AGE-RELATED CHANGES IN REDUCIBLE CROSS-LINKS OF HUMAN DENTAL PULP COLLAGEN

by

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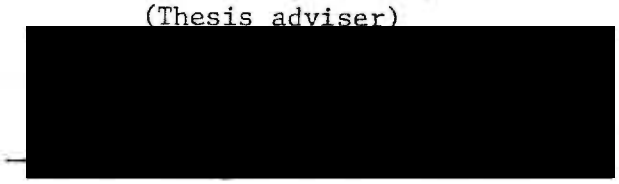

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To my wife, Judy, who helps to keep things in perspective.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

ABSTRACT

In the past fifteen years the elucidation of important post-translational modifications of collagen has added greatly to the understanding of structural and metabolic characteristics of this protein. A considerable body of evidence suggests that particular intermolecular cross-links formed as Schiff bases when present in the reducible form can be used as indicators of active collagen synthesis. The ability to measure these was employed in studying human dental pulp collagen. If continuing fibrosis of the dental pulp occurs with age one would expect to see high levels of the reducible form throughout the life of the individual. On the other hand if pulpal collagen does not increase in density after a certain age, then such cross-links might be expected to diminish as they become non-reducible by normal in-vivo processes. Our results indicated that the latter seemed to be the case. A significant decrease in the major cross-link of pulp collagen occurred between the ages of sixteen years and forty years. Various interpretations of this result were discussed and it was felt that the most probable explanation was a diminished collagen synthesis over the years included in this study. Additional investigation of collagen, protein, water, and calcium content supported this belief.

INTRODUCTION

It is a common contention, based upon histological and histochemical observations that pulpal fibrosis is a normal concomitant of aging. The belief that fibrosis does indeed occur, and occurs as a result of continuing collagen deposition throughout life, remains to be satisfactorily proven, and indeed, there are several alternative explanations to account for the apparent increase in the mass of collagen seen by histological examination. Interpretation is influenced by several factors. Pulpal volume is constantly diminishing with age and as a consequence, what might appear as increased collagen density might be more relative than absolute. Loss of water or ground substance might accentuate such an apparent increase. In performing biochemical assay procedures one is dealing with an extremely small amount of tissue which might be contaminated by either dentin fragments or pulp stones. Factors such as caries, restorations, and periodontal disease might all contribute to increased collagen deposition and would have to be considered along with the effects of age.

One manner of studying the question of pulpal fibrosis would be an indirect approach whereby one measures the rate of collagen synthesis over a period of time. If fibrosis occurs with age one might assume either that collagen synthesis continues at a steady rate or, in fact, increases. It is the intention of this work to investigate possible pulpal

fibrosis by measuring new collagen synthesis using a relatively new biochemical assay procedure. The technique identifies and quantifies a particular set of collagen cross-links which the author and others feel are indicative of new collagen synthesis. By determining changes in the rate of synthesis of the protein, it is felt that conclusions can be reached regarding whether or not collagen that is found in the pulp increases primarily during the developmental stages of the tooth or whether such an increase is a continuous process occurring throughout the life of the individual after the tooth has matured.

LITERATURE REVIEW

PULPAL CONNECTIVE TISSUE - MATURATION OF CELLULAR AND FIBROUS ELEMENTS

Historically it has been the belief of many that fibrosis of the dental pulp is a continous process throughout the life of the tooth (1-9). Recent evidence has accumulated to suggest that perhaps both the cellular elements responsible for collagen production and the protein itself reach maturity at an age roughly equivalent to physical maturity of the animal. Subsequent to this the cells enter into a period of diminished activity. Consequently collagen synthesis decreases and any changes in the physical and chemical reactivity of the protein reflect the final maturation of the protein.

Breyan and Schilder (10) in a comparison of continuously erupting rat incisors with nineteen year old human premolars described rat pulpal fibroblasts with prominent "intracellular machinery" necessary for collagen production (rough endoplasmic reticulum, Golgi apparatus, mitochondria) but relatively few extracellular fibers. Human pulps on the other hand demonstrated an abundance of extracellular fibers but few intracellular organelles, leading the authors to refer to such cells as fibrocytes. These observations also led to the conclusion that two stages of cellular collagen metabolism were being observed: young pulp tissue of rat incisors containing very active fibroblasts engaged in the synthesis

of collagen precursors with few extracellular fibers, and the more mature human pulp displaying the end stages of a chain of events in the maturation process with little new collagen formation.

Additional histological support for the idea that teeth at different stages of development may demonstrate differences in the capacity to produce collagen was offered by Avery and Han (11). They observed fibroblasts in molar tooth buds of new born hamsters and found that these cells had less rough endoplasmic reticulum and mitochondria with fewer cristae than mature rat fibroblasts of lymph nodes. While a direct comparison to Breyan and Schilder's study is difficult because of different species and different teeth the idea that tooth pulp may vary in its overall development from one age to another is evident. Unfortunately neither study compares teeth that might be considered "young" with older teeth of the same species. Such a comparison would give validity to the idea that the dental pulp does indeed mature with age.

Nevertheless if histologic evidence suggests an altered capacity to produce protein, one might expect to measure such differences in terms of protein concentration or rate of synthesis. By using ^{3}H-proline as a marker and separating bovine pulp protein into collagen and non-collagenous portions with protease-free clostridial collagenase, Patten et.al.(12) noted in-vitro that the ratio of collagen synthesis to total protein synthesis decreased in direct relation to the chron-

ological age of the pulp. Similar results have been obtained with human teeth where the ratio differed significantly between teeth with open apices and teeth with closed apices(13).

This type of study points out a difference between collagen and other proteins which needs to be considered at this time. Most cells synthesize protein throughout their lifetime which, since it does not accumulate, must clearly be degraded at some time after its synthesis. This dynamic equilibrium of continuous biosynthesis balanced precisely with continuous degradation gives rise to the concept of protein turnover, with each protein having a specific and measurable half-life. In most tissues collagen is normally considered to have a very long half-life and thus exhibits a negligible degree of turnover. It is this collagen synthesis unaccompanied by collagen degradation which gives rise to a net accumulation of the protein known as fibrosis.

Some specialized tissues such as the periodontal ligament are known to demonstrate a relatively high turnover rate for collagen in contrast to the negligible rate observed in most other interstitial tissue. It has been claimed by Orlowski (14) that pulp tissue exhibits a similar increased rate of turnover. By measuring the rate of loss of labeled hydroxyproline in rat incisor he found a turnover rate of 6.0 days compared to 13.5 days for the molar. Such very short turnover times for collagen are not supported by any other workers in any tissue studied. Orlowski's work is difficult

to interpret since it appears that he simply measured the specific activity of free amino acids in the pulp. Incredibly, no hydrolysis step is included in his protocol. Nevertheless, in support of Breyan and Schilder's work one might suggest that the continuously erupting incisor is a "younger" tooth more involved in collagen synthesis and degradation than the molar. Additional support for this idea is given by Shuttleworth et.al. (15) who found a higher metabolic activity of collagen in the rabbit incisor compared to the molar. The authors attribute this to the former's continuous eruption. (According to both Navia (16) and Miles (17) both teeth are continuously erupting!)

In discussing the "maturity" of collagen, the solubility of the protein in solutions of increasing ionic strength is often used as a measure of the degree to which one molecule is bound to another, i.e. cross-linked. An inverse relationship between cross-linking and solubility is known to exist (18) and is used to describe the maturity of the protein. Thus, recently synthesized collagen is sometimes referred to as "salt-soluble", whereas older, more highly cross-linked collagen is insoluble.

Slavkin and Bavetta (19) studied the pulps of rabbit teeth extracted during gestation, at birth, and shortly after birth and found increasing amounts of insoluble collagen and negligible amounts of salt-soluble collagen with the passage of time. Similarly in a study of bovine dental papilla and

pulp Shuttleworth (15) reported a progressive loss of solubility of the protein. They concluded that an age-related increase in cross-linking and fiber organization had occurred.

Zerlotti (4) in a comparison of human tooth pulps from different age groups (11-15,40-55,70 years) found fibers of young pulps to be easily digested by collagenase whereas in mature pulps these same fibers were somewhat resistant and in collagen fiber bundles of aged pulps the enzyme had no effect. When 0.1 M phosphate and 0.1 M citric acid buffers were used to test for changes in solubility it was noted that fibers of younger pulps were more soluble than older pulps as would be expected. This resistance to enzymatic degradation and decrease in solubility in older pulps led the author to conclude that the stability of old fibers resulted from masking of reactive groups through cross-linking.

Van Amerongen and Tonino (20) tested bicuspid teeth extracted for orthodontic purposes and found only limited solubilization of pulpal collagen using a variety of solvents. They concluded that the protein was highly cross-linked and could not be considered immature.

Thus there appears,on the basis of solubility studies, to be a concensus of opinion that pulpal collagen is similar to other tissue collagens, in that with the passage of time the protein becomes more insoluble as a result of cross-linking. The marked decrease in solubility makes it unlikely that pulp collagen has a high turnover rate.

QUANTIFICATION OF PULPAL COLLAGEN

In the last twenty years several studies have addressed the question of whether or not collagen in the dental pulp accumulates with age, and, to date, no clear consensus has emerged. Perhaps the work most often quoted is that of Stanley and Ranney (21) who used light microscopy to study the human dental pulp. They detected two patterns of collagen deposition, "diffuse" and "bundle". The former was characterized by delicate collagenous fibers lacking definite orientation while the latter had large coarse bundles of collagen running either parallel to nerve bundles or independently. They concluded that:

1. anterior tooth pulp had more collagen than posterior tooth pulp;
2. root pulp had more collagen than coronal pulp;
3. collagen in coronal pulp did not increase after twenty years of age;
4. "bundle" collagen of root pulp increased up to age thirty-nine, but not thereafter;
5. "diffuse" collagen of root pulp decreased between the ages of ten years and forty-nine years.

These authors made no attempt to provide alternative explanations for what might have been an apparent increase. Other authors (4,6,22,23) have referred to diminishing pulpal vol-

ume, decreased water content, and changes in ground substance. These might conceivably account for observed results.

Bernick et.al. (24) speculated that the fibrotic appearance of older pulps resulted from persistance of connective tissue of the adventitia following calcific degeneration of blood vessels, whereas the stroma of the pulp, regardless of age, consisted of fine collagenous fibers.

Possible interaction between the protein and other connective tissue components may eventually be shown to account in part for what is seen histologically. The demonstration by Tanzer et.al. (25) of a Schiff base condensation between an unspecified hexose and a hydroxylysine residue in reduced calf skin may be the first evidence of a covalent bond between collagen and other connective tissue macromolecules. The observation by Orlowski (26) of fine collagenous fibers in young bovine and porcine pulps associated with higher proportions of glycoproteins may prove to be a consequence of bonding such as this.

In a study by Pearson and Ainsworth (27) variations in the content of pulpal hydroxylysyl glycosides in unerupted and erupted bovine molar teeth were measured. (These are hexose units attached to collagen hydroxylysyl residues.) The authors found less hydroxylysine-bound carbohydrate in erupted teeth. Based on Grant's (28) suggestion that thickness and degree of organization of collagen fibers increases as the total hexose content decreases, one might accept this as

indirect evidence that pulpal collagen does indeed aggregate into thicker bundles with time, if not increase absolutely.

Attempts to measure absolute amounts of pulpal collagen have been relatively few. The results achieved to date are difficult to apply to the human situation because different animal models were used and results reported in differing units.

Uitto and Antila (29) studied rabbit molar and incisor pulps and found collagen to be 0.69% and 0.52% of wet weight respectively. The ratio of collagen to total protein was 12.0% and 10.3%. Orlowski (26) reported collagen as 12.7% of dry weight in porcine pulp and 16.8% in bovine pulp. Orlowski (14,30) subsequently found the rat incisor to have 3.3-4.7% collagen and the molar pulp, 7.0% collagen on a dry weight basis.

Van Amerongen and Tonino (20) studied human pulp specimens and determined collagen to account for "about 25% of the dry mass" and "about 3.5% of the wet weight."

In an unpublished study Frick (31) compared human pulp tissue of different ages. He measured the hydroxyproline/nitrogen ratio as an indication of collagen concentration and found that there was no significant increase between the age groups of 20-39 years and 40-59 years. Park (32) in another unpublished study of human bicuspid pulps, with or without closed apices, used methods similar to Frick's and concluded that there was a significant increase in collagen concentration

of pulps of teeth with closed apices compared to teeth with open apices. It is unfortunate that in both these studies the tissue samples were pooled into arbitrary age groups rather than being analyzed individually. A steadily decreasing rate of collagen synthesis from age 20 to 39 matched by an equal but opposite increase in rate from age 40 to 59 could give the same result yet be masked by the manner in which the data were presented.

Uitto and Ranta (13) in a similar study of human pulps, reported the collagen/protein ratio of teeth with incompletely formed roots to be 30.0% and of teeth with completed roots to be 37.1%. Wet weight percentages were 1.44 and 2.08 respectively. It is of interest that total collagen per pulp decreased from an average of 0.57 mg to 0.30 mg. The authors attributed this to overall reduction in the size of the pulp. A summary of the quantitative data is shown in Appendix A.

Unfortunately, most of the studies do not permit one to answer the question, "Does pulpal collagen synthesis continue throughout life?" The best measure of the rate of synthesis and turnover of a protein involves the injection of a radiolabeled precursor amino acid into the experimental animal. Tissue containing the protein is removed at varying times and the protein in question is isolated and purified. The amount of radioactivity incorporated is then measured. This type of experiment could not be done with human pulp

collagen for obvious reasons.

The study presented here uses a novel approach to ask the same question. It was decided to assay reducible collagen cross-links which are found in much higher concentrations in newly synthesized collagen (see below). It is appropriate at this point to digress from the central theme and briefly review the nature and biogenesis of these cross-links.

COLLAGEN CROSS-LINKING

The major stabilizing intermolecular links between adjacent collagen molecules result from a Schiff base condensation between the epsilon amino groups on either of the two aldehydic residues, allysine and hydroxyallysine. These latter two residues are formed by action of the connective tissue enzyme lysyl oxidase which oxidatively deaminates lysyl and hydroxylysyl residues yielding an aldehyde functional group on the number six carbon (Figure 1). Depending on which two of these four residues combine, one of three aldimines can be created: dehydrolysinonorleucine, dehydrohydroxylysinonorleucine, or dehydrodihydroxylysinonorleucine. These in turn when reduced with sodium borohydride in a test tube give rise to the reduced forms of lysinonorleucine (LNL), hydroxylysinonorleucine (HLNL), or dihydroxylysinonorleucine (DHLNL) (Figures 2,3 and 4). The proportion of different cross-links has been measured in different tissues by several authors.

