippossian G and Finot P-A (1987) Identification of N-carboxymethyllysine: a new Maillard reaction product, in rat urine. J Agr Food Chem 35, 427-431.

Milch RA (1963) Studies of collagen tissue aging: interaction of certain intermediary metabolites with collagen. Gerontologia 7, 129-152.

Moore S (1968) Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the

ninhydrin reaction. J Biol Chem 243, 6281-6283.

Moore S and Stein WH (1954) A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J Biol Chem 211, 907-913.

Nkhumeleni FS, Raubenheimer EJ, Dauth J, Van Heerden WFP, Smith PD and Pitout MJ (1992) Amino acid composition of dentin in permanent human teeth. Arch Oral Biol 37, 157-158.

Odetti P, Pronzato MA, Noberasco G, Cosso L, Traverso N, Cottalasso D and Marinari UM (1994) Relationships between glycation and oxidation related fluorescences in rat collagen during aging. Lab Invest 70, 61-67.

Pollock JJ, Lotardo S, Gavai R and Grossbard BL (1987) Lysozyme-protease-inorganic monovalent anion lysis of oral bacterial strains in buffers and whole stimulated saliva. J Dent Res 66, 467-474.

Rivera EM and Yamauchi M (1993) Site comparisons of dentin collagen cross-links from extracted human teeth. Arch Oral Biol 38, 541-546.

Sell DR and Monnier VM (1989) Structure elucidation of a senescence cross-link from human extracellular matrix; implication of pentoses in the aging process. J Biol Chem 264, 21597-21602.

Slight SH, Prabhakaram M, Shin DB, Feather MS and Ortwerth BJ (1992) The extent of N-(carboxymethyl)lysine formation in lens proteins and polylysine by the autoxidation products of ascorbic acid. Biochim Biophys Acta 1117, 199-206.

Uchiyama A, Inoue T and Fujimoto D (1981) Synthesis of pyridinoline during in vitro aging of bone collagen. J Biochem 90, 1795-1798.

Walters C and Eyre DR (1983) Collagen crosslinks in human dentin: increasing content of hydroxypyridinium residues with age. Calcif Tissue Int 35, 401-405.

Wells-Knecht KJ, Zyzak DV, Litchield JE, Thorpe SR and Baynes JW (1995a) Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. Biochemistry 34, 3702-3709.

Wells-Knecht MC, Thorpe SR and Baynes JW (1995b) Pathways of formation of glycoxidation products during glycation of collagen. Biochemistry 34, 15134-15141.

Chapter 6

A NOVEL PYRROLENINONE CROSS-LINK FROM BOVINE DENTIN*

GA Kleter, JJM Damen, JJ Kettenes-van den Bosch, RA Bank, JM te Koppele, R Fokkens, JR Veraart and JM ten Cate

Abstract. - Aim was to identify suspect cross-links eluting close to known cross-links in ion exchange HPLC. Bovine tooth roots as source of dentin were powdered, demineralized, reduced, and acid-hydrolyzed. Cross-linking amino acids were isolated from the acid hydrolyzate by size exclusion-, adsorption-, and sequential ion exchange-chromatography. In addition to dihydroxylysinonorleucine and hydroxylysylpyridinoline, two unknown cross-links were isolated (IV and V-2). Structures are proposed for these novel trifunctional amino acids, both with a five-membered ring. The structure of V-2 containing a pyrroleninone nucleus was confirmed by ultraviolet, mass, and nuclear magnetic resonance spectrometry.

Abbrevations. BuOH / HAc / H_2O, n-butanol / acetic acid / water = 4:1:1 by volume; CE, capillary electrophoresis; DAD, diode array detection; (Δ-) DHLNL, (didehydro)dihydroxylysinonorleucine; ES, electrospray ; FAB, fast atom bombardment; FMOC, 9-fluorenylmethyloxycarbonyl ; FMOC-Cl, 9-fluorenylmethyl chloroformate; HAL, histidinoalanine; (Δ-)HLNL (didehydro)hydroxylysinonorleucine; HP, hydroxylysylpyridinoline (pyridinoline); Hyl, hydroxylysine; IP, ion-pair; LAL, lysinoalanine; LP, lysylpyridinoline (deoxypyridinoline); MALDI-TOF, matrix assisted laser desorption ionization - time of flight; OPA, o-phtalic dialdehyde; RP, reverse phase; SCX, strong cation exchange.

INTRODUCTION

Dentin is a dental tissue consisting of mineral and organic matter, the latter mostly collagenous. The collagen molecules of dentin are covalently attached to each other through cross-linking amino acid residues. The cross-links reported in collagen of dentin fall into different categories.

One category comprises products of oxidized lysine or hydroxylysine amino acid residues with another lysine or hydroxylysine residue yielding difunctional cross-links. These cross-links may then react with another

oxidized residue to form trifunctional cross-links. The collagen cross-links in dentin are the difunctional didehydro-dihydroxylysinonorleucine (Δ-DHLNL) and didehydro-hydroxylysinonorleucine (Δ-HLNL), and the trifunctional hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) (Davis, 1973; Kuboki et al., 1981; Linde and Robins, 1988; Mechanic et al., 1971; Mechanic et al., 1974; Walters and Eyre, 1983; Yamauchi et al., 1992). Lysinoalanine and histidinoalanine fall into a different category and form links between collagen and phosphoproteins (Fujimoto et al., 1981; Fujimoto et al., 1982). Two additional categories, phosphate diesters and carbohydrates, have been postulated (Schlueter and Veis, 1964). Analogs of hydroxylysylpyridinoline lacking either the ring OH-group (Barber et al., 1982; Tilson et al., 1985) or both OH-groups (Heidemann et al., 1992; Rohn, 1990) have been reported. Another trifunctional cross-link, cyclopentenosine, has also been described. Cyclopentenosine is formed during acid hydrolysis from the condensation product of three oxidized lysine residues (Nakamura et al., 1992; Suyama et al., 1995). An oxidation product of the difunctional Δ-HLNL is believed to decompose into aminoadipic acid in acid hydrolyzates (Bailey et al., 1977; Ranta, 1978). The stabilization of mineralized tissues by cross-linked C-terminal collagen peptides (Light and Bailey, 1985) is brought in conjunction with compound M (Barnard et al., 1987). A trifunctional cross-link with a pyrrole nucleus is formed from the reaction between residues of Δ-DHLNL and oxidized lysine (Kuypers et al., 1992).

In our analyses of hydrolyzed bovine dentin, unidentified amino acid peaks appeared close to the established cross-links in cation exchange HPLC. Two suspect cross-links were purified by size exclusion-, adsorption-, and cation exchange chromatography. The two cross-links were postulated to have a pyrrolic nucleus. The identity of pyrrole cross-links has been deduced previously from positive reactions of cross-linked peptides with azo- and Ehrlich's reagent (Kuypers et al., 1992; Scott et al., 1981; Scott et al., 1983; Horgan et al., 1990). Since such reactions are not specific, it is little surprising that the Ehrlich's reaction has also been attributed to the presence of nucleic acids and covalently bound acidic polysaccharides (Steigmann, 1956; Steigmann, 1964). An Ehrlich-positive pyrrole-2-carboxylic acid is formed from sialic acid by acid hydrolysis of collagen (Deasy, 1961). We therefore employed several spectrometric methods for unequivocal confirmation of the hypothetical structures for the two unknown cross-links. One cross-link was thus identified as a trifunctional amino acid with a pyrroleninone nucleus.

MATERIALS AND METHODS

Chemicals. Sephadex G10 and SP Sephadex C25 were from Pharmacia, CF1 cellulose from Whatman, and silica gel-coated glass plates with concentration zones and the ninhydrin spray reagent from Merck. Dialysis bags were made from Spectra Por 4 dialysis tubing (Spectrum Medical, Los Angeles CA, USA). The ninhydrin reagent for manual determination was from Sigma. All other reagents were analytical grade unless mentioned otherwise.

Solutions were made in distilled water, but for HPLC they were prepared in demineralized water.

Bovine lower jaws were obtained from a local slaughterhouse. Teeth were extracted, adherent soft tissue was removed with a scalpel, and the teeth were incubated in a sodium hypochlorite solution (technical grade) for 2-3 hours. The tooth crowns and roots were separated with a water-cooled circular saw and the roots were kept in tapwater at 4°C until further use.

Root powdering and demineralization. Sixty-five frozen and pulp-free tooth roots were ground in a Waring blender and powdered under liquid nitrogen in a freezer mill (Spex, Edison NJ, USA). The sieved powder (< 450 µm) was demineralized in dialysis bags in 0.5 M EDTA, pH 7.4, at 4°C with regular replacement of the solution. Demineralization was continued until no further calcium release could be detected by atomic absorption spectrometry.

Reduction and hydrolysis. Reduction was necessary to stabilize the acid-labile difunctional collagen cross-links by converting Δ-DHLNL and Δ-HLNL into DHLNL and HLNL, respectively.

The reduction was done essentially as described previously (Robins, 1976). After several washings with 0.9% (w / v) sodium chloride, 0.05 M sodium phosphate, pH 7.4, at 4°C, the stirred demineralized root powder was reduced with 0.71 g sodium borohydride, an estimated one-twentieth of the collagen mass. After one hour, the solution was acidified (pH ≤ 4, pH paper) to inactivate remaining borohydride. Thereafter, the pH was readjusted to 7.5. The solution was centrifuged and the pellets were hydrolyzed by heating for 24 hours at 112°C in 6 M HCl under N_2 in bottles with Teflon-sided screw caps. The dark hydrolyzate was paper-filtered, evaporated under reduced pressure at 50°C, and mixed with 5 ml 0.1 M acetic acid. The hydrolyzate was assayed for hydroxyproline.

Size exclusion chromatography. The concentrated hydrolyzate was applied to a Sephadex G10 column (78 × 1.8 cm, exclusion limit 700 Da) and elut-

ed with 0.1 M acetic acid. The effluent was collected after the first 40-45 ml in twenty 4-ml fractions. Fractions were tested for ninhydrin-reactivity and alternate fractions for cross-links by TLC. Selected fractions were concentrated.

Adsorption chromatography. The concentrated sample in 30 ml eluent (n-butanol / acetic acid / water = 4:1:1 by vol. (BuOH / HAc / H_2O)) was applied to a column of 5 g CF1 cellulose preswollen in eluent. The column was first eluted with BuOH / HAc / H_2O and three 100-ml fractions collected, then with water and four additional 50-ml fractions collected.

The fractions were concentrated and subjected to the ninhydrin assay. Thereafter, fractions eluted with BuOH / HAc / H_2O were combined and concentrated, as were the fractions eluted with water. The presence of cross-links was verified by TLC.

Ion exchange chromatography. Samples were fractionated on SP Sephadex C25 (34 × 2.6 cm), a strong cation exchange medium containing propylsulfonate groups.

Three gradients of 0.0-0.5 M sodium chloride were run consecutively at 4°C in 0.05 M sodium acetate-acetic acid, 1 mM sodium azide, pH 5.25, followed by 0.05 M sodium acetate-acetic acid, 1 mM sodium azide, pH 3.5, and finally by 0.05 M sodium dihydrogen phosphate-disodium hydrogen phosphate (approx. 1:3), 1 mM sodium azide, pH 7.0. After sample application, the column was washed with the starting buffer to remove any non-bound compounds. Elution was continued with the high salt buffer. Fractions of 4 ml were collected and assayed for reactivity towards ninhydrin and for electric conductivity (salt concentration) after 75-fold dilution of a 100-µl aliquot. Ninhydrin-positive fractions were pooled for each peak, concentrated, and desalted by size exclusion chromatography (see above).

Ninhydrin assay. Ninhydrin reactivity was measured essentially as described previously (Moore and Stein, 1954; Moore, 1968): 100-µl aliquots were mixed with an equal volume of ninhydrin reagent in duplicate. After heating in a boiling water bath for 15 minutes and dilution with 0.80 ml ethanol / water (1:1 by vol.), the absorbance at 550 nm was measured with a Vitatron flow-through spectrophotometer. Standards comprised 0.0-0.5 mM leucine.

Hydroxyproline assay. Hydroxyproline was determined according to Jamall et al. (1981). The assay employs p-dimethylaminobenzaldehyde (Ehrlich's reagent), which forms colored products with pyrroles originating from hydroxyproline oxidation. The values thus determined for hydroxyproline mass were multiplied by 8.0 to obtain the corresponding collagen mass.

TLC. Aliquots of samples and standards were run on silica-coated thin layer chromatography (TLC) plates in either n-butanol / acetic acid / water (4:1:1 by vol. (BuOH / HAc / H_2O)) or n-propanol / concentrated ammonia / water (8:1:11, pre-equilibration). These eluents were previously described (Keller et al., 1984) for the two-dimensional TLC-separation of elastin cross-links.

The eluent front was marked on the glass plates prior to drying over a hot plate. Amino acids were visualized as purple spots by application of the ninhydrin spray reagent to the plate and drying with hot air. Amino acids with zero mobility ($R_f = 0$ in BuOH / HAc / H_2O) are assumed to be cross-links (Keller et al., 1984).

Strong cation exchange HPLC. The system consisted of two pumps for high performance liquid chromatography (HPLC), an injector, a Merck Polyspher AA NA 120 × 4.6 mm strong cation exchange (SCX) column (75°C), a pump for post-column reagent delivery, a mixing tee with a reaction coil, and a fluorescence detector (Waters). A two-buffer gradient modified after Pfeifer and Hill (1983) was applied: 0% B, 0 min; 45% B, 10 min; 52% B, 48 min; 70% B, 68 min; 82% B, 88 min; 87% B, 123 min; 95% B, 128 min; 100% B, 143 min; 100% B, 163 min; 0% B, 168 min; A+B = 0.20 ml / min. After separation on the negatively charged column resin, primary amino acids reacted with reagent added post-column (0.2 ml / min) to yield fluorescent peaks. Detector: λ_{ex} 330 nm, λ_{em} 440 nm; A: 67 mM sodium citrate, 0.3 mM thymol, pH 3.0 (HNO_3); B: 247 mM sodium nitrate, 24 mM boric acid, pH 10.2 (NaOH); reagent buffer: 0.5 M $K_2B_4O_7$, pH 10. Thymol and tetraborate were dissolved under heating. One litre of reagent contained 5 mM o-phtalic dialdehyde (OPA) in ethanol, 20 mM N-acetylcysteine, and 7 mM sodium N-lauroylsarcosinate in reagent buffer, and was protected from light and allowed to stabilize overnight.

Reverse phase HPLC of FMOC-amines. Amines were derivatized with 9-fluorenylmethyl chloroformate (FMOC-Cl) at pH 8. Products were extracted with pentane, diluted with 25% (v / v) acetonitrile in buffer pH 8, and analyzed by reverse phase (RP) HPLC on a Varian ODS-80TM column at 40°C with gradient elution and fluorescence detection (λ_{ex} 254 nm, λ_{em} 630 nm) as described elsewhere (Bank et al., 1996).

Ion-pair reverse phase HPLC. Aqueous samples containing pyridoxine as internal standard and 0.5% heptafluorobutyric acid as ion-pairing agent (IP) were injected in an RP-HPLC system with a Varian ODS-80TM column and eluted with 0.15% heptafluorobutyric acid in 24% methanol. Pyridinoline cross-link fluorescence was detected at λ_{ex} 295 nm and λ_{em} 400 nm (Bank et al., 1997).

Mass spectrometry. (+)Fast atom bombardment (FAB) mass spectrometry was carried out with a JEOL JMS-SX/SX102A mass spectrometer. Dried samples were dissolved in methanol-water, mixed with (thio-) glycerol, and applied to a direct insertion probe. During the high resolution FAB-MS measurements, a resolving power of 10,000 (10% valley definition) was used. Cesium iodide, glycerol, or polyethylene oxide (MW_{av} = 600) was used to calibrate the mass spectrometer.

Electrospray (ES) mass spectrometry was carried out with a Fisons platform quadrupole mass spectrometer coupled to a VG Masslynx data system. The samples were introduced into the source by direct injection via a valve loop system. Loop injection was accomplished with a Rheodyne 7125 injector valve, placing a 10-µl loop in the acetonitrile/water stream.

Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was carried out with a PerSeptive Biosystems Voyager-DE-RP MALDI-TOF mass spectrometer. A 337-nm UV nitrogen laser producing 3-ns pulses was used in the reflectron mode. The samples were prepared by mixing 10 µl of a 0.1 M HAc solution of the sample with 20 µl of a solution of 3 mg/l α-cyano-4-hydroxy cinnamic acid in water. One µl of that solution was loaded on the gold-sample plate.

Nuclear magnetic resonance spectrometry. ^{1}H-nuclear magnetic resonance (NMR) spectra of samples in deuterium oxide were run at 500 MHz (IV and V-2) with a Bruker AMX-500 and at 300 MHz (V-2 and D,L-hydroxylysine) with a Gemini-300 (Varian) with the HOD signal at δ 4.8 ppm.

Capillary electrophoresis with diode array detection. Ultraviolet spectra (190-350 nm) were recorded after separation of fractions III, IV, and V-2 by capillary electrophoresis with diode array detection (CE-DAD) in 30 mM phosphoric acid buffer pH 2.3 and pH 7.2. Separations were carried out with a Hewlett-Packard 3D CE equipped with a diode array detector. Samples were introduced under pressure (30 mbar, 10 sec) into a fused silica capillary (effective length 8 cm, total 64.5 cm, ID 50 µm, OD 375 µm) followed by separation at 30 kV.

RESULTS

Chromatographic purification of cross-links. The initial steps of the purification were to separate the cross-links from monomeric amino acids, followed by the separation of individual cross-links. The acid hydrolyzate of demineralized root powder (13.0 g collagen) was separated on a size exclusion column to yield the high molecular weight fractions.

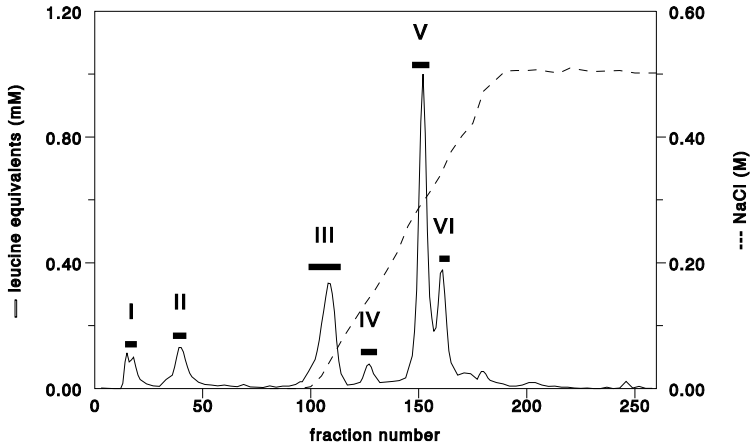


Figure 1
Separation of a purified hydrolyzate of demineralized bovine dentin by cation exchange chromatography at pH 5.25. Gradient 0.0 - 0.5 M NaCl in 0.05 M HAc/NaAc, 1 mM NaN_3, pH 5.25. Column SP Sephadex C25 (34 × 2.6cm). Flow rate approx. 30 ml/h. The 4-ml fractions were assayed for amino acids by ninhydrin reaction (—) and for NaCl by electric conductivity measurement after 75-fold dilution (– –). Fractions collected for further characterization are denoted by bars and Roman numerals.