In general LNL is a minor component while DHLNL is more prominent in hard tissue. The proportion of HLNL and DHLNL in soft tissue varies widely, depending on the tissue source.*

Definite age-related changes in the appearance and disappearance of these cross-links have been shown. Bailey and Shimokomaki (33) sampled rat, bovine, and human skin, tendon, and cartilage and found that the amount of HLNL decreased as the subjects aged. This change was virtually complete at physical maturity, causing the authors to conclude that such a change was part of the growth process rather than an aging phenomenon.

In a study of rat skin Allain et.al. (34) reported that DHLNL which was present at birth decreased and had disappeared by three months, whereas HLNL increased during this period and subsequently disappeared with further aging of the tissue.

Robins et.al. (35) found the major reducible cross-link in embryonic bovine skin to be DHLNL. This was replaced in later gestation and early post-natal growth by HLNL which increased during rapid growth then decreased until maturity

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* For the sake of simplicity this work will refer to the three cross-links as LNL, HLNL, and DHLNL. It should be understood that these designations properly refer to the reduced forms. By referring to them as such the author will often be actually referring to their unreduced forms as they exist as aldimines in-vivo.

when both cross-links were virtually absent. Interestingly, the authors found a direct correlation between solubility of the tissue at various ages and the amount of labile reducible cross-links. Their conclusion that the proportion of Schiff bases closely reflects the rate of growth, i.e. the amount of newly synthesized collagen present at any one time, provides the central theme of this paper. If pulpal collagen is being constantly synthesized throughout life, this should be indicated in the presence of reducible cross-links over the same course of time. If collagen synthesis accelerates with the onset of fibrosis, the content of reducible cross-links should increase.

Additional support for Robins' et.al. contention comes from studies of different tissues in different animals. Volpin et.al. (36) studied human dermis between the ages of two days and seventy-one years and obtained results similar to Robins et.al. in that DHLNL disappeared shortly after birth while HLNL was high during growth but diminished at maturity.

In a study of human placenta Kao and Leslie (37) found varying amounts of reducible cross-links with respect to development of the tissue. In younger placenta, as the tissue was in the process of reaching maturity, a high percentage of reducible cross-links was found. In full term placenta the percentage was significantly less.

Ranta (38) found the major cross-link of bovine periodontal ligament to be DHLNL while the amount and ratio of DHLNL to HLNL remained constant with age. Takagi et.al. (39), on the other hand, using the same animal model and the same tissue found the DHLNL/HLNL ratio to change over four years with the major component changing from DHLNL to HLNL. The difference in results of these two studies is difficult to explain. Ranta's work is reported as an abstract without any data while the Takagi et.al. paper is written in Japanese with only the figures being represented in English. Nevertheless, one fact was evident. In the periodontal ligagment, a tissue believed to be constantly producing collagen throughout the life of the animal, there was no appreciable decrease in the total amount of reducible cross-links.

All three of these last studies strengthen Robins' et.al. proposal (35) that the presence of reducible cross-links is indicative of collagen synthesis since both tissues, placenta and periodontal ligament, are known to be involved in production of the protein at specific times - the placenta during its early formation and the periodontal ligament throughout its existence. By contrast, any tissue not actively synthesizing collagen would not be expected to have high levels of reducible cross-links. It should be emphasized at this point that the cross-links do not truly disappear with age. Were this the case, collagenous tissue would soon become non-functional. What does disappear is the aldimine

double bond which permits these cross-links to be reduced in a test tube using an appropriate reducing agent such as radioactive sodium borohydride and subsequently isolated.

Numerous theories have suggested the mechanism whereby cross-links become no longer reducible in-vivo. The simplest manner in which the double bond could be reduced would be by the addition of hydrogen as proposed by Mechanic et.al. (40) in the case of dehydro-DHLNL. However, no in-vivo reduction of this type could be detected by Bailey and Peach (41).

Bailey et.al. (42) subsequently proposed a mechanism for HLNL and LNL whereby the aldimine form of these compounds are oxidized to a peptide linkage. Subsequent hydrolysis and isolation of products yield results consistent with this possibility.

The most recent hypothesis suggests the creation of a hydroxypyridinium compound as an intermolecular cross-link involving three peptide chains. Eyre and Oguchi (43) envision that the cross-link results from the spontaneous combination of two residues of hydroxylysino-5-ketonorleucine (the keto form of DHLNL, see Figure 3). Fujimoto et.al. (44) regard the compound as a condensation of one hydroxylysine and two hydroxyallysines. The value of Eyre and Oguchis' model is the predictable decrease in DHLNL with increasing hydroxypyridinium levels. This would explain results seen in many studies where the levels of DHLNL decrease with age yet the collagen becomes increasingly resistant to chemical and physical

denaturation. Theoretically DHLNL cross-links condense to form the more stable hydroxypyridinium compound and in the process become non-reducible and thus undetectable by the procedures used.

The timing of cross-link metabolism has not yet been determined. How long after the creation of the molecule is it before cross-linking can commence? Dodd and Carmichael (45) demonstrated the presence of reducible cross-links in bovine predentin and commented on the speed at which such bonding must take place in a tissue which in man exists no longer than thirty-one hours before it is totally replaced by new predentin (46). How long are cross-links in existence before they "disappear"? Deshmukh and Nimni (47) showed, in-vitro, by the addition of tritium to the double bond that cross-links in reaggregated collagen fibers disappear in as little as two to three weeks. However many authors question whether these bonds are reduced in-vivo by the simple addition of hydrogen.

The answers have not been found but the occurrence of reducible cross-links in tissues with active collagen synthesis nevertheless provides a technique for testing the hypothesis that with age pulpal collagen increases. If, as some suggest, fibrosis of the pulp is a continuous life-long phenomenon or if the tissue turns over rapidly, cross-links would be detectable throughout the life of the pulp. if how-

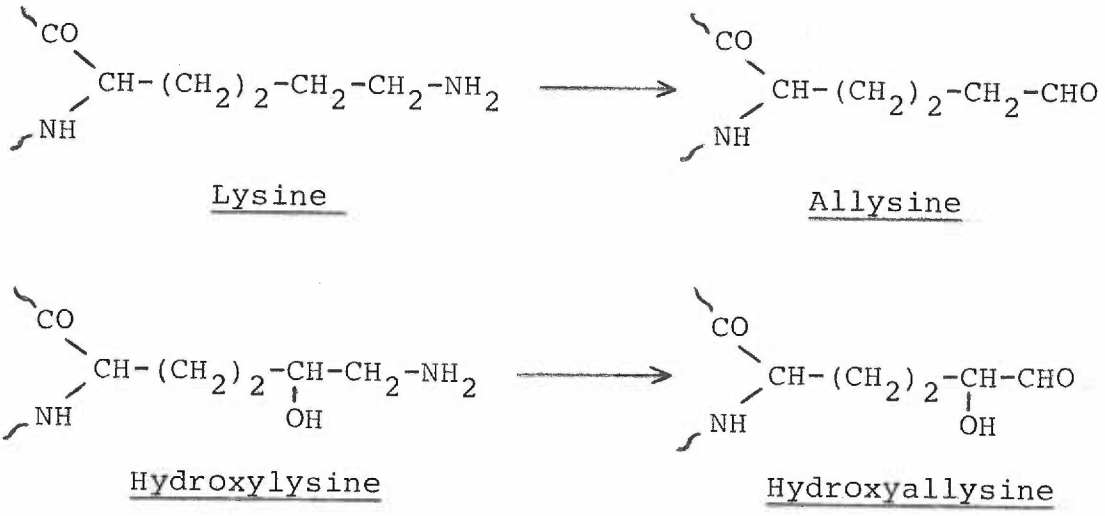


Figure 1. The origin of allysine and hydroxyallysine.

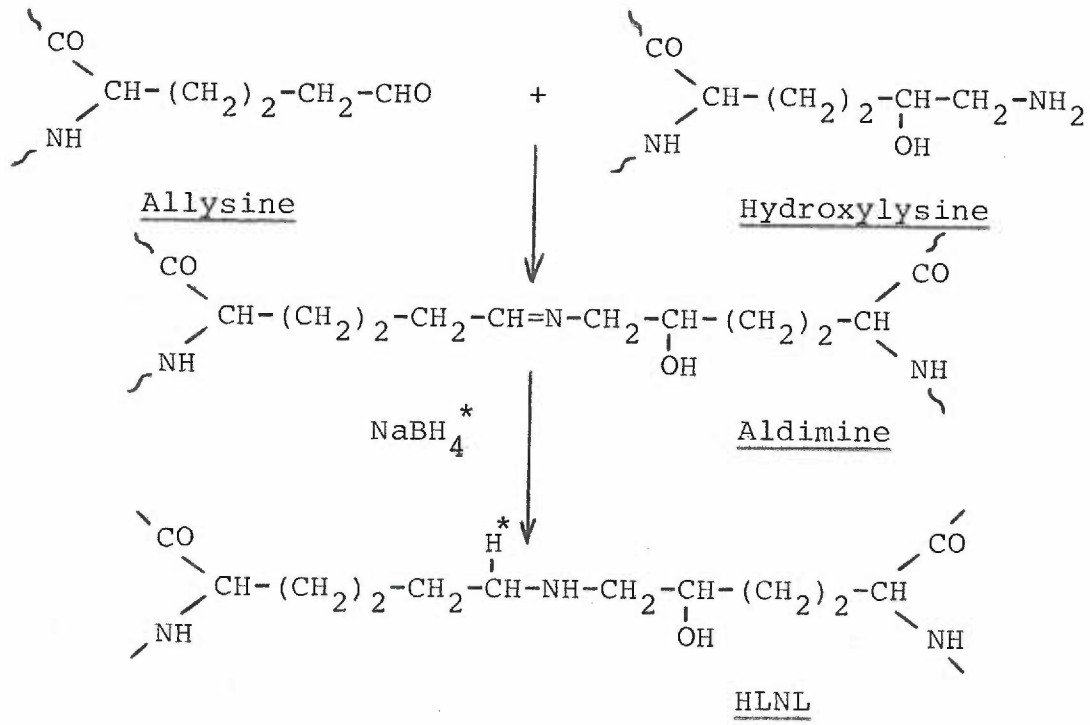


Figure 2. The origin of hydroxylysinonorleucine. The aldimine cross-link formed between allysine and hydroxylysine can be subsequently reduced with tritium-labeled sodium borohydride to form the reduced cross-link hydroxylysinonorleucine (HLNL). The asterisk represents a tritium atom that would occur in HLNL after reduction with the radioactive marker.

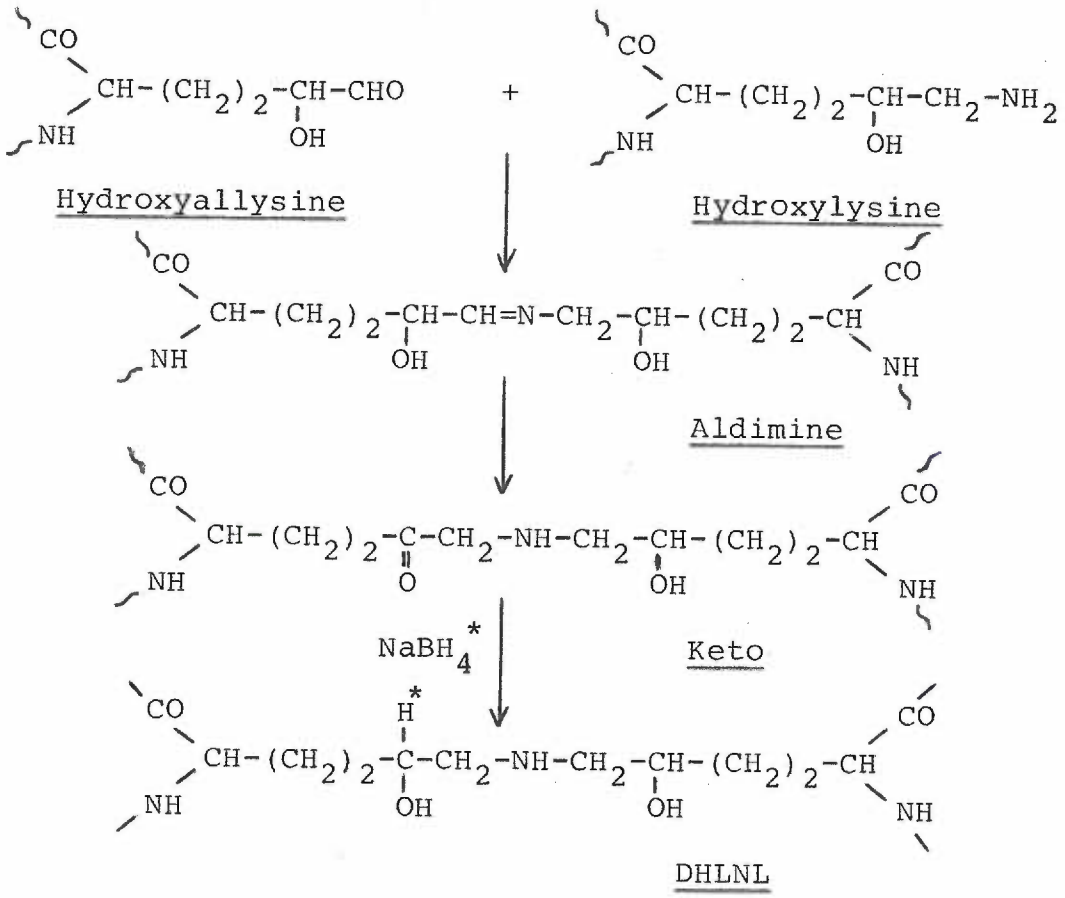


Figure 3. The origin of dihydroxylysinonorleucine. The aldimine cross-link between hydroxyallysine and hydroxylysine can undergo an Amadori rearrangement resulting in the Keto form. This can be reduced with tritium-labeled sodium borohydride to form the cross-link dihydroxylysinonorleucine (DHLNL). The asterisk represents a tritium atom that would occur in DHLNL after reduction with the radioactive marker.