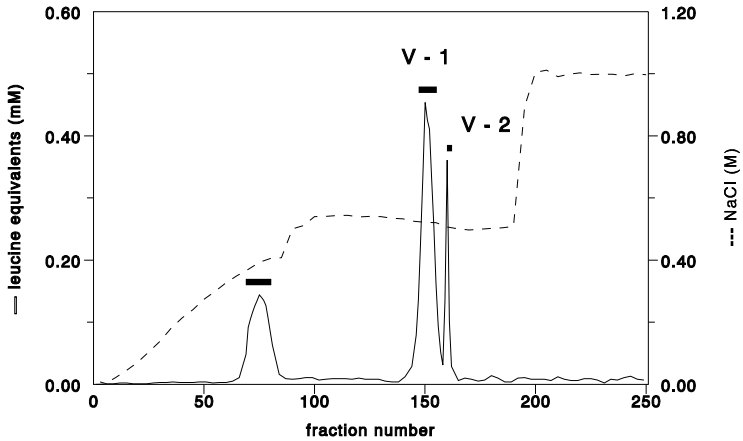


Figure 2
Ion exchange separation of V (fig. 1) at pH 3.5. Same conditions as in figure 1, except pH = 3.5, plus final washing with 1.0 M NaCl in same buffer.

Part of the high molecular weight fractions contained ninhydrin-positive compounds displaying $R_f \approx 0$ on TLC-plates (BuOH / HAc / H_2O), characteristic of cross-links. To remove contaminating common amino acids, the selected fractions were further purified by adsorption chromatography on cellulose. Most of the amino acids eluted with BuOH / HAc / H_2O, accounting for about three quarters of the total reactivity towards ninhydrin. The amino acids eluted with BuOH / HAc / H_2O displayed spots with widely varying mobilities after TLC separation with the same eluent, whereas those eluted with water showed zero and very low mobility.

Ion exchange chromatography at pH 5.25 of the aqueous fractions yielded six peaks, I - VI (fig. 1). Peak V contained a mixture of amino acids (TLC with n-propanol / ammonia / water = 8:1:11 by vol.) (table 1). Therefore, the pooled fractions of V were separated at pH 3.5 (fig. 2). Three peaks were observed, the first probably representing ammonia formed from azide. The second peak (V-1) still displayed a minor impurity on TLC, whereas the third peak (V-2) appeared as a single spot. Peak V-1 could not be separated further by ion exchange chromatography at pH 7.0 (peak V-1-1) .

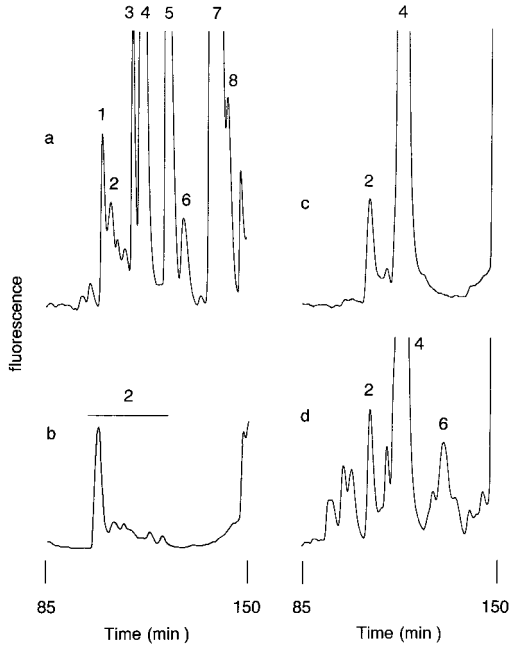


Figure 3
V-2 from acid and alkaline hydrolyzates, SCX-HPLC of amino acids. a: mixture of purified cross-links and hydroxylysine; b: purified cross-link V-2; c: amino acids from an acid hydrolyzate (6 M HCl) of reduced bovine dentin retained on a phenylboronate agarose column after purification as high molecular weight fractions by repeated size exclusion chromatography; d: as c, alkaline hydrolyzate (2 M KOH). Injections (c, d) resulted from 18 and 52 mg collagen originally hydrolyzed, respectively. 1 = III (HP); 2 = V-2; 3 = IV; 4 = V-1-1 (DHLNL); 5 = HLNL (bovine tendon); 6 = VI (histidinoalanine?); 7 = hydroxylysine; 8 = VI (lysinoalanine).

Desalted peak fractions were analyzed by both IP-RP-HPLC and RP-HPLC (derivatized with FMOC-Cl) to investigate the presence of pyridinoline- and lysinonorleucine type cross-links, respectively. Two fractions, III and V-1-1, were found to contain the cross-links HP and DHLNL, respectively. Additional analyses by cation exchange HPLC showed IV and V-2 as closely eluting peaks and VI as a mixture of lysinoalanine and an unidentified amino acid (fig. 3a).

The results of the combined analyses are summarized in table 1.

Fractions III, IV, and V-2 were subjected to mass spectrometry and fractions IV and V-2 to NMR spectroscopy.

Table 1
Chromatographic characteristics of fractions obtained by ion exchange chromatography of dentin hydrolyzate (cf. figures 1 and 2).

Peak #	TLC[a] R_f values		HPLC			Identification
	butanol / acetic acid / water	propanol / ammonia / water	FMOC-amine reverse phase	ion-pair reverse phase	strong cation exchange	
I	~0	0.63	-	-		
II	~0	0.65	unident.	-		
III	~0	0.66	-	HP		HP
IV	~0	0.78	unident.	-		
V	~0	0.55; 0.63				
VI	~0	0.63	-	not sign.	LAL, unident.	LAL, HAL?
V-1		0.54				
V-2		0.61	-	-		
V-1-1		0.50	DHLNL	not sign.		DHLNL

[a]Relative mobility (R_f - value) for each sample is given; FMOC = precolumn derivatization with FMOC-Cl, unident. = unidentified peak, not sign. = not significant peak, - = no fluorescent peak, DHLNL = dihydroxylysinonorleucine, HAL = histidinoalanine, HP = hydroxylysylpyridinoline, LAL = lysinoalanine.

Mass spectrometry. The FAB, ES (fig. 4a), and MALDI-TOF mass spectra of III showed a molecular ion at m/z 429, which confirms the identity as hydroxylysylpyridinoline (HP) (MW 429 Da), as found by IP-RP-HPLC. The (+)FAB mass spectrum clearly shows a signal at m/z 429, where ES-MS further confirmed the presence of HP in III by a molecular ion at m/z 429 and its doubly charged ion. In addition, the MALDI-TOF-MS confirmed the presence of a compound with a MW 429.

High resolution (+)FAB-MS confirmed the molecular ion for HP ($C_{18}H_{29}N_4O_8$ $mass_{calc}$ 429.1985, $mass_{exp}$ 429.1964, diff. -4.9 ppm).

The FAB, ES, and MALDI-TOF mass spectra of V-2 show a large peak at m/z 453, indicating the presence of a sodium cationized molecule with a MW 430. As an example the ES-MS mass spectrum of V-2 is presented in figure 4. High resolution FAB-MS of the $[M+Na]^+$ ion at m/z 453 confirmed the elemental composition of V-2 as $C_{18}H_{30}N_4O_8Na$ ($mass_{calc}$ 453.1961, $mass_{exp}$ 453.1949, diff. -2.8 ppm).

Although the fragmentation patterns of V-2 and IV show some similarities in the (+)FAB-MS spectra, no signal was found at m/z 453 for IV. IV also proved very labile in ES-MS and MALDI-TOF-MS, yielding a large signal at m/z 279 and a signal at m/z 243 in ES-MS, probably due to fragmentation products.

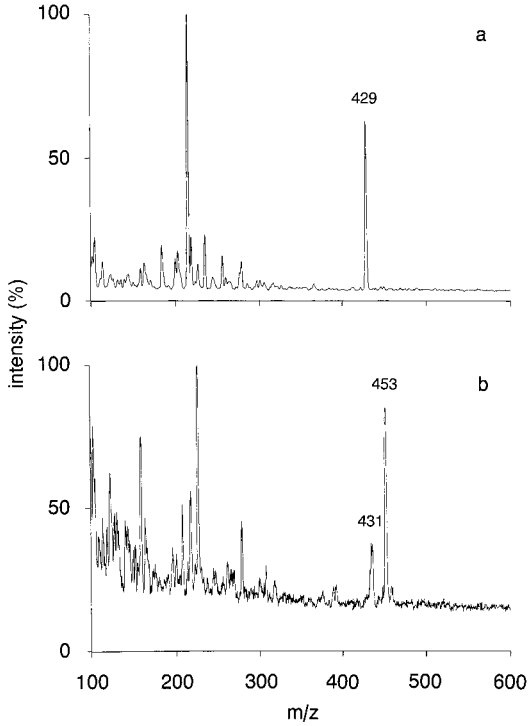


Figure 4
(+) Electrospray mass spectra of III (a) and V-2 (b).

NMR. The 300 MHz ^{1}H-spectrum of V-2 in D_2O is shown in figure 5a. The three multiplets between 1.4 and 2.05 ppm, the two double doublets between 3.4 and 3.7 ppm, and the multiplet between 3.6 and 3.8 ppm comply well with a hydroxylysine substituent at a ring nitrogen (fig. 5b, table 2). No definite conclusion as to the total number of protons can be drawn from the spectrum, since impurities seem to be present, for example singlets at 2.2, 2.05 and 1.95 ppm. The compound V-2 was purified further by ion exchange- and adsorption chromatography and a 500 MHz 1D ^{1}H- (fig. 6b) and a 2D TOCSY-spectrum (in D_2O) were run. Also here, the main spin system present is the hydroxylysine residue (fig. 5b).

Figure 6a shows the 500 MHz spectrum of IV in D_2O with doublets at 7.69 (or 2 overlapping singlets) and 4.46 ppm; a singlet at 7.08 ppm and multiplets at 4.00, 3.22 (dt, J = 15 and 7 Hz) and 3.09 ppm (J = 15 and 4 Hz). The integral ratio for these signals is roughly 1:2:1:2:1:1. In addition, there is a broad signal between 3.7 and 3.9 ppm, and double doublets at 3.68 (J = 12 and 4 Hz) and 3.60 ppm (J = 12 and 7 Hz); the integral ratio for these signals is 1.3:1:1, but the integral of a single signal is about one sixth of that of the signal at 3.09 ppm.

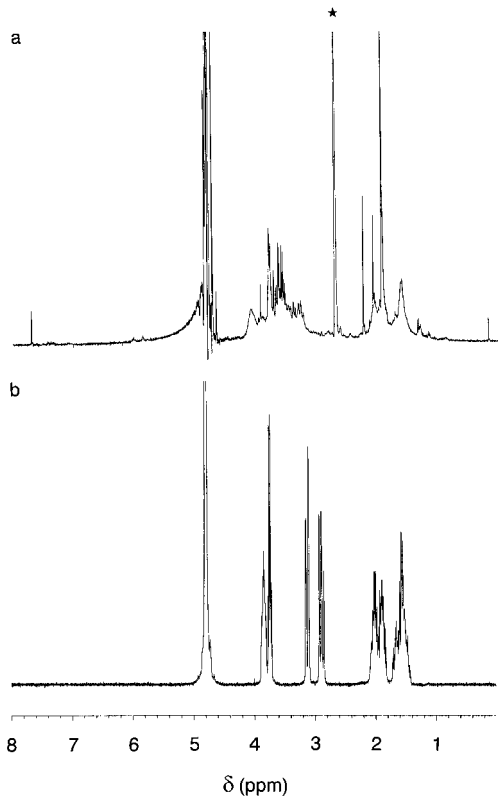


Figure 5
^{1}H-NMR spectra (300 MHz) of samples dissolved in D_2O. a: V-2 (HOD signal suppressed); b: DL-hydroxylysine; ★ residual DMSO signal from an unsuccessful attempt to dissolve V-2 in DMSO-d_6.

Table 2
Assignment 300 MHz spectrum of compound V-2 (numbering of protons, see figure 8, compound V-2). The assigned protons are also found in the 500 MHz spectrum.

δ (ppm)	multiplicity	J (Hz)	assignment
1.4-1.8	m		H_3
1.9	m		H_{4A}
2.0	m		H_{4B}
3.34	dd	12.0 (AB), 6.7 (1,2)	H_{1A}
3.46	dd	12.0 (AB), 4.0 (1,2)	H_{1B}
3.60	m		H_2
3.60	m		H_5

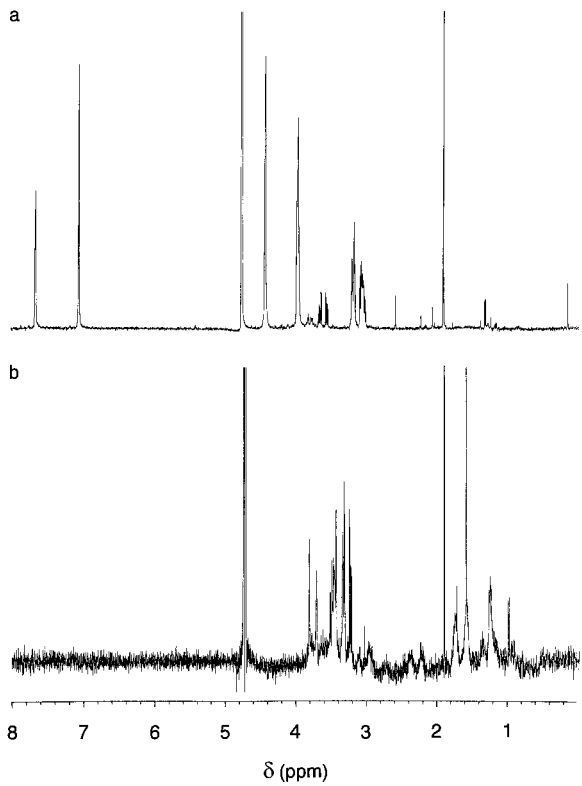


Figure 6
NMR spectra as in figure 5, but spectra sampled at 500 MHz (HOD signal suppressed). a: IV; b: V-2, purified from the sample used for the 300 MHz spectrum.

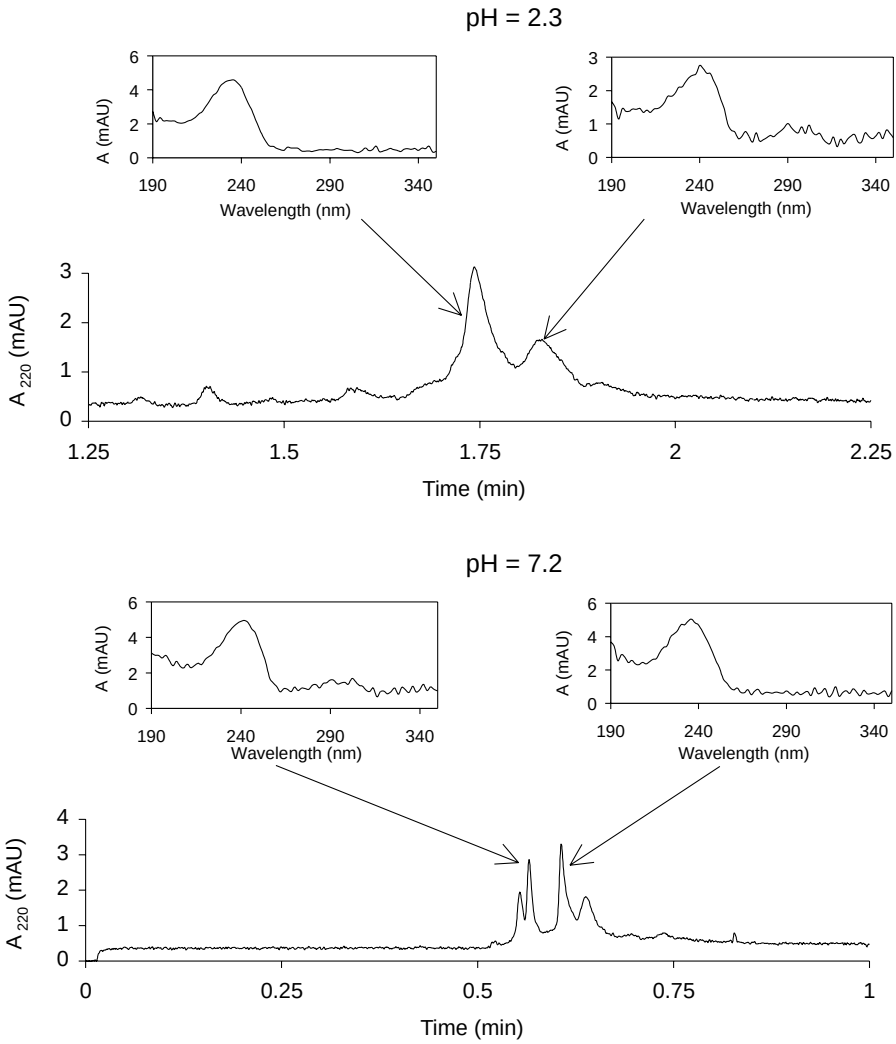


Figure 7
Capillary electrophoresis of V-2 at pH 2.3 and pH 7.2. Inserts: ultraviolet spectra (190-350 nm) of selected fractions. Capillary separation at -30kV with diode array detection.

CE-DAD. Both at pH 2.3 and at pH 7.2 compounds III, IV, and V-2 migrated at comparable velocities, indicative of closely resembling charge densities. The spectra of III at different pH-values were consistent with those found for HP (Sakura et al., 1982). An additional minor fraction preceding HP at pH 2.3 was noted, probably LP. At pH 2.3 IV exhibited a peak at 216 nm, which disappeared at pH 7.2 due to a hypsochromic

shift. Compound V-2 was resolved into closely migrating fractions (fig. 7), with more fractions at pH 7.2 than at pH 2.3. At both pH values one fraction absorbed at 236 nm while another fraction absorbed at 240 nm with a less intense maximum at 290-300 nm (fig. 7). The other two fractions at pH 7.2 did not show a clear maximum above 200 nm.

DISCUSSION

The occurrence of four collagen cross-links in bovine dentin has been described previously (Linde and Robins, 1988; Walters and Eyre, 1983; Yamauchi et al., 1992): didehydro-hydroxylysinonorleucine (Δ-HLNL), didehydro-dihydroxylysinonorleucine (Δ-DHLNL), lysylpyridinoline (LP), and hydroxylysylpyridinoline (HP). In this investigation, only two of these established cross-links were purified: HP (peak III, fig. 1) and DHLNL (peak V-1-1).

In addition, peak VI (fig. 1) contained two compounds, one identified as lysinoalanine (table 1). Lysinoalanine is a well-known artefact of alkaline protein treatment but is supposed to be formed in dentin by the reaction between a collagen lysine- and a phosphoprotein phosphoserine residue (Fujimoto et al., 1981). Both compounds were not detected by HPLC after FMOC-derivatization, most likely because of fluorescence quenching inherent to the close vicinity of several FMOC groups attached to one molecule . Thus the unknown compound seems rather similar to lysinoalanine. We suggest the unknown compound is histidinoalanine, which is present in dentin (Fujimoto et al., 1982) and likely shows fluorence quenching in its FMOC derivate.

Both peak IV (fig. 1) and peak V-2 (fig. 2) resemble cross-links in TLC (R_f = 0 in BuOH / HAc / H_2O). IV gave one main peak in SCX-HPLC, but V-2 was separated into several fractions with one major peak (fig. 3b). The yields were relatively low compared with that of HP, namely 1.0, and 1.4 µmoles leucine equivalents for IV and V-2, respectively, versus 10.1 for III (HP). This is not due to instability under acid hydrolysis conditions, since 80% of IV and 100% of V-2 survived acid hydrolysis in a preliminary test.