Components	Non-reduced Cross-link	Reduced Cross-link
Lys + Allys	Dehydrolysinonorleucine	LNL
Hyd-Allys + Lys	Dehydrohydroxylysinonorleucine	HLNL
Hylys + Allys	Dehydrohydroxylysinonorleucine	HLNL
Hyd-Allys + Hylys	Dehydrodihydroxylysinonorleucine	DHLNL

Figure 4. Nomenclature of collagen cross-links. LNL, Lysinonorleucine; HLNL, Hydroxylysinonorleucine; DHLNL, Dihydroxylysinonorleucine.

ever collagen deposition within the pulp is a matter of maturation, cross-links should only be detectable during that specific time interval in the tooth's development.

COLLAGEN TYPES

The picture is clouded by the existence of at least five distinct collagen types (I-V), each characterized by a distinct amino acid composition and sequence and anatomical specificity.

Within the last ten years investigators of pulpal collagen have isolated at least two and possibly three of the five types. With the use of disc electrophoresis and amino acid analysis Hirschmann and Shuttleworth (48) demonstrated both type I and type III collagen in bovine dental follicle and papilla. In a solubility study of the same species Shuttleworth et.al. (49) found that 18% of follicle collagen, 24% of papilla collagen, and 28% of pulp collagen were type III. A cyanogen bromide analysis showed percentages to be 20%. 20%, and 31% respectively. They concluded that there was a small increase in type III collagen with age in the tissues associated with a developing tooth.

By use of limited pepsin digestion followed by gel electrophoresis Hayakawa et.al. (50) reported that in the teeth of cows, the ratio of type III to type I changed from 0.15 in the partly formed crown stage to 0.46 by the time the teeth were showing signs of attrition.

Van Amerongen and Tonino (20), using techniques similar to Hayakawa's on young human premolars, showed that besides type I collagen a small amount of type III was present.

Gotoh et.al. (51) studied odontogenic cells of the rabbit tooth germ with respect to the synthesis of procollagen precursor types and found that 60% of the secreted precursor was type III procollagen and 40% was type I procollagen.

Cournil and Pomponio (52) demonstrated a moderate reaction of rat incisor pulp tissue to both types I and III collagen antiserum with the latter particularly evident on the walls of pulp capillaries.

In an immunofluorescent study of different collagen types in the mouse tooth primordium, Miller and Carmichael (53) located type I in the dental papilla and type IV along the entire dentin-enamel junction and in all basement membranes that delineated epithelial-mesenchymal interfaces. Whether these junctions and interfaces can be considered representative of primordial pulp would be open to question. Theslaff et.al. (54) used the same animal and techniques and found only a weak reaction to anti-collagen I antibodies in dental mesenchyme during the time of tooth formation, whereas the reaction to procollagen type III was lost during odontoblast differentiation but reappeared with advancing vascularization of the dental pulp.

Through the use of carboxymethyl cellulose chromatography Trelstad and Slavkin (55) found type I but no type IV collagen in the mesenchymal tissue of rabbit molar papilla.

Takagi et.al. (39) used cyanogen bromide digestion of bovine pulp and reported that 44% of total collagen was type III and that this percentage significantly increased from age one to four years. Takagi et.al. (39) and Takagi (56) are the only authors to date to have analyzed cross-links present in pulpal collagen (bovine). They found the ratio of DHLNL to HLNL increased from 0.82 to 1.33 between the ages of one and four. Similar studies on human pulp collagen have not been performed and will be an important contribution of this project.

The existence of several types of collagen in the pulp could conceivably influence any interpretation of cross-link analysis. A correlation between types of collagen and types of cross-links has yet to be established. This study was based on the premise that collagen synthesis is characterized by the presence of reducible cross-links. The study will not specify which collagen is being produced. The possibility of cross-links arising from one or more collagen types will have to be considered, but regardless of which collagen type exists in the pulp, the fact remains that the cross-links in all connective tissue are virtually all DHLNL and/or HLNL. Therefore, the concept that collagen synthesis is characterized by increased levels of reducible cross-links should be valid.

SUMMARY

Histological studies of the dental pulp have demonstrated that collagen concentration may increase with age and/or "mature" in the sense of becoming more aggregated into larger discrete bundles. This reported increase in collagen aggregation along with the evidence of diminished solubility with time, seems to indicate that pulpal collagen becomes more cross-linked as the tissue ages. Most of these studies however do not distinguish between normal growth and development, i.e. maturation, and the aging process. Nevertheless, there are those who believe that pulpal collagen does indeed undergo a time of maturation during development of the tooth and changes subsequent to that are only minimal. It is the author's impression, upon extensive investigation of the literature, that much of the evidence for this concept is based primarily on animal, not human, models, and involves teeth in various stages of immaturity without adequate comparison to stages later in life.

There have been few attempts to quantify pulpal collagen at various ages. The data that have been reported are distributed among studies of different animal species and reported in one of three ways: as percentage of dry weight, of wet weight, or of total protein. To date there has been no conclusive quantitative study to show that collagen does indeed increase with age once a tooth has reached maturity (i.e. three years post-eruption or the time that the apex closes (57).

The correlation of reducible cross-link concentration with active collagen synthesis provides a model for testing the hypothesis that with age pulpal collagen increases. The ability to measure cross-links using very small amounts of tissue permits the experiment to be performed with individual human teeth. If one assumes that as the dental papilla matures into the dental pulp, collagen is laid down as part of the extracellular matrix, then one would expect to measure cross-links in these stages of tooth development. Similarly, if collagen is constantly produced throughout life as suggested by many authors, such reducible cross-links should be detectable throughout the life of the pulp. Conversely, if collagen deposition ceases or slows with maturity these same cross-links should disappear or diminish at that time. We therefore propose to quantify the reducible collagen cross-links in human coronal pulp from subjects of varying ages (16-40 years).

METHODS AND MATERIALS

Abstract: Thirty coronal pulps of human third molars of patients ranging in age from 16 to 40 years were collected, weighed wet, desiccated, and dry weights recorded. Twenty of these pulps were reduced with tritiated sodium borohydride, hydrolyzed, and subjected to cross-link analysis and hydroxyproline assay. An estimate of hard tissue (dentin and/or pulp stones) contained within each hydrolyzate was made by use of colorimetric determination of calcium (Group A).

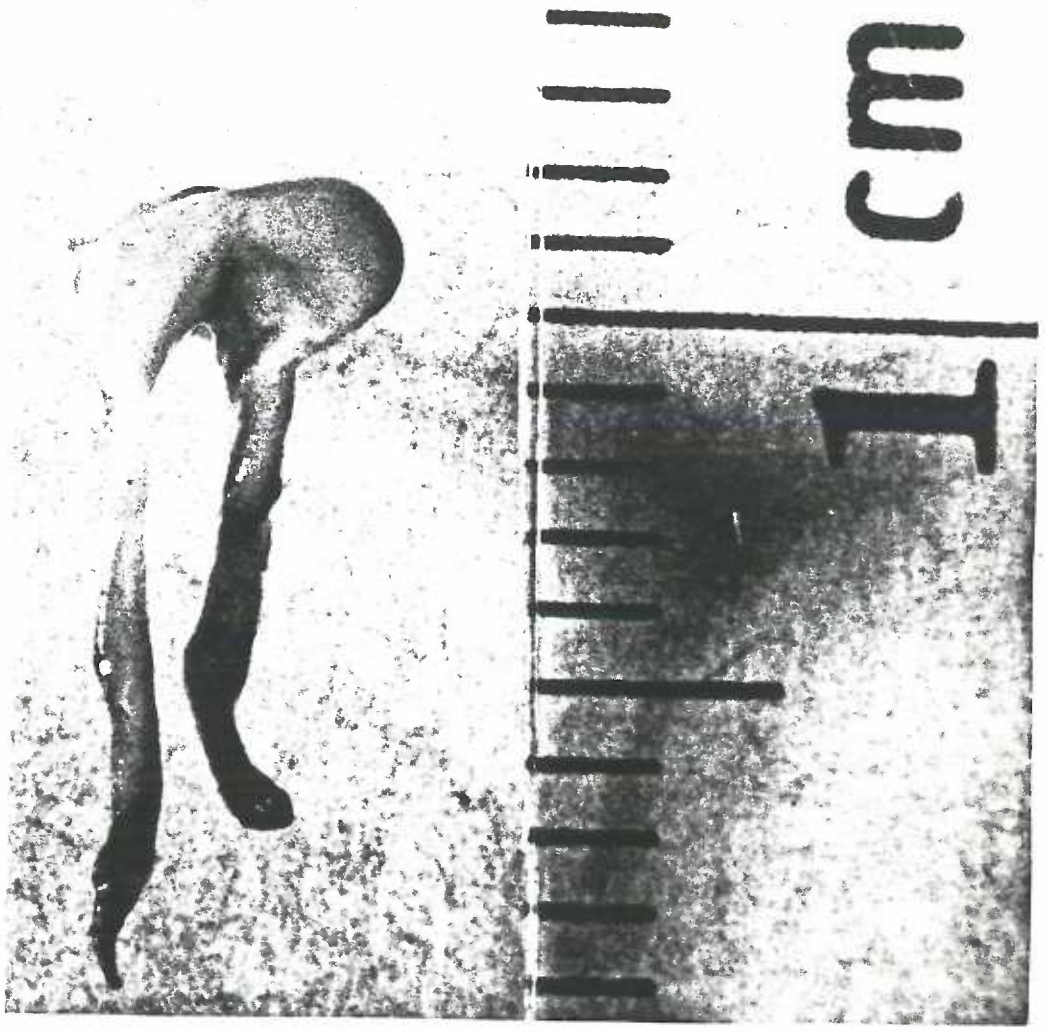
The coronal pulp of forty-eight additional third molars including ages 16 through 30 were dried, weighed, hydrolyzed and subsequently analyzed for hydroxyproline and nitrogen. Results were reported as collagen, protein, and collagen/protein percentages (Group B).

Only teeth that were shown to be free of caries, restorations, periodontal disease or other conditions that might affect the pulp were used in this study.

PREPARATION OF TISSUE

1. All teeth used in this work were third molars extracted from human subjects and immediately frozen at -15° to -20° C until required for use.

2. Teeth were grooved with a high-speed fissure burr using water coolant, split open with a chisel, and the pulps removed. Coronal pulp was obtained by sharp dissection with a scalpel blade at what corresponded to the radicular orifice (Figure 5). When wet weights were to be recorded, the tissue sample was immediately placed in a pre-weighed



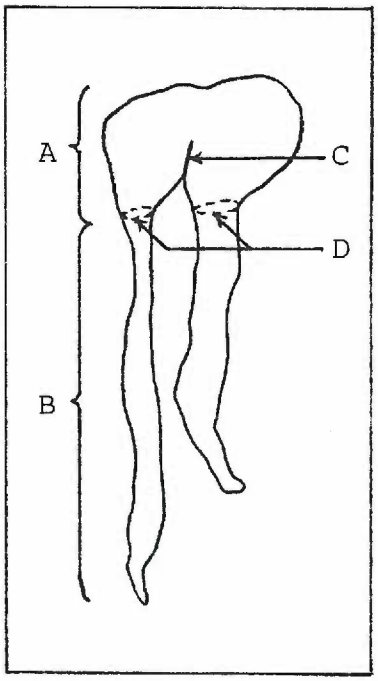


Figure 5. Human dental pulp.

Top: Dental pulp taken from mandibular third molar of twenty year old individual. This particular pulp demonstrates two individual radicular portions which join at the coronal pulp. To the right of the pulp is a centimeter ruler where one division equals one millimeter.

Right: Diagrammatic representation of pulp seen in top photograph. (A) coronal pulp (B) radicular pulp (C) supporting needle (D) lines of dissection corresponding to radicular orifices.

0.5 ml glass vial with a ground glass stopper and weighed on a Mettler Type H balance. The difference in weights of the pre-weighed vial and the vial plus sample was considered wet weight.

3. The sample was then placed in a desiccator at 4° C until a constant dry weight was achieved and recorded.

CROSS-LINK ANALYSIS

A two column system (long and short) for resolution of reduced cross-links as described by Mechanic (58) was employed.

1. The twenty dried samples in Group A were placed in 2.0 ml 0.02 M potassium phosphate buffer, pH 7.4, and agitated for 3 hours at 4° C, then washed three times with 1.0 ml distilled water and lyophilized overnight. This procedure removed soluble serum proteins.

2. Samples were again placed in 1.0 ml 0.02 M potassium phosphate buffer, pH 7.4, at 4° C and agitated for 2½ hours. One drop of Anti-Foam A Concentrate (1:1000, Appendix G) was added to each sample which was reduced with 5.0 µl 0.08 M tritiated sodium borohydride (250 mCi/mM, Appendix G) using a stir plate at room temperature for 3 minutes. Two additional increments of reducing agent, 5.0 µl each, were subsequently added for 3 and 5 minutes respectively. Following the last addition the sample was dialyzed overnight against running water at 4° C.

3. Samples were lyophilized and then hydrolyzed in 2.0 ml of 3 N para-toluene sulfonic acid (pTSA) at 105° C for 20 hours in vacuum sealed vials. (Samples in Group B were hydrolyzed in vacuum sealed vials containing 1.0 ml 6 N HCl for 23 hours. Hydrolyzates were evaporated to dryness under nitrogen and re-dissolved in 2.0 ml distilled water.)

4. Hydrolyzates were run through glass wool and stored in air-tight containers at 4° C until required for use.

LONG COLUMN CHROMATOGRAPHY

1. The long column was packed to 58 cm with a spherical cation exchange resin (9-12 microns, Appendix G).

2. A 25 µl aliquot of hydrolyzate was counted using Packard Insta-Gel scintillation fluor (Appendix G) on a Packard Tri-Carb Liquid Scintillation Spectrometer. From this determination, a volume of hydrolyzate was applied to the column so that approximately 2×10^6 cpm were used. A 0.2 ml amino acid "spike" consisting of glycine, lysine, phenylalanine, hydroxylysine, and histidine in concentrations of 3 mg/ml was added to aid in localization of constituents.