Based on the first FAB-MS data, we assumed IV and V-2 to be two isomers with a pyrrolemethanol and a pyrroleninone nucleus, respectively (fig. 8), originating from the condensation of Δ-DHLNL with an oxidized hydroxylysine residue (fig. 9). The proposed formation of IV is a Knorr-Paal condensation, which has been proposed for the formation of an other pyrrolic cross-link analogous to the heme-precursor porphobilinogen (Scott et al., 1981). In addition, both IV and V-2 had migration speeds comparable to HP (III) in capillary electrophoresis. The presence

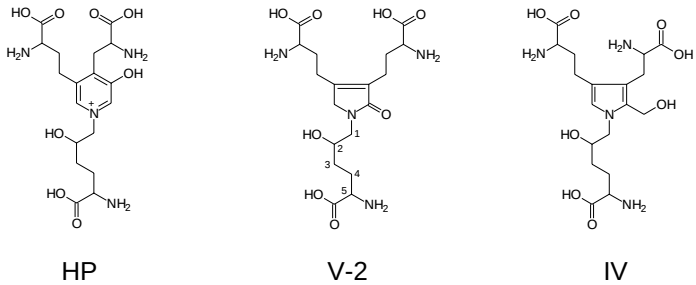


Figure 8
Proposed structures for IV and V-2. HP = hydroxylysylpyridinoline.

of pyrrolic cross-links in collagen has been assumed, based on a reaction with Ehrlich's reagent (Scott et al., 1981; Scott et al., 1983; Horgan et al., 1990). However, IV and V-2 gave no Ehrlich's reaction in a spotting plate assay (Grdinic and Medic-Saric, 1984). For V-2 this is however consistent with nil reactivity of 1,3,4-trimethylpyrrolenin-2-one (Falk et al., 1977; Ribó et al., 1981). Moreover, the pyrroleninone nucleus of V-2 was expected to survive both acid and alkaline conditions. This was confirmed by HPLC conducted on purified alkaline and acid hydrolyzates from another dentin batch (fig. 3c,d).

V-2 is separated into several fractions in CE-DAD (fig. 7) as seen in HPLC (fig. 3b). This separation of V-2 is most likely caused by the presence of V-2 isomers with slightly different physicochemical properties. For example, the pyrroleninone nucleus of V-2 can tautomerize (Baker and Sifniades, 1979; Mondelli et al., 1971), yielding the 2-hydroxypyrrole, Δ^3- and Δ^4-pyrrolenin-2-one forms (fig. 10). In addition, acid hydrolysis causes formation of D- and L-epimers from L-hydroxylysine (Hamilton and Anderson, 1955). It can be expected that this occurs also with the N-substituent hydroxylysine chain of V-2. This alone, however,would not explain the additional separation of V-2 tautomers, since DHLNL, HP, and LP with the same hydroxylysine side chain do not give a separation in SCX-HPLC. The pyrroleninone nucleus of V-2 should therefore reinforce the difference in physicochemical properties between the D- and L-epimers, for example by H-bonding of either pyrroleninone C=O or hydroxypyrrole OH with αCOOH of only one epimer. The spectra of the fractions separated by CE-DAD of V-2 at pH 7.2 indicate that a separation of tautomers has indeed occurred. For example, one of the fractions exhibited a UV-maximum at 236 nm as expected for an α,ß-conjugated acrylamide like the Δ^3-pyrrolenin-2-one. Another fraction with a maximum at 240 nm and a weaker band at 290-300 nm probably accounted for the Δ^4-pyrrolenin-2-one tautomer. These UV-maxima were consistent as

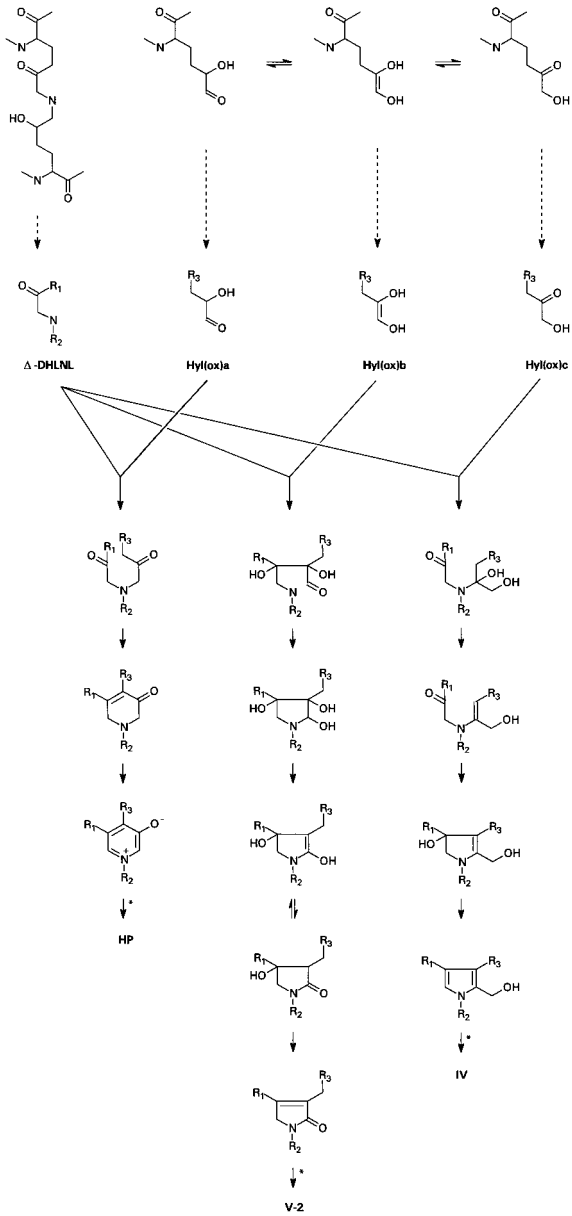


Figure 9
Proposed mechanism of formation of the putative pyrrolic cross-links. The ketoamine form of a Δ-dihydroxylysinonorleucine (Δ-DHLNL) residue reacts with either an oxidized hydroxylysine residue (Hyl(ox)a) or its tautomers (Hyl(ox)b and Hyl(ox)c) yielding two novel cross-links, which are recovered as IV and V-2 upon acid protein hydrolysis (*).

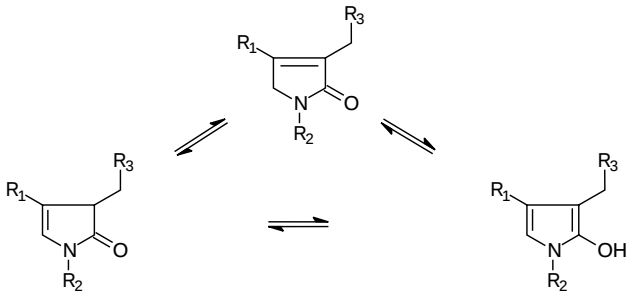


Figure 10
Tautomerism of the proposed pyrrolenin-2-one nucleus of V-2, resulting in proton exchange at C3 and C5 of the five membered ring. R_1, R_2, and R_3 as in figure 9.

they were also observed for two fractions separated at pH 2.3.

The FAB, ES (fig. 4b), and MALDI-TOF mass spectra of V-2 show the $[M+Na]^+$ ion at 453 corresponding with $C_{18}H_{30}N_4O_8Na$ (high resolution FAB, $mass_{calc}$: 453.1961, $mass_{exp}$: 453.1949).

From the NMR spectra of V-2, in particular the 500 MHz spectrum (fig. 6b), the presence of a hydroxylysine residue is clear: two double doublets between 3.2 and 3.4 ppm ($H_{1A, B}$), and multiplets between 3.4 and 3.5 (H_2, H_5), and between 1.2 and 1.8 ppm (H_3, H_4). The corresponding correlations are found in the TOCSY spectrum (not shown). The 300 MHz spectrum (fig. 5a, table 2) shows additional signals in the 3 to 5 ppm region, which may correspond with the substituents at the 3- and 4-position in the ring of the proposed structure for V-2. The 300 MHz spectrum does not allow unequivocal assignment for the protons of the substituents in the 3- and 4-positions of the pyrroleninone ring. Their signals will overlap with the protons of the hydroxylysine residue. The protons α to the carboxyl groups are expected around 3.6 ppm, the ß protons will be in the 1.5-2 ppm region, whereas the chemical shift of the γ protons (next to the double bond) is expected to be around 3 ppm. The 500 MHz spectrum shows less signals than the 300 MHz spectrum. However, it is known from the literature, that Δ^3-pyrroleninones exchange the protons at the allyl position when dissolved in D_2O (Baker and Sifniades, 1979; Mondelli et al., 1971). In the proposed structure V-2, exchange is possible at all allyl positions (fig. 11). Not being aware of this, the sample was stored in D_2O for at least a week prior to running the 500 MHz spectrum. Therefore, extensive deuterium exchange may explain the missing protons in the 500 MHz spectrum.

For compound IV we propose a pyrrolemethanol structure. The 190-350 nm UV-spectrum of IV run after electrophoretic separation shows a maximum at 216 nm (pH 2.3), which is absent in the spectrum at pH 7.2.

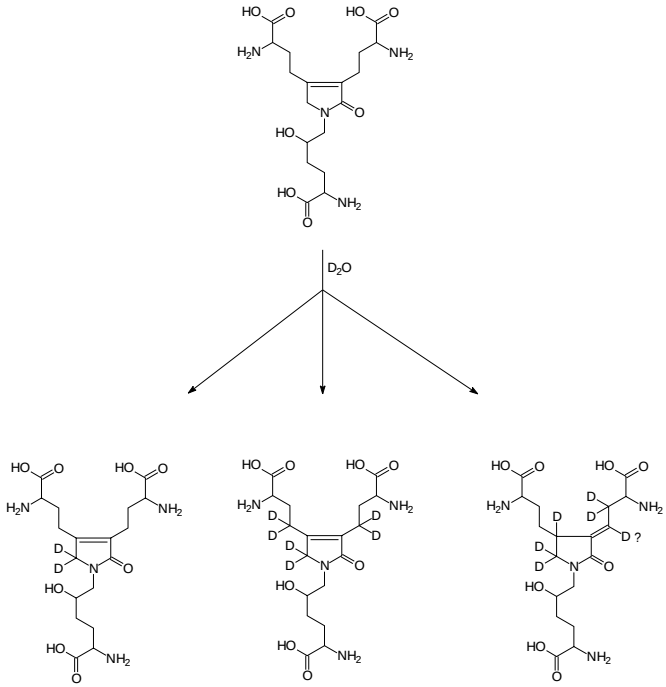


Figure 11
Exchange of H- against D-atoms at allylic positions in V-2 during storage in D_2O.

This behavior is consistent with that published for pyrroles (Chiang and Whipple, 1963; Hinman and Theodoropulos, 1963; Bullock, 1958; Cookson, 1953). The instability of IV may be explained by reactions of the substituents of the pyrrole ring: Scissions in the alkyl chain attached to the nitrogen of the ionized pyrrole ring have been reported previously (Duffield et al., 1965). The OH-group at C2 of the hydroxylysine chain (fig. 8) likely enhances chain breakage at C1-C2. The protonated hydroxymethyl group at the pyrrole ring may dehydrate, yielding a reactive azafulvenium ion (Tietze et al., 1996). The proposed structure was not confirmed by ^{1}H NMR. In addition to two singlets in the aromatic region (7.08 and 7.69 ppm; ratio approx. 1:1), the spectrum shows a doublet at 4.46 ppm and multiplets at 4.00, 3.22 and 3.09 ppm (ratio 2:2:1:1). Minor signals between 3 and 4 ppm may belong to a hydroxylysine residue: two double doublets for C$\underline{H}_2$CHOH, and multiplets between 3.65 and 3.75 ppm. These signals may belong to an impurity or IV may be in equilibrum with, or decompose into, a compound in which this group is present.

However, SCX-HPLC chromatograms of IV before and after running the ^{1}H NMR spectrum are identical. No definite explanation can be given yet for the observed phenomena.

In conclusion, the present study reveals the formation of a novel cross-link (V-2). To our knowledge, this is the first time that the structure of a pyrrolic cross-link has been elucidated. The analogous trifunctional pyridinoline cross-links are common diagnostics for collagen degradation in diseases affecting mineralized tissues. We therefore expect the novel cross-link to be a potential marker for collagen breakdown.

Acknowledgement. The authors wish to thank for their valuable contribution: Dr JGM Bolscher, Dr JW Hagen, AC Hogenboom, Dr H Lingeman, P Potman, J Slager (Free University, Amsterdam), ThL Snoeck (University of Amsterdam), A Brands, W Verwaal (Inspectorate for Health Protection, Amsterdam), and A Lammens. The authors also thank Dr BR Leeflang (Bijvoet Center for Biomolecular Research, University of Utrecht) for running the 500 MHz NMR spectra, Dr H Weenen (Quest International, Naarden) for his advise on the reaction mechanisms in figure 9, and Prof Dr NMM Nibbering (Institute of Mass Spectrometry, Amsterdam) for his advise.

REFERENCES

Bailey AJ, Ranta MH, Nicholls AC, Partridge SM and Elsden DF (1977) Isolation of α -amino adipic acid from mature dermal collagen and elastin. Evidence for an oxidative pathway in the maturation of collagen and elastin. Biochem Biophys Res Comm 78, 1403-1410.

Baker JT and Sifniades S (1979) Synthesis and properties of pyrrolin-2-ones. J Org Chem 44, 2798-2800.

Bank RA, Beekman B, Verzijl N, De Roos JADM, Sakkee AN and Te Koppele JM (1997) Sensitive fluorimetric quantitation of pyridinium and pentosidine crosslinks in biologic samples using a single HPLC-run. J Chromatogr (in press).

Bank RA, Jansen EJ, Beekman B and Te Koppele JM (1996) Amino acid analysis by reverse-phase high-performance liquid chromatography: improved derivatization and detection conditions with 9-fluorenylmethyl chloroformate. Anal Biochem 240, 167-176.

Barber M, Bordoli RS, Elliott GJ, Fujimoto D and Scott JE (1982) The structure(s) of pyridinoline(s). Biochem Biophys Res Comm 109, 1041-1046.

Barnard K, Light ND, Sims TJ and Bailey AJ (1987) Chemistry of the collagen cross-links. Origin and partial characterization of a putative mature cross-link of collagen. Biochem J 244, 303-309.

Bullock E (1958) The structure of pyrrole salts and the basic strengths of some simple pyrroles. Can J Chem 36, 1686-1690.

Chiang Y and Whipple EB (1963) The protonation of pyrroles. J Am Chem Soc 85, 2763-2767.

Cookson GH (1953) The ultra-violet absorption spectra of some pyrroles. J Chem Soc 2789-2795.

Davis NR (1973) A method for the determination of lysine-derived collagen crosslinks. Biochem Biophys Res Comm 52, 877-883.

Deasy C (1961) Occurrence of 2-pyrrolecarboxylic acid in hide hydrolyzates and its bearing on the determination of hydroxyproline. J Am Leather Chem Assoc 56, 357-361.

Duffield AM, Beugelmans R, Budzikiewicz H, Lightner DA, Williams DH and Djerassi C (1965) Mass spectrometry in structural and stereochemical problems. LXIII. Hydrogen rearrangements induced by electron impact on N-n-butyl- and N-n-pentylpyrroles. J Am Chem Soc 87, 805-810.

Falk H, Grubmayr K, Höllbacher G, Hofer O, Leodolter A, Neufingerl F and Ribó JM (1977) Beiträge zur Chemie der Pyrrolpigmente, 18. Mitt.: Pyrromethenone-Partialstrukturen von Gallenpigmenten: Struktur und Eigenschaften in Lösung. Monatsh Chem 108, 1113-1130.

Fujimoto D, Hirama M and Iwashita T (1981) Occurrence of lysinoalanine in calcified tissue collagen. Biochem Biophys Res Comm 103, 1378-1383.

Fujimoto D, Hirama M and Iwashita T (1982) Histidinoalanine, a new crosslinking amino acid, in calcified tissue collagen. Biochem Biophys Res Comm 104, 1102-1106.

Grdinic V and Medic-Saric M (1984) Cation-exchange method for the detection of pyrrole derivatives. Fresenius Z Anal Chem 319, 306.

Hamilton PB and Anderson RA (1955) Hydroxylysine: isolation from gelatin and resolution of its diastereoisomers by ion exchange chromatography. J Biol Chem 213, 249-258.

Heidemann E, Seba J, Tanay I, Lentze C, Rohn M and Kisselbach Th (1992) Untersuchungen über die reifen Quervernetzungen der Rindshaut. Leder 43, 75-85.

Hinman RL and Theodoropulos S (1963) The methylpyrroles. Synthesis and characterization. J Org Chem 28, 3052-3058.

Horgan DJ, King NL, Kurth LB and Kuypers R (1990) Collagen crosslinks and their relationship to the thermal properties of calf tendons. Arch Biochem Biophys 281, 21-26.

Jamall IS, Finelli VN and Que Hee SS (1981) A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. Anal Biochem 112, 70-75.

Keller S, Ghosh AK, Ghosh AK, Turino GM and Mandl I (1984) Separation of the cross-linking amino acids of elastin on thin-layer plates. J Chromatogr 305, 461-464.

Kuboki Y, Takagi T, Sasaki S, Saito S and Mechanic GL (1981) Comparative collagen biochemistry of bovine periodontium, gingiva, and dental pulp. J Dent Res 60, 159-163.

Kuypers R, Tyler M, Kurth LB, Jenkins ID and Horgan DJ (1992) Identification of the loci of the collagen-associated Ehrlich chromogen in type I collagen confirms its role as a trivalent cross-link. Biochem J 283, 129-136.

Light N and Bailey AJ (1985) Collagen cross-links: location of pyridinoline in type I collagen. FEBS Lett 182, 503-508.

Linde A and Robins SP (1988) Quantitative assessment of collagen crosslinks in dissected predentin and dentin. Coll Rel Res 8, 443-450.

Mechanic GL, Gallop PM and Tanzer ML (1971) The nature of crosslinking in collagens from mineralized tissues. Biochem Biophys Res Comm 45, 644-653.

Mechanic GL, Kuboki Y, Shimokawa H, Nakamoto K, Sasaki S and Kawanishi Y (1974) Collagen crosslinks: direct quantitative determination of stable structural crosslinks in bone and dentin collagens. Biochem Biophys Res Comm 60, 756-763.

Mondelli R, Bocchi V, Gardini GP and Chierici L (1971) NMR spectra of Δ^3- and Δ^4-pyrrolin-2-one. Org Magn Reson 3, 7-22.

Moore S (1968) Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J Biol Chem 243, 6281-6283.

Moore S and Stein WH (1954) A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J Biol Chem 211, 907-913.

Nakamura F, Yamazaki K and Suyama K (1992) Isolation and structural characterization of a new crosslinking amino acid, cyclopentenosine, from the acid hydrolysate of elastin. Biochem Biophys Res Comm 186, 1533-1538.

Pfeifer RF and Hill DW (1983) High-performance liquid chromatography of amino acids: ion-exchange and reversed-phase strategies. Adv Chromatogr 22, 37-69.

Ranta H (1978) Age-related changes in collagen cross-linking. Changes in bovine dentine and periodontal ligament and description of a new type of non-reducible cross-link. Proc Finn Dent Soc 74 (Suppl. II), 3-64.

Ribó JM, Masip MD and Vallès A (1981) Reaktivität der Pyrrolpigmente, 2.Mitt.: Deprotonierung von 3-Pyrrolin-2-onen. Monatsh Chem 112, 359-368.

Robins SP (1976) The separation of cross-linking components from collagen. In: The methodology of connective tissue research (ed. Hall DA), Chap. 6, pp. 37-52. Joynson-Bruwers, Oxford UK.

Rohn M (1990) Versuche zur Identifizierung von mit Chondroitinase spaltbaren Quervernetzungen in alter Haut (thesis). Technical University, Darmstadt, Germany.

Sakura S, Fujimoto D, Sakamoto K, Mizuno A and Motegi K (1982) Photolysis of pyridinoline, a cross-linking amino acid of collagen, by ultraviolet light. Can J Biochem 60, 525-529.

Schlueter RJ and Veis A (1964) The macromolecular organization of dentine matrix collagen. II. Periodate degradation and carbohydrate cross-linking. Biochemistry 3, 1657-1665.

Scott JE, Hughes EW and Shuttleworth A (1981) A collagen-associated Ehrlich chromogen: a pyrrolic cross-link? Biosc Rep 1, 611-618.