3. The sample was applied to the resin surface and washed in with starting buffer. The column was eluted with a complex gradient generated by a Technicon gradient mixer (Appendix G) and a Mark Instruments pump (Appendix G). The starting buffer was 0.25 M sodium citrate, pH 3.15, while the limiting buffer was 0.4 M sodium citrate, pH 9.1. The complete analysis

took 6 hours at a flow rate of approximately 68 ml/hr. The back pressure of the column was generally 340 p.s.i.. Fractions of 1.5 minute duration were collected on a Gilson Micro-Fractionator fraction collector (Appendix G).

4. From each fraction, aliquots of 0.2 ml were pipetted into 7 ml scintillation vials in addition to 0.4 ml distilled water and 5.0 ml Insta-Gel scintillation fluor and counted for 15 minutes. Counts per minute were converted to disintegrations per minute by use of the sample channel ratios method.

5. Other aliguots of 0.2 ml were mixed with 0.2 ml ninhydrin reagent and the color developed by heating in a water bath at 100^{o} C for ten minutes. The positions of marker amino acids were recorded on the radioactivity elution profile.

SHORT COLUMN CHROMATOGRAPHY

The long column procedure did not adequately separate the major reducible cross-links from low levels of other non-cross-link reducible components. To do this, the fractions containing the three major cross-links were pooled and applied to a second ion exchange column. By this means the contaminents which gave rise to noisy base lines were eliminated.

1. A sample consisting of 5-6 pooled fractions representing a radioactive peak from the initial long column chrom-

atography was acidified by the addition of 6 N HCl (4 drops/ fraction) was added to an amino acid "spike" identical to that used on the long column. This was placed on a short column packed to 28 cm with identical resin as used in the long column.

2. Elution was performed with a 0.35 M citric acid buffer of pH 5.25. Fractions were collected for 1 minute at a flow rate of 70 ml/hr. Back pressure generally ran 300 p.s.i.

3.Radioactivity and ninhydrin analyses were performed as before.

STANDARDIZATION OF PULP REDUCTIONS

Because the test population of Group A consisted of twenty pulps reduced with two separate batches of sodium borohydride some means of standardization of the radioassay results was required. Rat tail collagen was divided into equal portions and reduced simultaneously with each pulp reduction. The specific activity of the resulting cross-links for each rat tail was compared and an appropriate correction factor determined.

HYDROXYPROLINE ASSAY

1. An appropriate dilution of the sample hydrolyzate was analyzed on a Technicon Autoanalyzer for hydroxyproline content using the method devised by Grant (59) as a modification of Stegemann's technique (60). This involved oxidation

of the hydroxyproline with chloramine T; destruction of excessive chloramine T with perchloric acid and reaction of the hydroxyproline chromagen with p-dimethylaminobenzaldehyde to develop a pink color. Absorption was read at 550 mu.

2. Collagen content was computed as hydroxyproline content x 7.4 (61) and expressed as a percentage of pulpal dry weight for Group A samples. Group B samples had collagen content expressed as a percentage of dry weight and as a collagen/protein ratio.

NITROGEN ASSAY BY THE KJELDAHL TECHNIQUE

1. An appropriate dilution of the sample hydrolyzate was digested in boiling sulfuric acid to which a copper catalyst had been added. Conversion of organic nitrogen to ammonium sulfate was followed by alkalinization and distillation of liberated ammonia into a standard acid medium. Titration of excess acid with standard base was achieved.

2. Protein content was computed as nitrogen content times 6.25 (62) and expressed as a percentage of pulpal dry weight.

CALCIUM ASSAY

1. Determination of the calcium concentration in pulp hydrolyzates for Group A (plus one additional sample) was by the method of Williams and Wilson (63) modified by addition of 3 N NaOH to correct for acidic conditions result-

ing from para-toluenesulfonic acid.

2. Standards ranging from 0.5 to 10.0 µg/ml calcium in distilled water were prepared.

3. Hydrolyzates were diluted with water by preparing 0.1 ml sample and 0.2 ml 3 N NaOH to 2.0 ml water.

4. A solution of 2.0 ml 0.4% glyoxal bis(2-hydroxy-anil) (Appendix G) in absolute ethyl alcohol was added and the resulting solution mixed for three seconds.

5. A solution of 0.2 ml containing 10.0 g NaOH and 0.5 g sodium carbonate in 100 ml water was added and the resulting solution again mixed for three seconds and taken up to 3000 r.p.m. centrifugation and immediately brought down.

6. Chloroform (5.0 ml) was added and the solution mixed well by inverting the test tube 10 times.

7. The solution was clarified by centrifugation (3000 r.p.m.) for 10 seconds.

8. Color was read at 535 nm using a Bauschand Lomb colorimeter (Appendix G).

BONE ELUTION PROFILE

Dog bone collagen, previously prepared and reduced, was used as a standard in identification and localization of various components in both long and short chromatograms because of its relative freedom of contamination within the

chromatogram and its ability to produce distinct evidence of all three cross-links.

RESULTS

WATER CONTENT

To determine whether water content of the pulp decreased over the time period of the experiment, linear regression equations were developed. In Figure 6 line A represents the regression line of all data points where $y = -0.522x + 91.74$; $r = -0.293$, insignificant at $p \leq 0.05$. For reasons listed in the following paragraph, a second regression equation represented by line B in Figure 6 was calculated which excluded the lowest data points at ages 17, 36, and 38 years. Here $y = -0.285x + 88.96$; $r = -0.411$, significant at $p \leq 0.05$. In this case the conclusion is reached that there is a significant change (approximately 7% decrease) in water content of pulp tissue between the ages of 16 years and 40 years. This agrees with the findings of other workers on the subject (4,22).

It was noted that upon opening some teeth that the pulps appeared desiccated. These teeth were not included in the study. This desiccation probably resulted from storage in the freezer and it is conceivable that many of the pulps used could have suffered from some degree of desiccation. However, the remarkably consistent water content shown in Appendix B and Figure 6 suggest that this was not the case. Three samples used in the study at ages 17, 36, and 38 years exhibited a low water content and it was considered that they could be excluded from the study. As shown in Figure 6 the water

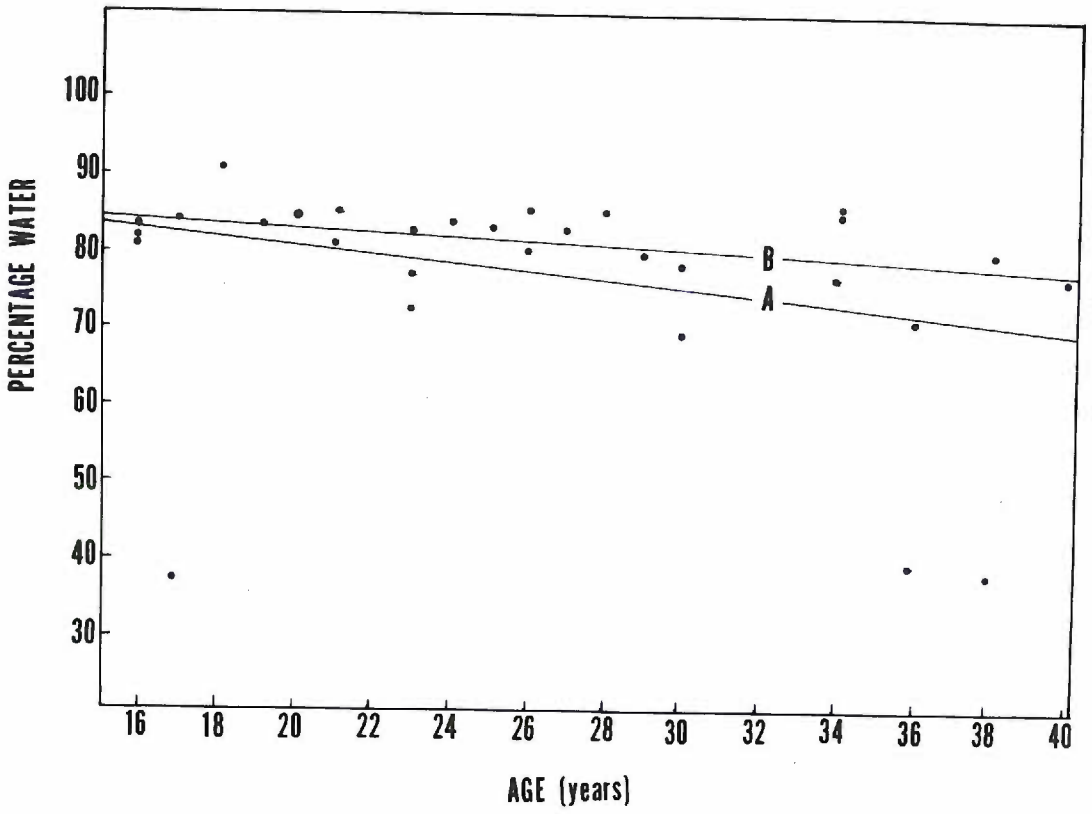


Figure 6. Water content of human pulp.

Line A represents a regression line including all 30 data points where $y = -0.552x + 91.74$; $r = -0.293$, insignificant at $p \leq 0.05$.

Line B represents a regression line excluding low values seen at ages 17, 36, and 38 years where $y = -0.285x + 88.96$; $r = -0.411$, significant at $p \leq 0.05$.

content of these three samples was approximately one-third that of the majority of the samples which we believe could not result from biological variation.

CHROMATOGRAPHY

An example of a long column chromatogram is shown in Figure 7 and a short column chromatogram in Figure 8. With regard to the former it can be seen that the reduced precursors, dihydroxynorleucine, DHNL, and hydroxynorleucine, HNL, eluted around fractions 34 and 52 respectively. (DHNL and HNL correspond to the reduced forms of allysine and hydroxyallysine prior to their becoming involved in aldimine formation.) Peaks eluting in fractions 125, 131, 151, and 156 correspond to hexosylamines (a combination of hexose unit and amine via a Schiff base condensation) reported by numerous authors (58,64,65). A large peak of unknown origin was consistently found at fraction 159 between the hexosylamine peak at 156 and the first cross-link, DHLNL, at fraction 165.

The reduced cross-link DHLNL was consistently the largest of the three cross-links studied. Nevertheless, much of the radioactivity in this fraction could be attributed to contamination. Upon rechromatography of fractions 163-167 on the short column (Figure 8) peaks other than the cross-link were found. Similar results were seen in the rechromatography of HLNL and LNL (Figures 9 and 10). These results are in agreement with Mechanic (58) who devised the system we used and

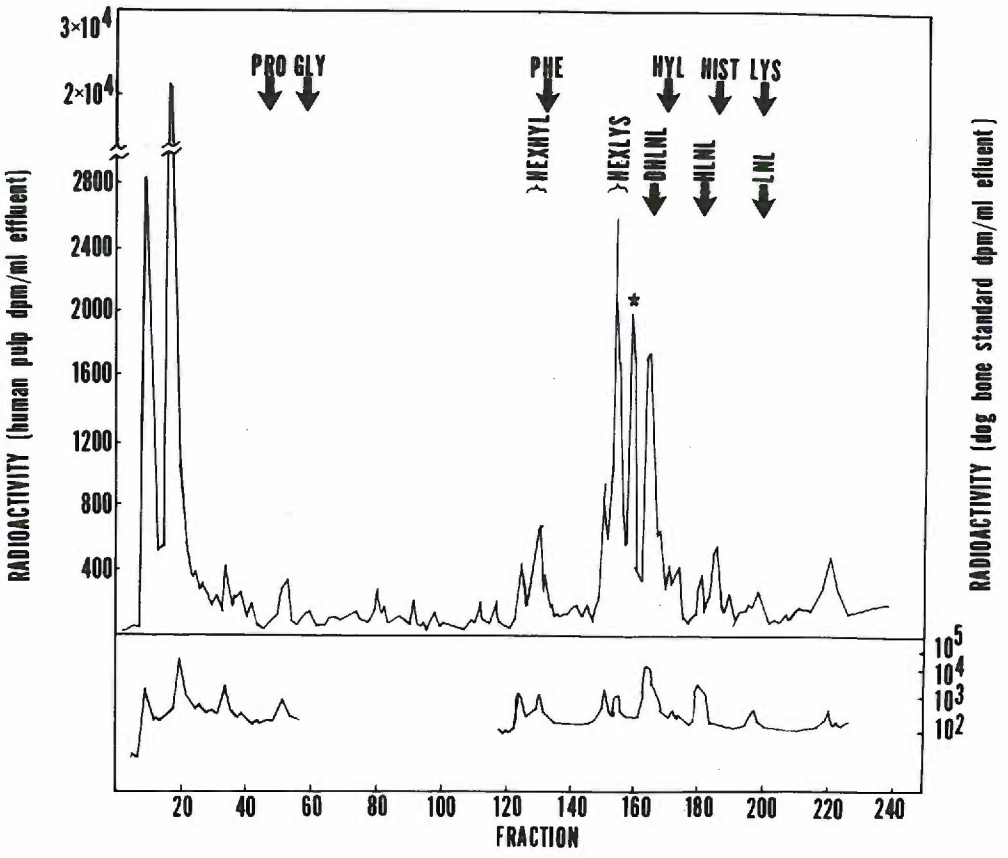


Figure 7. Long column chromatogram.

Typical elution pattern (long column) of reducible components from acid hydrolyzates of human pulp (upper portion of graph) and dog bone standard (lower portion of graph). See text for description of peaks.

Radioactivity measured in disintegrations per minute (dpm) per milliliter of effluent.