Scott JE, Qian R-G, Henkel W and Glanville RW (1983) An Ehrlich chromogen in collagen cross-links. Biochem J 209, 263-264.

Steigmann A (1956) Réactions colorées spécifiques des gélatines. Sci Ind Phot 27, 422-426.

Steigmann A (1964) Microconstituents of photographic gelatin. Desoxyribonucleic acid and an unknown polysaccharide. Sci Ind Phot 35, 145-157.

Suyama K, Yamazaki K and Nakamura F (1995) Cyclopentenosine, trifunctional crosslinking amino acid of elastin and collagen, characterization and distribution. Spec Publ - R Soc Chem 151, 425.

Tietze LF, Kettschau G and Heitmann K (1996) Synthesis of N-protected 2-hydroxymethylpyrroles and transformation into acyclic oligomers. Synthesis 851-857.

Tilson MD, Dreyer RN, Hudson A, Cotter RJ and Tanzer ML (1985) Partial purification of an analog of pyridinoline isolated from human skin. Biochem Biophys Res Comm 126, 1222-1227.

Walters C and Eyre DR (1983) Collagen crosslinks in human dentin: increasing content of hydroxypyridinium residues with age. Calc Tiss Int 35, 401-405.

Yamauchi M, Chandler GS and Katz EP (1992) Collagen cross-linking and mineralization. Excerpta Med Int Congr Ser 1002, 39-46.

Chapter 7

CONCLUDING REMARKS

In the course of dentin caries, both demineralization and reactions with the organic matrix take place. Matrix reactions include proteolysis and covalent modifications. From the introduction (Chapter 2) and the review on discoloration in caries (Chapter 3), it becomes clear that there are still few reports on the effect of matrix modifications on dentin caries. In Chapters 2, 4, and 5, the investigations were aimed at filling the information gap concerning the effect of reactions of dentin matrix on caries. To this end, degradation and modification of dentin were studied in demineralized specimens in vitro. In addition, specimens placed in dentures in situ and caries lesions in extracted teeth were analysed for modifications.

In Chapter 2, the proteolysis of demineralized organic matrix of bovine dentin promoted demineralization of both erosive and subsurface lesions in vitro, especially in advanced lesions. In contrast to previous investigations, the organic matrix was destroyed enzymatically, not chemically.

One conspicuous histological feature of dentin attacked by the proteolytic enzymes is the apparent resistance of peritubular matrix to degradation compared with intertubular matrix. One example from collagenase-degraded bovine dentin is shown in figure 1. Obviously, it reflects the different composition of the peritubular matrix, high in proteoglycans and free of collagen. It would be interesting to find out if this also increases the resistance of the intertubular matrix to invading bacteria after noncovalent binding of mucopolysaccharides.

In a parallel preliminary experiment, hypochlorite instead of collagenase was employed for matrix degradation. After hypochlorite treatment, tubules in sound dentin beneath the lesions contained material positive for PAS- and Richardson's stains, which was not observed after the buffer- and collagenase treatments. The bacterial degradation of the organic matrix was, therefore, simulated enzymatically.

Based on the results in Chapter 2, one might speculate that inhibition of bacterial proteases promotes caries arrestment. Patent literature mentions both the application and inhibition of proteases for caries arrestment. Proteases may serve either the lysis of bacteria or the removal of infected dentin prior to restoration placement.

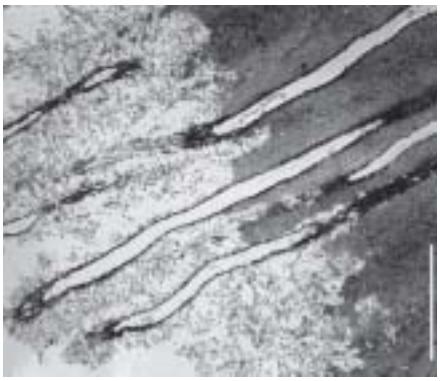


Figure 1
Lesion in bovine dentin with tubules protruding from degraded intertubular matrix (left: degraded matrix; right: intact matrix). Demineralization in 0.1 M acetic acid pH 4.0, with subsequent exposure to bacterial collagenase. Fixed and demineralized with glutardialdehyde-acetic acid, post-fixed with osmium tetroxide; ultrathin sections stained with uranyl acetate - lead citrate.

From the literature reviewed in Chapter 3, it can be concluded that the Maillard reaction between carbohydrates and proteins is the likely cause of the discoloration of caries lesions. Research in the following chapters focused on this reaction.

Chapter 4 describes the in vitro reaction of glucose with demineralized dentin. Preliminary tests revealed that use of distilled instead of deionized water accelerated browning, consistent with the effect of trace metals on the Maillard reaction. The yellow discolored slices were more resistant than controls to pepsin-mediated breakdown, but not to trypsin-mediated breakdown. It would be worthwhile to investigate proteolysis of dentin collagen covalently bound by the Maillard reaction to proteins, which penetrate into a caries lesion.

In addition to this in vitro work, an in situ experiment was carried out (table 1, unpublished results). Demineralized slices of bovine dentin were exposed intraorally in partial prostheses of human volunteers for four or five weeks. After exposure, the color of the slices varied from beige to brown. The fluorescence specific for the Maillard reaction increased, but not as much as it did in vitro (Chapter 4). In addition, the Maillard product pentosidine was not detected (table 1). No significant change in the content of the physiological cross-link hydroxylysylpyridinoline (HP, mol/mol collagen) was found, contrary to increases in vitro as reported in literature. In a preliminary in situ experiment employing mineralized instead of demineralized dentin, an increase in hydroxylysylpyridinoline had been noted in the developing lesion (Kleter et al.,

Table 1
Characteristics of demineralized slices of bovine dentin after 4-5 weeks of oral exposure[a].

	exposed (n=22)	control (n=6)
Collagen content (mg)	2.80 ± 0.34	3.96 ± 0.12[b]
Fluorescence (a.u.)[c]	4.3 ± 1.2	1.8 ± 0.4[b]
Amino acids		
hydroxylysylpyridinoline[d]	0.33 ± 0.04	0.32 ± 0.02
lysylpyridinoline[d]	ND	ND
pentosidine[e]	0.7 ± 0.5	0.7 ± 0.1

ND = non-detectable
[a] mean values ± SD, methods as in Chapter 4; [b] P<0.001; [c] diluted collagenase digests (200 pmol collagen in 3 ml, pH 7.4), λ_{ex} 370 nm, λ_{em} 440 nm, one arbitrary unit (a.u.) is one thousandth of the fluorescence of 3.0 µM tyrosine, pH 7.4, λ_{ex} 275 nm, λ_{em} 303 nm; [d] mol/mol collagen; [e] mmol/mol collagen.

1995). HP formation may have been promoted by the acidic environment and stabilization of HP precursors through complexation with phosphate released during demineralization. Altogether, therefore, the one-month in situ model appeared unsuitable for simulating the Maillard reaction. Longer exposures would have enhanced the risk of bacterial degradation of the slices.

Direct evidence for the Maillard reaction in caries lesions is scarce (Chapter 3). Therefore in Chapter 5 we compared specific Maillard products in hydrolysates of carious and sound human dentin. The advanced products carboxymethyllysine and pentosidine increased in caries, but furosine (a marker of initial products) was found occasionally in low amounts, but not in sound dentin. In addition, fluorescence (λ_{ex} 370 nm, λ_{em} 440 nm), specific for the Maillard reaction, increased. These results suggest that the Maillard reaction in caries lesions takes place with carbonyl compounds other than C_6 aldoses. Formation of carboxylate groups in collagen (such as carboxymethyllysine from lysine) increases cation binding by collagen, which could influence de- and remineralization in a dentin lesion by binding calcium. The decrease in HP in carious dentin can be the result of either the formation of new collagen matrix, which is not very likely, or the oxidation of HP's pyridinol moieties. In the latter case, the original HP cross-linking of the peptide chains would be maintained.

In Chapter 6, an attempt was made to identify suspect physiological cross-links, tentatively assigned from HPLC data, in addition to those already established in literature. Two novel cross-links, denoted chromatographic fractions IV and V-2, were purified from bovine root dentin, and the structure of V-2 was elucidated. During the analysis of human dentin as described in Chapter 5, V-2 appeared below detection level. A peak was observed for IV. Part of the material with the same retention as IV was not retained on cellulose in butanol / acetic acid / water = 4:1:1 (vol.). Co-elution of a non-cross-linking amino acid with IV could therefore not be excluded, and the results were omitted.

The general finding that the Maillard reaction occurs in caries lesions both raises new questions about and provides new insights into the treatment and arrestment of caries.

One of the questions evolving from the results of Chapters 3 and 5 is: If C_6 sugars are not involved in the Maillard reaction, which compounds are? In addition, is the glycosylated dentin as resistant to proteolysis by cariogenic bacteria as it is to pepsin in vitro?

In Chapter 5, it is concluded that release of intracellular bacterial metabolites after cell lysis may have been responsible for the increases in advanced Maillard products. Lysis of bacteria can deliberately be induced by, for example, lysogenic enzymes and phages. In addition to the direct targeting of cariogenic microorganisms, lysis could thus contribute indirectly to caries arrestment by causing an extensive Maillard reaction. However, the problem of the concomitant unaesthetic discoloration will need to be considered before practical application becomes feasible.

Aside from the Maillard reaction, other covalent modifications of amino acids and proteins are possible within the caries lesion, which merit future investigation. For example, certain oral microorganisms excrete γ-glutamyl transferases. These enzymes catalyse the formation of cross-links between glutamic acid and lysine residues of proteins. In addition, N-acyl amino acids are present in plaque, which adsorb to mineral surfaces.

In conclusion, evidence has been gathered for a role of the Maillard reaction in caries. This reaction can cause inhibition of matrix degradation, which in turn inhibits lesion demineralization. Further research is needed to elucidate the pathways and the importance of this reaction in in vivo caries pathology.

References

Kleter GA, Damen JJM and Ten Cate JM (1995) Purification of cross-linking amino acids for their analysis in carious dentine. Caries Res 29, 309.

REACTIES VAN DE ORGANISCHE MATRIX TIJDENS DENTINECARIES

Samenvatting (Dutch summary)

Het laboratorium- en literatuuronderzoek in dit proefschrift betreft de mogelijke effecten van reacties van de organische matrix van dentine op cariës.

Dentine vormt het grootste deel van de tandwortel en is in de kroon bedekt met glazuur. Zowel glazuur als dentine bestaan voor het grootste deel uit mineraal, maar dentine bezit tevens een aanzienlijk precentage organische matrix, voornamelijk bestaand uit collageen. Cariës in dentine wordt beschreven als een proces van opeenvolgende gebeurtenissen. Demineralisatie, bacteriële infiltratie en afbraak van de organische matrix volgen elkaar op.

Het valt te verwachten dat een verbeterde mondgezondheid tot minder cariës van de tandkroon zal leiden. Hierdoor zal tevens het percentage bejaarden dat nog de beschikking over (een deel van) het gebit heeft, toenemen. Dit is een populatie waarin wortelcariës veel voorkomt. Hoewel het probleem van cariës in dentine belangrijk zal blijven in de toekomst, is maar een beperkt aantal wetenschappelijke artikelen gewijd aan de rol van matrixafbraak in het verloop van cariës in dentine.

Een overzicht van zowel de fysiologie van dentine als de pathologie van dentine tijdens cariës wordt in hoofdstuk 1 gegeven.

In hoofdstuk 2 werden monsters runderdentine in vitro elke dag gedurende een beperkte tijd gedemineraliseerd in zure oplossingen, zodat laesies in of net onder de buitenste mineraallaag ontstaan. De demineralisatie verliep sneller wanneer de matrix, die door mineraalverlies bloot is komen te liggen, enzymatisch werd verwijderd in de periodes tussen de incubaties met zuur. Dit effect was nog duidelijker in een extra groep met diepe laesies die niet waren afgedekt met een gemineraliseerde laag aan het oppervlak. Met behulp van microscopie kon worden vastgesteld dat de veranderingen in de enzymatisch behandelde matrix verschilde tussen laesies met, respectievelijk zonder gemineraliseerde oppervlaktelaag.

De bruin tot zwarte verkleuring van cariëslaesies is een bekend fenomeen. Er is een verband tussen de mate van verkleuring en de fase waarin cariës verkeert. Zo is een harde zwart verkleurde laesie niet meer actief. Deze verkleuring en de inactivering van cariës houden waarschijn-

lijk verband met reacties van de organische matrix in laesies. Hoofdstuk 3 beschouwt de mogelijke mechanismen voor deze verkleuring en concludeert dat de Maillard-reactie de meest aannemelijke verklaring is. Door deze reactie tussen koolhydraten en eiwitten worden zijketens van basische aminozuren in eiwitten gemodificeerd en ontstaan covalente bindingen ("cross-links") tussen eiwitmoleculen. Zulke veranderingen kunnen carieus dentine resistent maken tegen enzymatische afbraak. In de tandheelkundige literatuur worden vooral Maillard reacties beschreven in vitro tussen tandweefsels en carbonylverbindingen, zoals suikers en hun afbraakprodukten. Daarnaast zijn er slechts enkele berichten gepubliceerd waarin specifieke produkten van de Maillard reactie in carieuse tanden worden aangetoond.

De Maillard-reactie in dentine werd getest in vitro door incubatie van gedemineraliseerd runderdentine met een glucose-oplossing gedurende 10 weken, zoals beschreven in hoofdstuk 4. Duidelijke toenames van indicatoren van de Maillard-reactie werden waargenomen na de reactie met glucose, zoals een fluorescentie bij 440 nm na excitatie bij 370 nm, het heterocyclische aminozuur furosine, dat gemaakt kan worden uit initiële produkten van de reactie, en het difunctionele aminozuur pentosidine, één van de later gevormde reactieprodukten. Het collageen van vergeeld dentine bleek minder gevoelig voor afbraak door pepsine dan dat van gezond dentine. Voor afbraak door trypsine kon echter geen verschil worden vastgesteld. Tijdens incubaties met pepsine vindt afbraak van het in de zure oplossing gedenatureerde collageen plaats, zoals het ook in cariëslaesies voorkomt. De Maillard-reactie kan dus inderdaad bijdragen aan resistentie van de dentinematrix tegen afbraak.

Markers van de Maillard-reactie werden gemeten in carieus dentine in het onderzoek beschreven in hoofdstuk 5. De vermelde fluorescentie, kenmerkend voor de Maillard-reactie, was hoger in oplossingen van collageenfragmenten uit carieus dentine dan in die van gezond dentine. De gehaltes aan de geavanceerde Maillard-produkten carboxymethyllysine en pentosidine waren eveneens hoger. Daarentegen kwam de marker voor initiële reactieprodukten met glucose, furosine, slechts in enkele monsters carieus dentine in kleine hoeveelheden voor. Tevens werd het gehalte aan normaal aanwezige covalente bindingen ("cross-links") tussen collageenmoleculen bepaald. De cross-links hydroxylysinonorleucine en dihydroxylysinonorleucine waren lager in carieus collageen. Dit kan het gevolg zijn van het verbreken van covalente bindingen in zuur milieu. Geconcludeerd kon worden dat de Maillard-reactie optreedt in carieus dentine, maar niet met C_6-suikers zoals glucose.

In hoofdstuk 6 wordt verslag gedaan van de poging twee cross-links te isoleren en karakteriseren. Doel was verbindingen te vinden die bijdragen aan de stabiliteit van dentinecollageen. Cross-links werden

gezuiverd uit worteldentine van runderen met behulp van vloeistofchromatografie. Twee fracties met onbekende aminozuren, voorlopig aangeduid met de nummers IV en V-2, werden onderworpen aan massaspectrometrie, kernspinresonantie en UV-spectroscopie. Een structuur voor V-2 kon hiermee vastgesteld worden.

Hoofdstuk 7 is een nabeschouwing van de resultaten uit vorige hoofdstukken en ongepubliceerd werk. De bevinding dat de Maillardreactie optreedt tijdens cariës roept nieuwe vragen op en biedt nieuwe inzichten in de pathologie van cariës in dentine.

NAWOORD

(Dutch epilog)

"Last but not least" wil ik hierbij al diegenen bedanken, die met hun werk en adviezen een niet onaanzienlijke bijdrage aan het onderzoek geleverd hebben. Allereerst gaat mijn dank uit naar mijn promotor Prof. J.M. (Bob) ten Cate en co-promotor Dr. J.J.M. (Jan) Damen. Niet zelden zijn Bob's heldere inzichten en adviezen over de te volgen weg onontbeerlijk gebleken. Jan's nuchtere visie, ervaring en kritiek maken hem tot een rots in de branding van de woeste zee genaamd onderzoekstraject.
De volgende personen wil ik graag bedanken:

kamergenoten:
Maxim Lagerweij, Cor van Loveren en Guus van Strijp;
Maxim en Guus tevens bedankt voor jullie bijdrage
aan het in situ onderzoek (hoofdstuk 7),

voor technische ondersteuning op de vakgroep:
Mark Buijs, Alfons Lammens, Rob Exterkate en Jos Buijs,

coauteurs:
R.A. Bank, V. Everts, R. Fokkens, J.J. Kettenes-van den Bosch,
J. Niehof, J.M. te Koppele en J.R. Veraart,

voor hun werkzaamheden, die helaas niet beschreven zijn:
J.G.M. Bolscher, J.W. Hagen, R.K. Kerkhoven, B.R. Leeflang,
P. Potman, A.N. Sakkee, J. Slager, Th.L. Snoeck,
F. van der Stadt, W. Verwaal, N. Verzijl en K.L. Weerheijm,

voor hun adviezen en stimulerende discussies:
A. Brands, B. Fasting, F.W. Jansen, A.C. Hogenboom, C.M. Kreulen,
H. Lingeman, Ch.R. Mol, N.M.M. Nibbering, G. van de Werken,
A.H.M.S.M. van Kuppevelt, J. Verouden en H. Weenen,

voor het onderzoeksmateriaal in hoofdstuk 5:
Y. Klompmaker, M. Kuyck, D.M. Stroink, en de overige
medewerkers van VU Mondziekten en Kaakchirurgie,

voor de collegiale sfeer:
collega's van de vakgroep Cariologie Endodontologie Pedodontologie, in het bijzonder Linda Peters, Margriet Rudolphy en Willem van der Borden voor hun bijdrage aan hoofdstuk 5,

special thanks to:
Rebeccah Kahama for her expert discussions.

Tevens wil ik voor de financiering van dit onderzoek bedanken:
de Interuniversitaire Onderzoeksschool Tandheelkunde (IOT).

Beebs van Riessen, Jan Couwenberg en Magda Bredschneijder hebben aan dit proefschrift vormgegeven.

De paranimfen Albert de Bie en Guus van Strijp wil ik alvast bedanken voor hun inzet om deze operatie tot een goed einde te brengen.

CURRICULUM VITAE

Gijsbertus Anthonius Kleter werd 1 november 1965 te Ede (Gelderland) geboren. Na zijn middelbare school (VWO, Het Wagenings Lyceum te Wageningen, 1978-1984) studeerde hij van 1984-1990 Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. De nadruk lag hierbij op toxicologie, hetgeen uitmondde in twee afstudeervakken: biochemie (Prof. Dr. Ir. I.M.C.M. Rietjens) en toxicologie (Ir. R.M. Hempenius) met aansluitend een stage toxicologie aan het Rijks-kwaliteitsinstituut voor Land- en Tuinbouwprodukten te Wageningen (Dr. M.J.B. Mengelers). In 1991 werd hij als assistent in opleiding aangenomen voor onderzoek naar de rol van eiwitafbraak in wortelcariës bij de vakgroep Cariologie en Endodontologie (Academisch Centrum Tandheelkunde Amsterdam). Promotor was Prof. Dr. J.M. ten Cate, begeleider was Dr. J.J.M. Damen.