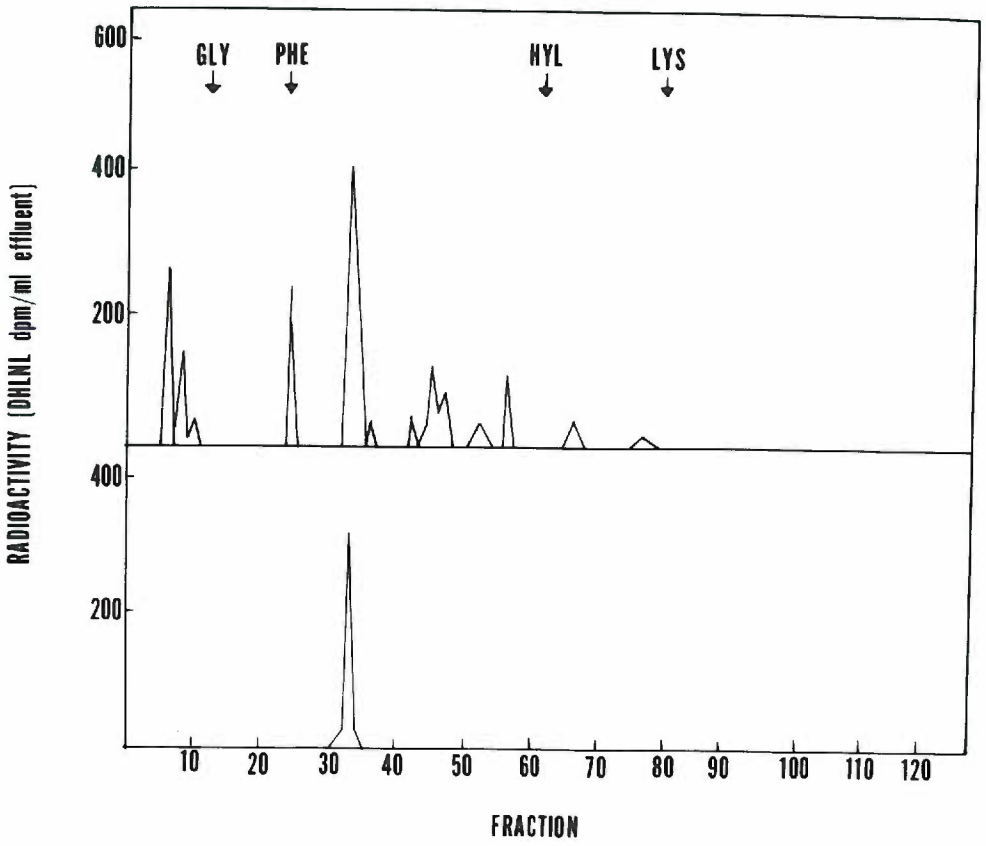


Figure 8. Short column chromatogram (DHLNL).

Typical elution pattern (short column) of rechromatography of pooled fractions corresponding to DHLNL (fractions 163-167, Figure 7) on long column. Human pulp upper portion of graph. Dog bone standard lower portion of graph.

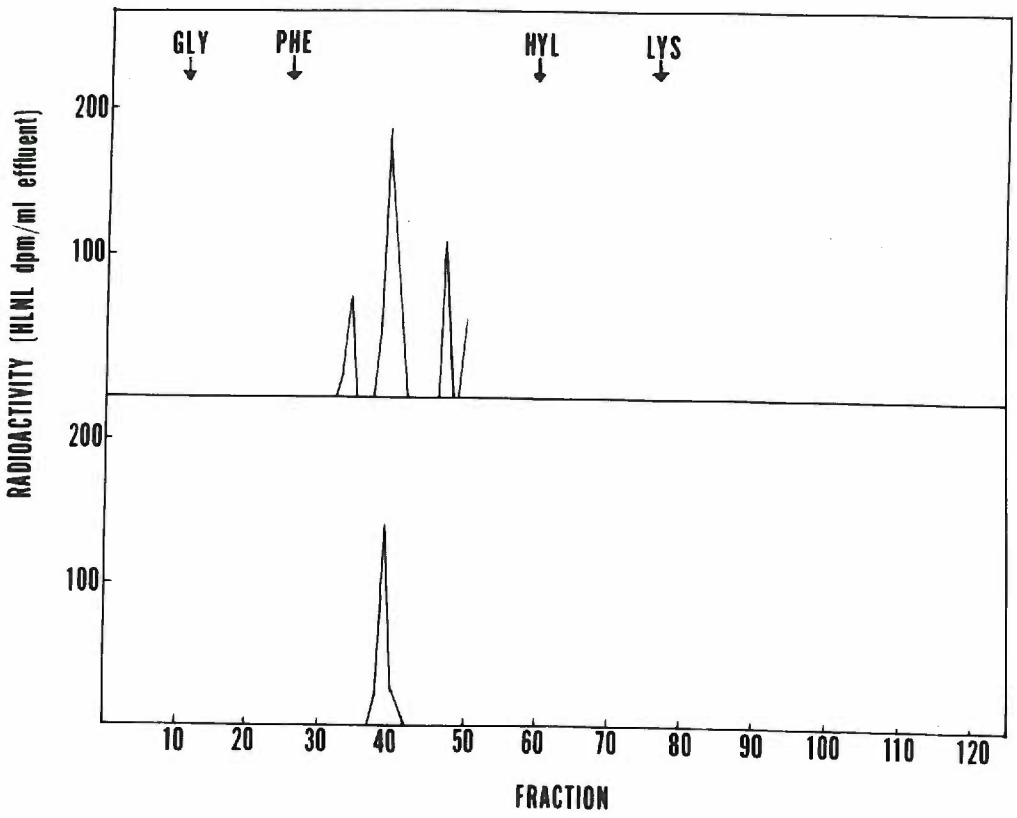


Figure 9. Short column chromatogram (HLNL).

Typical elution pattern (short column) of rechromatography of pooled fractions corresponding to HLNL (fractions 177-182, Figure 7) on long column. Human pulp upper portion of graph. Dog bone standard lower portion of graph.

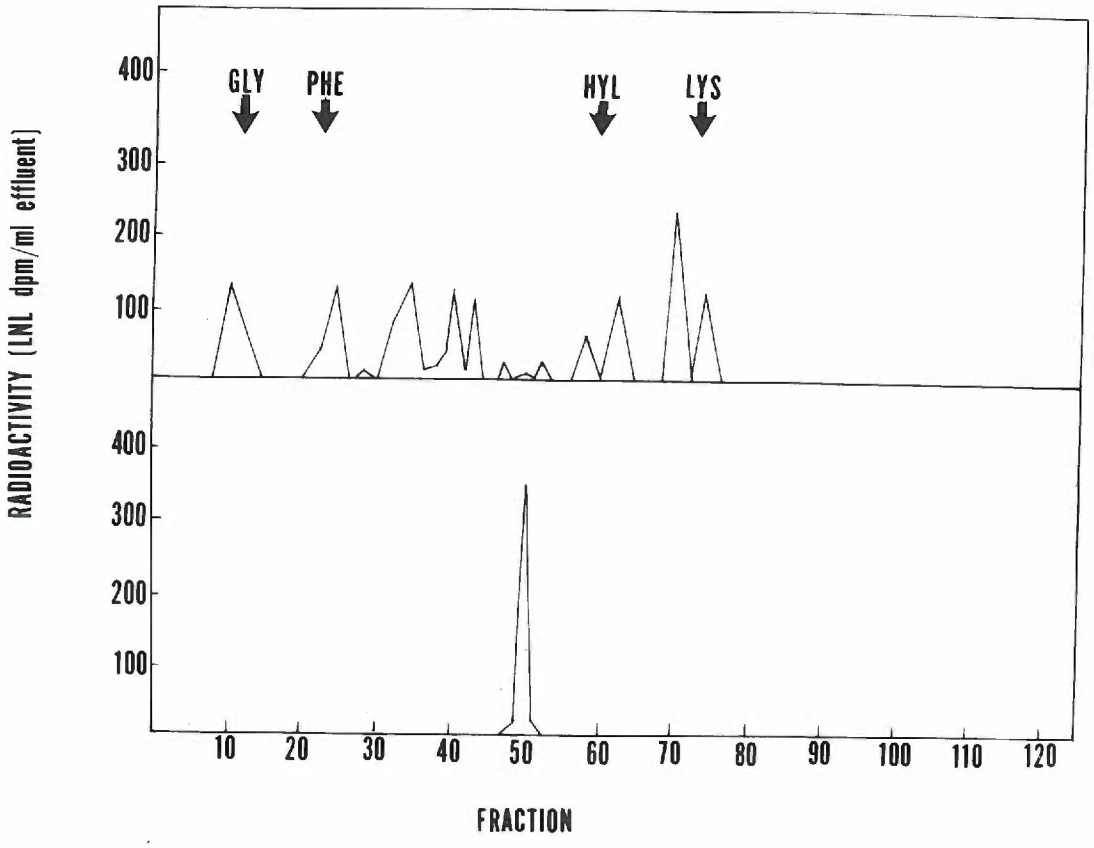


Figure 10. Short column chromatogram (LNL).

Typical elution pattern (short column) of rechromatography of pooled fractions corresponding to LNL (fractions 197-200, Figure 7) on long column. Human pulp upper portion of graph. Dog bone standard lower portion of graph.

showed co-elution of many components on the long column which could only by separated by rechromatography on the short column. It is obvious from Figure 7 and Figure 11 that DHLNL is the major cross-link while HLNL and LNL are very low and difficult to quantify. The quantitative aspects of this study are based upon an assay of the amount of radioactive DHLNL present in each pulp. The results are expressed either as DHLNL dpm/mg dry pulp or DHLNL dpm/mg collagen.

Because peaks corresponding to HLNL and LNL on the long column chromatogram were generally very small or even absent, the only means of pooling fractions corresponding to these cross-links was by location of amino acid markers. This was done by computing the proportionate distance of a particular cross-link between phenylalanine and lysine on a dog bone collagen standard chromatogram and applying this same proportion to amino acid markers on the chromatogram of pulp hydrolyzate. A total of 5-6 fractions were collected around the indicated fraction in order to ensure the inclusion of the HLNL and LNL on subsequent short column chromatography. In general the amount of DHLNL far exceeded that of HLNL while the amount of LNL was barely detectable (Figure 11).

AGE-RELATED CHANGES IN CROSS-LINKS

DHLNL demonstrated a definite age-related decrease over the ages included in this study. It was seen to diminish from a high of approximately 21,000 dpm/mg collagen at age 16

to approximately 11,000 dpm/mg collagen at age 40 (Figure 11). Statistically the data for DHLNL dpm/mg collagen was best represented by a linear equation line (line A) of equation $y = -390.72x + 26,145.44$. The correlation coefficient, r, equaled -0.578 and was significant at $p \leq 0.01$. When expressed as dpm/mg dry weight a similar relationship to age for DHLNL was found (Figure 12, line A) where the regression line was represented by $y = -159.64x + 7854.74$. The correlation coefficient, r, equaled -0.658 and was significant at $p \leq 0.01$.

HLNL and LNL showed concentrations on the average of approximately 2000 and 500 dpm/mg collagen respectively and remained relatively constant with time. Regression lines for HLNL and LNL were represented by $y = -31.08x + 2694.01$ and $y = 16.40x - 108.74$ respectively (Figure 11, lines B and C). The values of the correlation coefficients proved not to be significant at $p \leq 0.05$. Because of the considerations already discussed regarding the difficulty in accurately obtaining the pooled sample and the insignificance of correlation coefficients, these data are considered unreliable. The most that can be said is the HLNL and LNL are far less abundant in the pulp than DHLNL, that HLNL seemed to be more abundant than LNL, and that both cross-links appear to be present over the age range studied.

Allowing for questionable interpretation of results regarding HLNL and LNL, one might tentatively conclude that because of the decreasing amount of DHLNL, the ratio of DHLNL

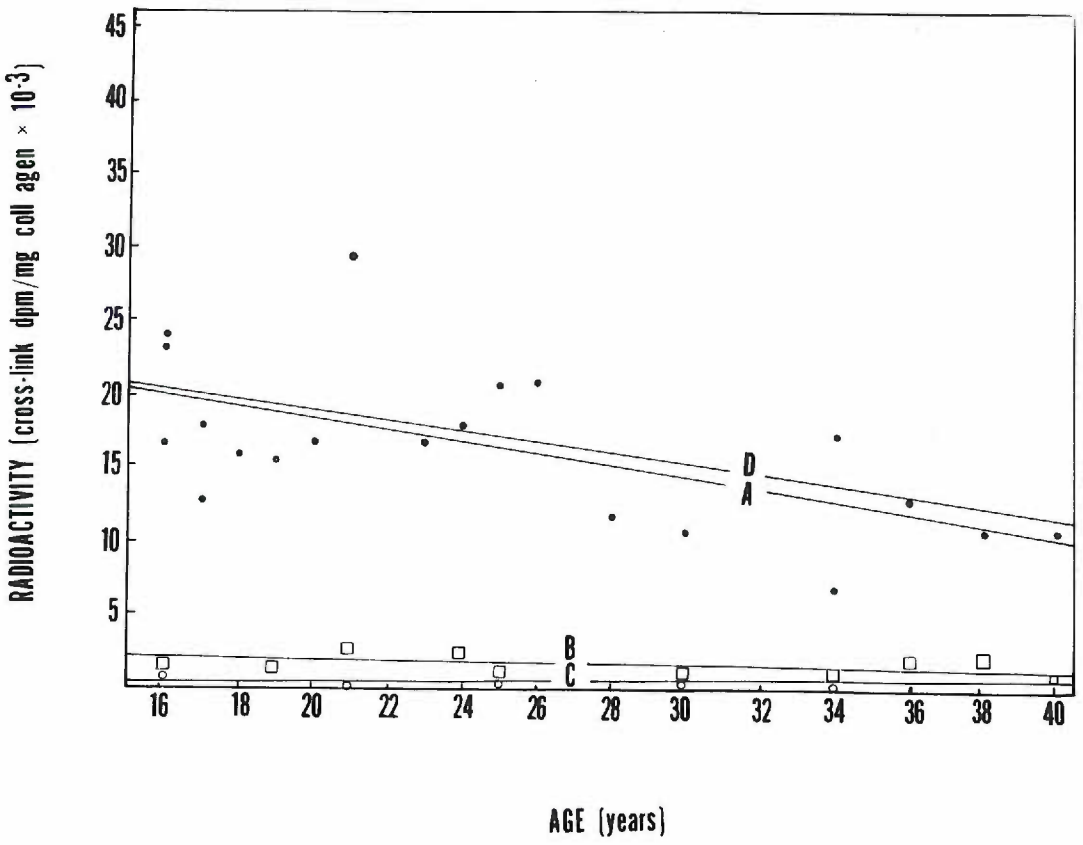


Figure 11. Reducible cross-links per milligram collagen as a function of age.

Closed circles represent individual data points for DHLNL dpm per milligram collagen without consideration of pulp calcium and correspond to line A where $y = -390.72x + 26{,}145.44$; $r = -0.578$, significant at $p \leq 0.01$.

Squares represent individual data points for HLNL dpm per milligram collagen without consideration of pulp calcium and correspond to line B where $y = -31.80x + 2694.01$; $r = -0.456$, insignificant at $p \leq 0.05$.

Open circles represent individual data points for LNL dpm per milligram collagen without consideration of pulp calcium and correspond to line C where $y = 16.40x - 108.74$; $r = 0.294$, insignificant at $p \leq 0.05$.

Line D represents DHLNL dpm per milligram collagen with consideration of pulp calcium where $y = -358.20x + 26{,}108.70$; $r = -0.513$, significant at $p \leq 0.05$.

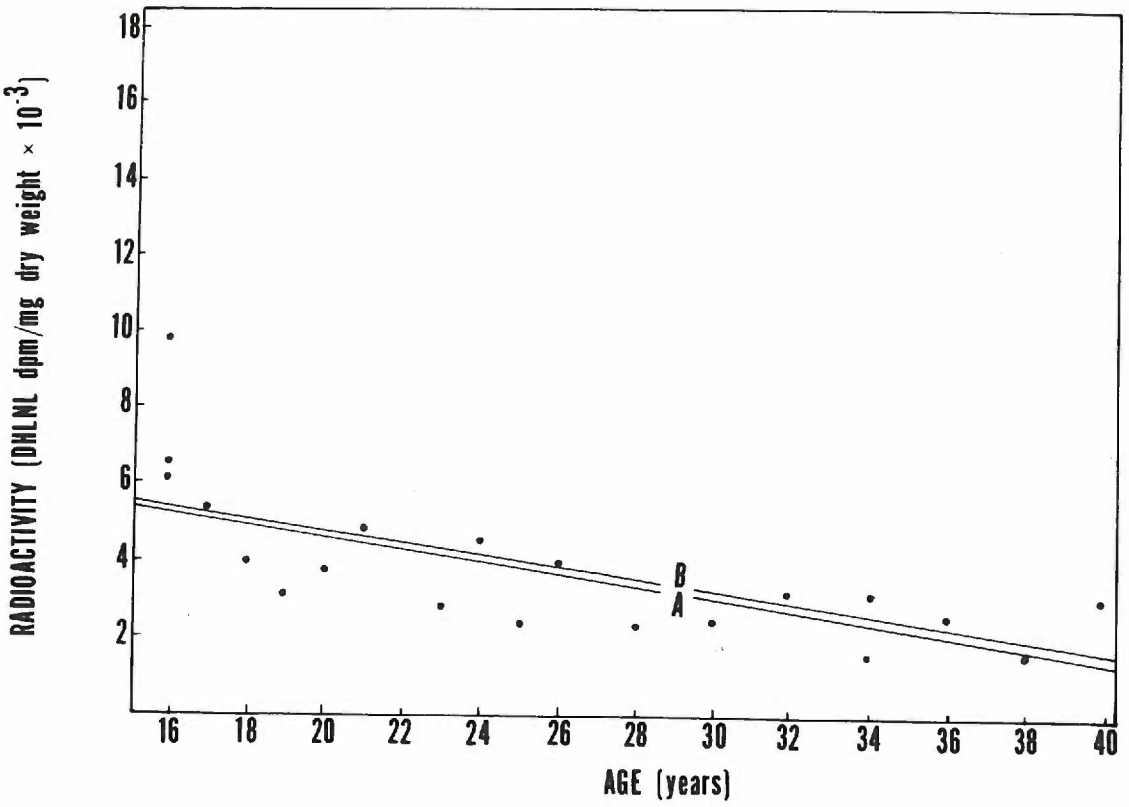


Figure 12. DHLNL dpm per milligram dry weight as a function of age.

Closed circles represent individual data points for DHLNL dpm per milligram dry weight without consideration of pulp calcium and correspond to line A where $y = -159.64x + 7854.74$; $r = -0.658$, significant at $p \leq 0.01$.

Line B represents DHLNL dpm per milligram dry weight taking into consideration pulp calcium where $y = -153.56x + 7881.48$; $r = -0.630$, significant at $p \leq 0.01$.

to HLNL decreases over the age range studied. This diminishing ratio leads to interesting speculation regarding a possible association between cross-link ratio, pulpal calcification, and the role of calcium hydroxide in dental treatment (see DISCUSSION, CROSS-LINKING AND CALCIFICATION).

COLLAGEN CONCENTRATION

The collagen content of the twenty specimens subjected to cross-link analysis (Group A) is seen in Figure 13, line A. In general a slight diminution of collagen on a dry weight basis is apparent between the ages of 16 and 40 years although this relationship proved insignificant at $p \leq 0.05$. The data were represented by a regression equation of $y = -0.37x + 32.82$ where $r = -0.397$.

Three different age groups (16-17, 23-25, and 28-30 years; Group B) were chosen for further study for several reasons. Firstly, the years from approximately 16 through 25 are a critical period in the development of the third molar as eruption, occlusion, and final root development are taking place. Secondly, evidence has accumulated suggesting changes in collagen concentration during the time of final root development (13,32). Finally, it was thought that this study could either confirm or refute the finding reported in the above paragraph where collagen content was seen to diminish slightly over the ages of 16 through 40 years.

When these different age groups were analyzed for collagen content (Figure 14, Appendix C) and for significant

differences between groups using analysis of variance for independent data (Appendix D) it was found that pulps of teeth in the age group 16-17 years had a significantly higher percentage of collagen than either of the older groups at a 0.01 probability level. The two older age groups did not differ significantly. The conclusion is reached that coronal pulp collagen content does decrease between the ages of 16-17 and 23-25 years, but no change is seen between the latter age group and 28-30 years.

Based on these results, a more thorough investigation of the data in Figure 13 was felt justified. When taken alone data points for ages 16 through 24 years produce a regression line B where $y = -1.86x + 61.36$ and $r = -0.654$. This coefficient was significant at $p \leq 0.05$. Data points of ages 24 through 40 years give regression line C where $y = 0.25x + 12.89$ and $r = 0.274$, insignificant at $p \leq 0.05$. This interpretation of the data agrees with the general conclusion that there is a significant decrease in coronal pulp collagen content in the years immediately following apical closure.

PROTEIN CONCENTRATION

When the same samples represented by the three different age groups (Group B) were analyzed for protein concentration (Figure 15, Appendix C) and significant differences between groups using analysis of variance for independent data (Appendix E), it was found that pulps of teeth in the

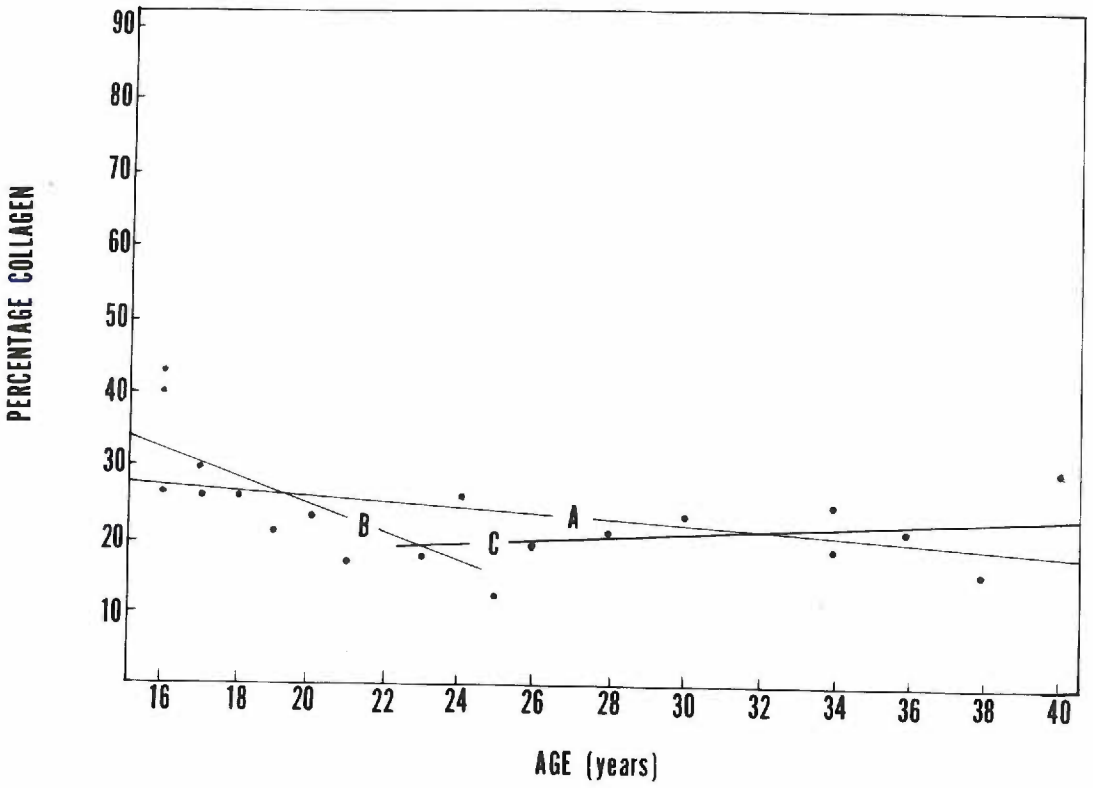


Figure 13. Collagen concentration of human pulp as a function of age.

$$\text{Percentage collagen} = \frac{\text{milligrams collagen}}{\text{milligrams dry weight}} \times 100$$

Individual data points of twenty samples subjected to cross-link analysis are shown.

Line A was computed on the basis of all twenty points where $y = -0.37x + 32.82$; $r = -0.397$, insignificant at $p \leq 0.05$.

Line B was computed on the basis of points including ages 16 through 24 years where $y = -1.86x + 61.36$; $r = -0.654$, significant at $p \leq 0.05$.

Line C was computed on the basis of points including ages 24 through 40 years where $y = 0.25x + 12.89$; $r = 0.274$, insignificant at $p \leq 0.05$.

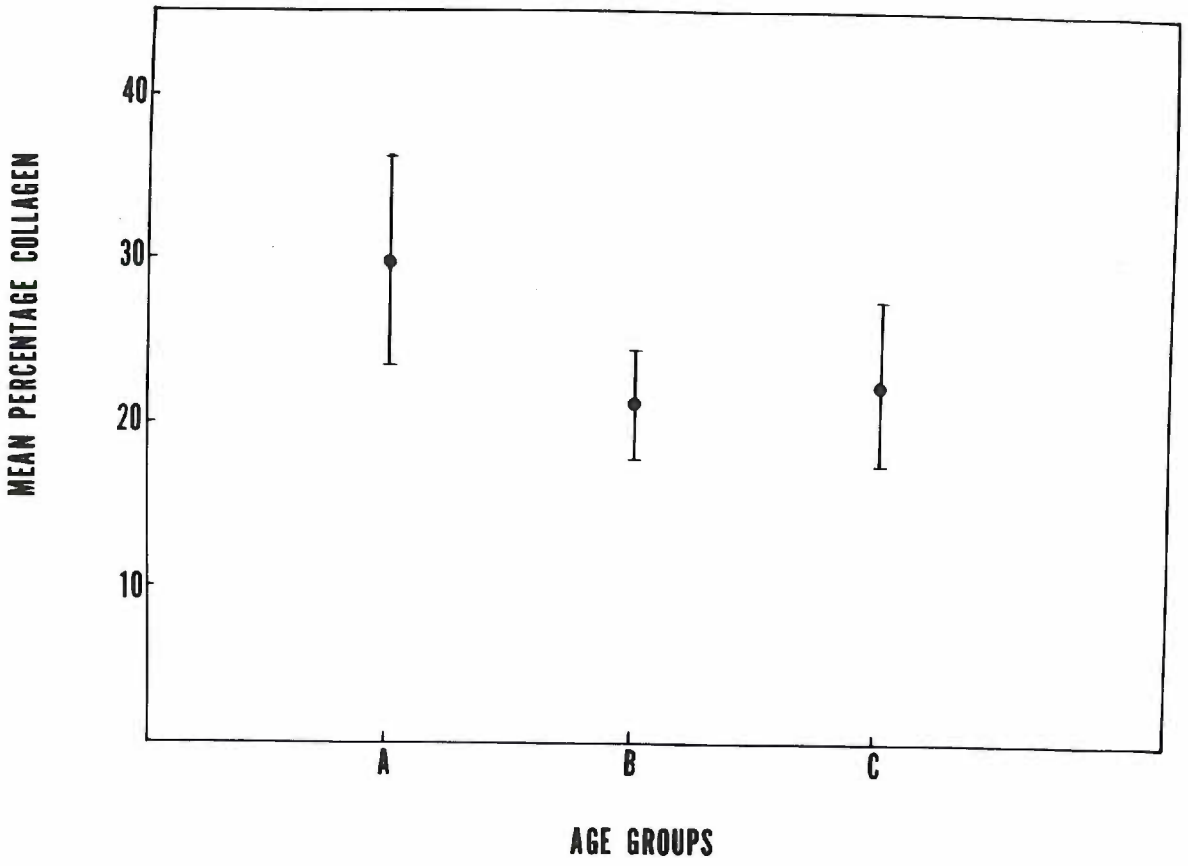


Figure 14. Mean percentage collagen of three different age groups.

$$\text{Percentage collagen} = \frac{\text{milligrams collagen}}{\text{milligrams dry weight}} \times 100$$

Brackets indicate standard deviations (s).

A: age group 16-17 years, $\bar{X} = 29.88$, $s = 6.54$, $n = 20$

B: age group 23-25 years, $\bar{X} = 21.01$, $s = 3.43$, $n = 19$

C: age group 28-30 years, $\bar{X} = 22.37$, $s = 5.22$, $n = 9$

Analysis of variance for independent data revealed a significant difference between A and B or C, but no difference between B and C at $p \leq 0.01$ (see Appendix D).

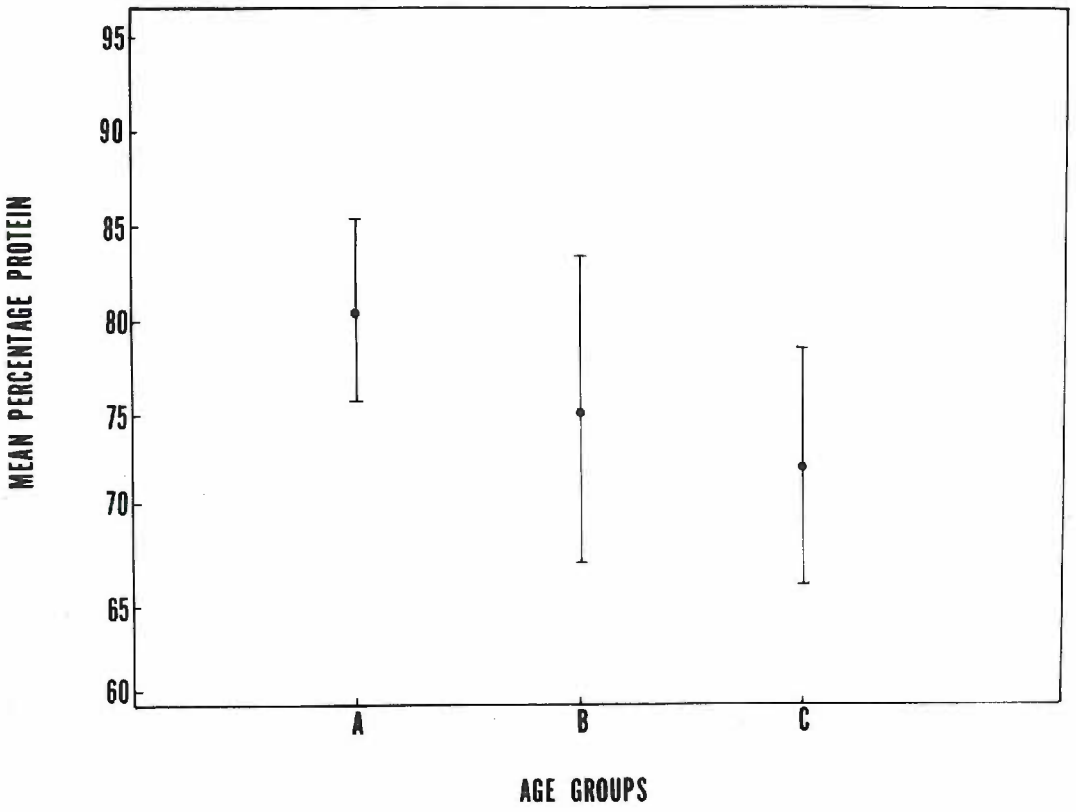


Figure 15. Mean percentage protein of three different age groups.

$$\text{Percentage protein} = \frac{\text{milligrams protein}}{\text{milligrams dry weight}} \times 100$$

Brackets indicate standard deviation (s).

A: age group 16-17 years, $\bar{X} = 80.59$, $s = 4.92$, $n = 20$

B: age group 23-25 years, $\bar{X} = 75.11$, $s = 8.46$, $n = 19$

C: age group 28-30 years, $\bar{X} = 72.17$, $s = 6.59$, $n = 9$

Analysis of variance for independent data revealed a significant difference between A and C, but no difference between A and B or B and C at $p \leq 0.05$ (see appendix E).

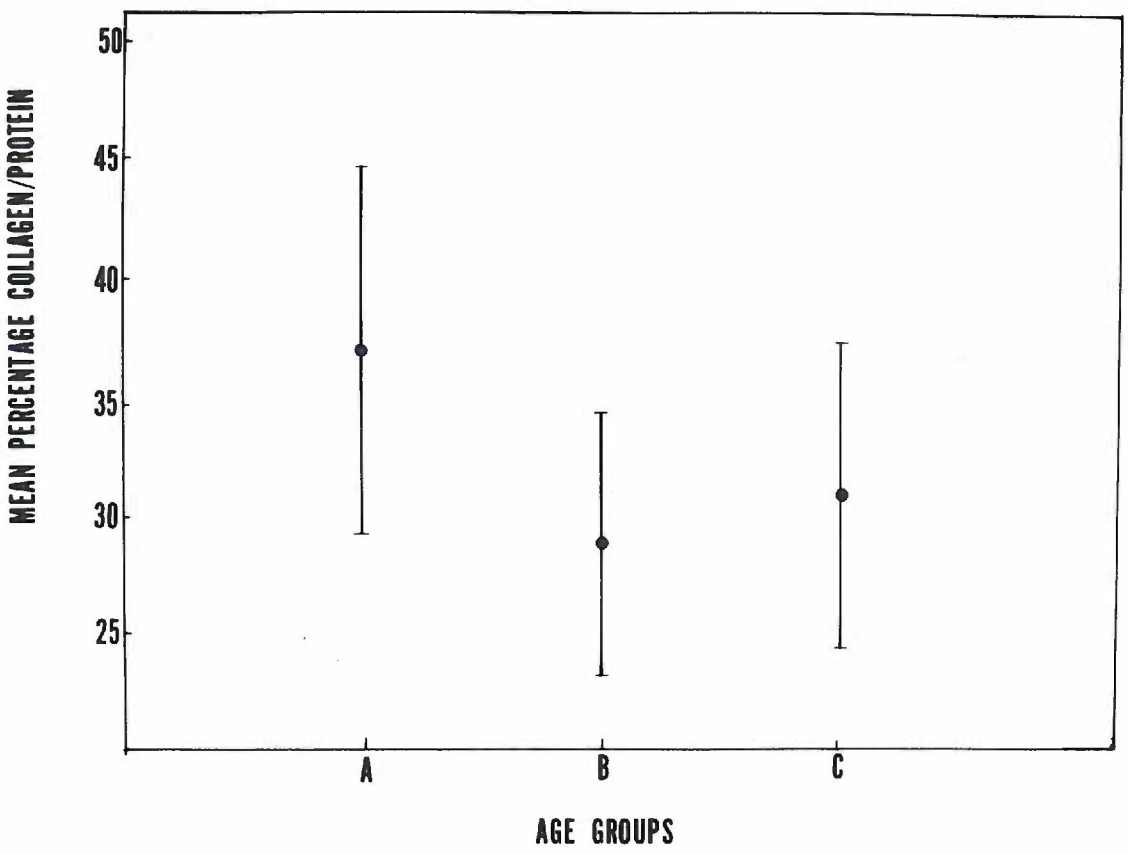


Figure 16. Mean percentage collagen/protein of three different age groups.

$$\text{Percentage collagen/protein} = \frac{\text{milligrams collagen}}{\text{milligrams protein}} \times 100$$

Brackets indicate standard deviations (s).

A: age group 16-17 years, $\bar{X} = 37.00$, $s = 7.75$, $n = 20$

B: age group 23-25 years, $\bar{X} = 28.80$, $s = 5.63$, $n = 19$

C: age group 28-30 years, $\bar{X} = 30.86$, $s = 6.52$, $n = 9$

Analysis of variance for independent data revealed a significant difference between A and B, but no difference between A and C or B and C at $p \leq 0.05$ (see Appendix F).

age group 16-17 years had a significantly higher protein content than the age group 28-30 years at $p \leq 0.05$. We conclude that like collagen concentration, protein concentration also diminishes at the time of final root development or soon thereafter.

When collagen and protein concentrations were considered as a ratio (Figure 16, Appendix C) and evaluated with analysis of variance for independent data (Appendix F) the only statistically significant difference was seen between age groups 16-17 and 23-25 years. No differences were seen between 16-17 and 28-30 or 23-25 and 28-30 years. However, if one applies the analysis at $p \leq 0.10$, a significant difference is found between ages 16-17 and 28-30. We conclude from this that there is a tendency towards a diminishing collagen/protein ratio over the age range studied and offer this as support that collagen concentration does indeed decrease during final root development in the coronal portion of the pulp.

CALCIUM CONCENTRATION

The calcium concentration of twenty pulp hydrolyzates (Group A) subjected to cross-link analysis (plus one additional sample) was seen to increase over the age range studied from less than 1% at age 16 years to over 4% at age 40 years. This is a close approximation of results achieved by other workers on the subject (22,66). Data were represented by a

regression line of $y = 0.10x - 0.98$, $r = 0.809$, significant at $p \leq 0.001$ (Figure 17).

Based on the facts that dry dentin is approximately 30% calcium (67-69) and 18% collagen (69,70), an estimation of the effect of hard tissue contamination (dentinal debris and/or pulp stones) might have had on the computation of DHLNL dpm/mg dry weight is presented in Figure 12 by regression line B where $y = -153.56x + 7881.48$, $r = -0.630$, significant at $p \leq 0.01$. Obviously both regression lines, one considering the effect of such contamination (line B), and one not considering the effect (line A), are very similar. As would be expected the effect of such contamination becomes larger with age as seen by the slight divergence of regression lines. Similar results are seen in Figure 11 where regression line D ($y = -358.20x + 26{,}108.70$, $r = -0.513$, significant at $p \leq 0.05$) represents DHLNL dpm/mg collagen taking into consideration contamination by hard tissue. Again, similarity between line A, where hard tissue contamination is not considered, and line D is evident. It is therefore concluded that contamination by hard tissue does not significantly alter results presented in this work.

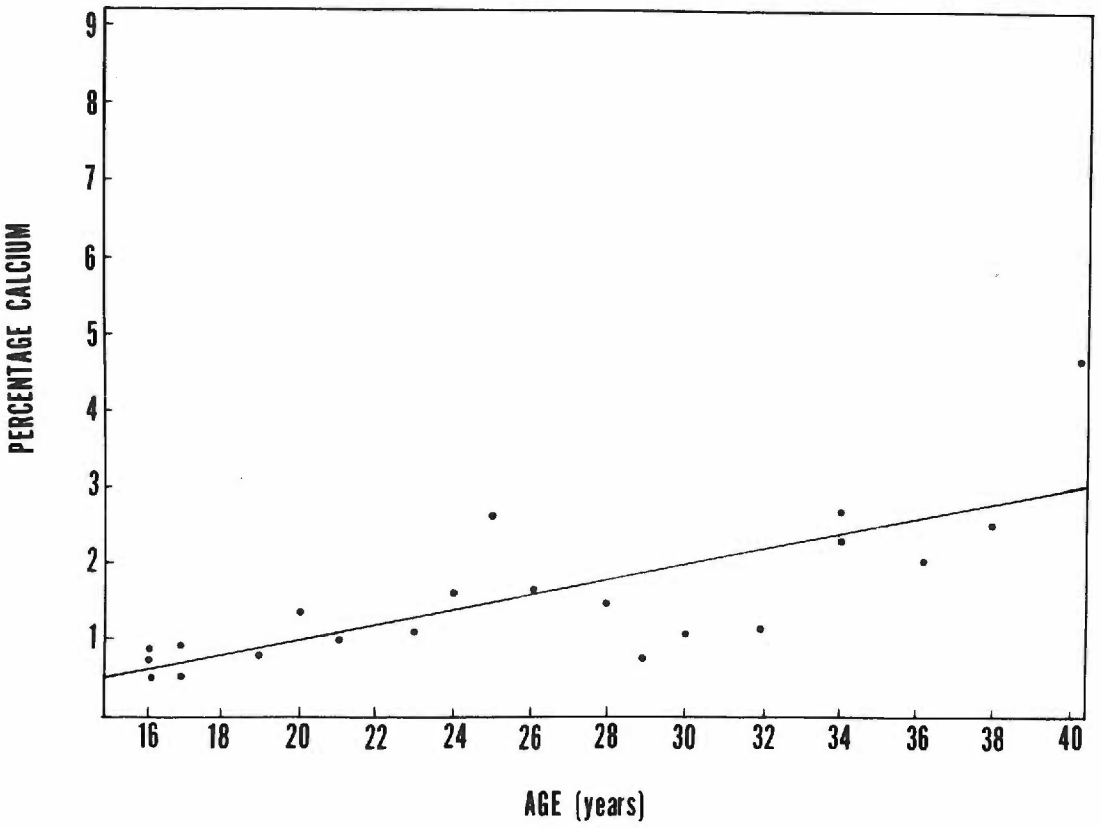


Figure 17. Calcium content of human pulp as a function of age.

$$\text{Percentage calcium} = \frac{\text{milligrams calcium}}{\text{milligrams dry weight}} \times 100$$

Equation of the regression line is $y = 0.10x - 0.98$, $r = 0.809$, significant at $p \leq 0.001$.

DISCUSSION

This study was limited by its selection of the pulp tissue studied. Oral surgeons were asked to provide only teeth without caries, periodontal disease, restorations, or evidence of possible pulpal pathology. Most of the teeth submitted proved to be third molars. Therefore, these were the only teeth included in the investigation. The study further limited itself to coronal pulp. This was for several reasons. Studies of pulpal reaction, including fibrosis, to restorative procedures and materials are most often restricted to investigation of the coronal pulp response. In addition, there is evidence to support the proposal that coronal pulp differs from radicular pulp in its lesser tendency towards fibrosis and increased cellularity. To analyze the entire pulp might hide important differences between the two, hence it was decided to concentrate on just the coronal segment. Future research might well consider radicular pulp.

An assumption upon which much of this research was based is that the collagen being studied represents "pulpal" collagen. Other sources of the protein which would be considered contaminants would include both predentin and the organic matrix of dentin itself. Pincus (66) stated that upon opening teeth "with light hammer blows" the "pulp can be torn away without odontoblasts which are presumably kept in place by their processes". More to the point was the finding of

Karjalainen et.al. (71) who showed histologically that if the coronal pulp is gently removed after the tooth is equilibrated at 0° C, whole odontoblasts remain attached to the predentin surface. For this reason it was felt that neither of these two possible collagen sources were contaminants in this study.

CROSS-LINKING AND PULPAL FIBROSIS

Starting with the premise that new collagen synthesis is characterized by the presence of reducible cross-links as suggested by Robins et.al. (35) the primary purpose of this research was to study changes in cross-links of human pulp with age and thus attempt to answer the question of whether fibrosis was occurring. This information would help to clarify whether changes in the pulp could be considered a result of maturation in its earlier years or aging throughout its life span.

DHLNL proved to be the major cross-link found in the pulp. And although not an unexpected finding, this work is the first to report such a fact. This cross-link was quantified and used as an indication of new collagen synthesis. When expressed in terms of dry weight (Figure 12) from the ages of 16 to 40 years it is apparent that the ratio of newly synthesized collagen to existing tissue is initially high, but diminishes until in the latter years of the study where it levels off.

The high levels of DHLNL dpm/mg dry weight seen in the earlier ages can be explained in two ways. There may be an increased level of cross-link synthesized per milligram collagen and/or the total percentage of collagen may be higher in pulps of these earlier ages. The results suggest that both occur. There can be no doubt that in the earlier ages the level of cross-link per milligram collagen is higher than in later ages (Figure 11). However, the higher ratio of DHLNL dpm/mg dry weight also appears to be a reflection of the data presented in Figure 13 where the concentration of coronal collagen at these earlier ages is apparently higher than at later ages.

To verify the finding of increased collagen concentration in younger coronal pulps, forty-eight pulp samples were analyzed for collagen concentration at the ages of 16-17, 23-25, and 28-30 years (Figure 14). Results indicated that in terms of dry weight, collagen concentration did indeed diminish between the ages of 16-17 and 23-25 years and that it remained constant between the ages of 23-25 and 28-30 years. These results are supported by the tendency seen in the collagen/protein ratio to diminish over the same age range. Thus one can assume that the markedly high values of DHLNL dpm/mg dry weight seen in the earlier ages of this study are a result of both a higher rate of collagen synthesis and a higher collagen concentration.

The interpretation of the results leads to the conclusion that the amount of new collagen being synthesized represented by DHLNL dpm/mg dry weight at the earlier ages of this study is double that seen at the later ages, or, to put it another way, that the rate of collagen synthesis seen during the later stages of root development is considerably higher than that seen at age 40. This finding supports Uitto and Rantas' (13) observation that the rate of collagen synthesis based on prolyl hydroxylase activity is highest during root formation. Patten et.al. (12), measuring collagen synthesis relative to protein synthesis, found similar results in that collagen synthesis decreased in direct relation to the degree of maturation of the tooth.

The observation that collagen concentration decreases between the ages of 16 and 30 years conflicts with earlier reports which state an increase in concentration during root development. Uitto and Ranta (13) reported concentration on the basis of wet weight but failed to take into account possible water loss which we have shown to occur and was also seen by others (4,22). In addition, while we studied coronal pulp Uitto and Ranta studied whole pulp. Park's study (32) also reported on whole pulp, failed to consider water loss, and stated data in terms of total protein, yet failed to discuss changes in total protein per se. Hence, any increase in collagen/protein ratio might be the result of decreased protein rather than increased collagen.

The continued presence of DHLNL, albeit much reduced, in the later years could be interpreted in several ways. First, the cross-link may not disappear and what is being observed is the continued presence long after its formation at an earlier age. While the basic premise of this work is that new collagen synthesis is characterized by the existence of reducible cross-links, the reverse of this, that reducible cross-links are only present during collagen synthesis, remains to be demonstrated. Nevertheless, evidence against such a proposal are those studies (35,36,65) which demonstrated an increase in reducible cross-links during growth of the tissue in question, followed by a decrease after growth ceased. While a detailed in-vivo analysis of the actual timing of disappearance has yet to be done, Deshmukh and Nimni (47) have shown disappearance in-vitro to occur by addition of tritium to the double bond in as little as two to three weeks. (Whether disappearance of the Schiff base is by simple addition of hydrogen in-vivo remains to be demonstrated.)

A second interpretation of Figures 11 and 12 might be that collagen synthesis is continuing at a lower level than seen at an earlier age and in fact contributes to an increased collagen density of the pulp, i.e. fibrosis is occurring. Frick's (31) report of no change in the ratio of collagen to total protein over the ages of 20 through 60 and Stanley and Ranneys' (21) histologic observation that collagen content of coronal pulp did not increase after the age of 20 years

would argue against this as well as our finding of no increase in collagen content.

A third interpretation of the results is possible insofar as the decrease in ratio of DHLNL dpm/mg dry weight may be a result of increased dry weight from contaminating sources. Pulp stones might contribute significantly to both dry weight and collagen concentration without being representative of true pulpal collagen. (Any cross-links within the organic matrix of the stone presumably would not be reduced by the assay procedure without prior decalcification.) Figure 17 shows an obvious increase in pulpal calcium levels with age which is interpreted to represent increased pulpal calcification. Under the assumptions that all such calcium arises from pulp stones and/or dentinal debris and that pulp stones are similar in composition to dentin (30% calcium and 18% collagen by dry weight) (67-70) it was found that the amount of contamination could not cause an appreciable difference in the conclusions of this study.

A fourth possibility and the author's preferred interpretation is that the smaller, almost constant ratio of DHLNL to tissue weight in the later ages of this study represents a small, albeit real, amount of collagen synthesis accounted for by normal pulpal collagen turnover. That collagen of the pulp does turnover quicker than collagen in other tissues has been suggested by others (14,30,72). Even in the studies already quoted where levels of reducible cross-links

increased with growth and then decreased with maturity, the levels never fell to zero but presumably maintained themselves at a point represented by turnover of that particular tissue.

From the discussion to this point it is obvious one cannot conclude that collagen synthesis ceases during the age range studied. Since results from this work and others indicate that a continued accumulation of collagen does not occur, it must be maintaining itself by synthesis at a level equal to its own degradation. We can conclude however that an increased rate of collagen synthesis does not occur through age 40, but rather that the pulp is characterized by a steadily declining rate of synthesis.

HEXOSYLAMINES

While it was our original intent to use the presence of reducible cross-links as an indication of new collagen synthesis it was felt that a large part of the significance of the results of this work resided in the demonstration and quantification of the reducible Schiff bases per se. What are believed by others (58,64,65) to be reduced Schiff bases (fractions 125,131,151, and 156 in Figure 7)between lysine and hydroxylysine and one or more free hexose units may have physiologic significance. Zerlotti (4), using a histochemical procedure, demonstrated a decrease in the number of free amino groups within collagen of the pulp and suggested that this was a reflection of increased masking of epsilon-amino

groups of lysine and hydroxylysine through cross-linking during aging. In light of the fact that hexosylamine content in many tissues is known to increase with age (35,73,74), one might expand on Zerlotti's observation and conclusion to include the suggestion of Robins et.al. (35) and Tanzer et.al. (25) that complexes between reactive amino groups and hexose units might be a reflection of increased binding between collagen and polysaccharides. The possibility that such complexes are artifact has not been ruled out, however.

CROSS-LINKING AND COLLAGEN TYPE

Recent evidence with regard to bovine pulp (39,49,50) has suggested that with age there is an increase in type III collagen. This particular type was considered by Robins et.al. (35) and others to represent a highly hydroxylated, embryonic form of the protein which decreased with age in bovine skin. Speculation included the possibility that type III collagen had a characteristically high amount of DHLNL. Bailey and Sims (75) account for Robins' et.al. results on the basis of higher levels of lysyl hydroxylating enzymes in embryonic tissue rather than the presence of two genetically distinct collagens (I and III).

Whether any correlation exists between cross-linking patterns and collagen types is unknown. The only work to date in this regard is that of Fujii et.al. (76) who separated, purified, and reconstituted foetal calf skin collagen in-vitro.

Their work suggested that the major cross-links of types I and III collagens were LNL and HLNL respectively. Excluding this work however, the literature reports data exclusively in terms of correlations between specific cross-links and anatomic tissue rather than between cross-links and collagen type. For example, DHLNL is the major cross-link of cartilage yet the statement cannot be made that type II collagen, characteristic of cartilage, is characterized by DHLNL as the major cross-link. In general, it appears that all types of collagen display the ability to form the necessary precursors leading to intermolecular bonding and in fact are found to have all three cross-links perhaps in varying amounts. For this reason no conclusion can be drawn from results of this work with regard to types of collagen which may or may not exist in human pulp and types of cross-links present.

One must consider, therefore, what biologic significance might be attributed to our finding of high pulp levels of DHLNL, normally a marker for hard tissue. It has been said that dentin and pulp should be considered as one tissue, where the former is the final morphogenic expression of structural and metabolic changes occurring in the latter (77). Whatever the factor(s) might be in determining what cross-link is finally produced - be it level of hydroxylating enzymes, type of collagen, or something else - it is apparent that odontoblasts, undifferentiated mesenchymal cells, and mature pulpal fibroblasts have the genetic make-up necessary for producing a collagen with DHLNL as its major cross-link. The finding

that pulp, predentin, and dentin all have high levels of DHLNL (78) adds further support to the concept that all three are components of one tissue and that the pulp is ultimately programmed to become hard tissue itself.

CROSS-LINKING AND SOLUBILITY

The presence of DHLNL in any tissue will often have a bearing on that tissue's solubility. Because of its ability to undergo an Amadori re-arrangement to the keto form (Figure 3), the cross-link imparts a degree of stability to the tissue higher than does HLNL. The example most often cited (Robins et.al.,35) is the observation that while collagenous tissues generally become more insoluble with age by increased cross-linking, embryonic bovine skin demonstrates a resistance to extraction higher than does skin from a calf several months old. This is accounted for by the existence of high levels of DHLNL in the former tissue which decrease shortly after birth to be replaced by HLNL as the major cross-link. The solubility difference between the two ages would be a result of the relative amounts of DHLNL in the tissue at any given time.

Seen in these terms, the results of Van Amerongen and Tonino (20), who found the collagen of human pulp to be non-extractable, might not only be a result of the protein being highly cross-linked as the authors suggest, but also a result of the high percentage of DHLNL cross-links within the protein. Similar reasoning can be applied to the results

of Stenstrom and Oreland (79) who found rat incisor pulp to be non-extractable and interpreted this to signify a low turn-over rate. Here again, the protein's ability to stabilize by shifting to the keto form might be affecting its metabolic activity.

CROSS-LINKING AND CALCIFICATION

We are about to propose a novel hypothesis which will offer an alternative explanation to the mode of action of calcium hydroxide, a compound often used in dental treatment to stimulate calcification. The hypothesis involves the ability of this substance to inhibit the enzyme lysyl hydroxylase and, as a consequence, alter the relation of the protein to surrounding molecules in such a way as to induce apatite formation.

Within the past ten years an interest in a possible association between tissue calcium levels, cross-link ratio, and abnormal ossification has arisen. Mechanic et.al. (80) showed an elevated ratio of DHLNL/HLNL in vitamin D-deficient chicks and in a follow-up study (81) showed the ratio to be highest in vitamin D-deficient chicks and lowest in animals fed high doses of vitamin D when compared to controls. Because a diminishing ratio of DHLNL to HLNL may be normally associated with maturation of bone collagen (40,73) the authors concluded that the vitamin might be directly responsible for inhibiting the hydroxlation of lysine thereby shifting the balance in favor of increased HLNL synthesis. Consequently the DHLNL/HLNL

ratio would drop and maturation of the protein could proceed. Support of this concept was given by Toole et.al. (82) who found that rachitic osteoid was distinguished not by a change in collagen type, but rather by increased hydroxylation of existing collagen. In effect, by keeping hydroxylation high in the rachitic condition, the ratio of DHLNL to HLNL in Mechanics' et.al. study 80) remained abnormally high, and the protein was not maturing as it was normally seen to do.

The same tendency towards higher ratios of DHLNL/HLNL in bone collagen was seen in patients with osteogenesis imperfecta (83). Again, the question of maturity of the protein was brought to mind. It was suggested that bone matrix collagen of osteogenesis imperfecta was a younger type because of the increased amounts of hydroxylysine (The correlation of increased hydroxylation and younger tissue is supported by findings of Miller et.al. (84), Barnes et.al. (85), and Bailey and Sims (75)). The presence of more woven bone, a histologically younger bone type, in osteogenesis imperfecta (86) also gave credence to the idea that there seemed to be an association between immature tissue, immature cross-link ratios, and failure to calcify the organic matrix.

While Mechanic et.al. (80) discarded the possibility that calcium was a determining factor, other authors were not as prone to dismiss that idea. An inverse relationship between plasma calcium levels and the degree of hydroxylation was found in several studies. Fujimoto et.al. (87) found elevated

levels of pyridinoline, a suggested intermolecular bond arising from hydroxylated precursors, in rachitic chicks, and suggested that hypocalcemia accelerated hydroxylation of lysine residues and resulted in an excess amount of hydroxylysine derived cross-links including DHLNL and pyridinoline. Ryhanen (88) demonstrated the inhibition of lysyl hydroxylase by calcium ion and stated that its mode of action was to compete with iron for the binding site in the hydroxylase enzyme. Similar support that hypocalcemia was at least a factor in control of cross-link ratios was given by Barnes et.al. (89) and Dickson et.al. (90).

The role of this regulation in calcification remains unclear but Toole et.al. (82) suggested that carbohydrate normally associated with hydroxylysine may interfere with the deposition of calcium apatite crystals in the "hole" region of the collagen fiber.

Of what concern then is this with results reported in this paper? For many years it has been the observation of dentists that by the judicious use of various forms of calcium hydroxide one could hope for, and indeed expect:

(1) increased secondary dentin formation and/or tubular sclerosis in areas approximating earlier pulpal insult,

(2) partial or complete bridging of exposed pulp by calcification akin to dentinogenesis,

(3) apical closure of root ends by renewed or accelerated apposition of dentin and/or cementum,

(4) cessation of external root resorption following traumatic injury, and

(5) accelerated healing of apical lesions by new bony tissue.

Calcium hydroxide would appear to be the panacea for most, if not all, that ails the pulp and surrounding tissue, yet for years, its mode of action has remained in the realm of speculation.

In light of the previous discussion and current results, the speculation can be pushed even farther. The major cross-link within the pulp is DHLNL. Its ratio to HLNL appears to decline with age, corresponding to a time that pulpal calcification is known to increase (7,9,24,91). Just as maturing bone osteoid demonstrates a diminishing DHLNL/HLNL ratio prior to calcification, the dental pulp might also be preparing the proper environment for eventual calcification by gradually decreasing its DHLNL/HLNL ratio. This would be part of the normal physiologic aging of the tissue.

What might be the effect of calcium hydroxide on this process? Could the application of the compound increase the local level of calcium to a point sufficient to inhibit lysyl hydroxylase activity? Any decrease in enzymatic activity would presumably affect, i.e. decrease, the DHLNL/HLNL ratio by diminishing the hydroxyallysine and hydroxylysine residues necessary for DHLNL formation. In effect this would accomplish what occurs naturally with age as demonstrated in the results of this paper. Taken a step farther, the younger tissue spoken

of in osteogenesis imperfecta (86) might be roughly equivalent to the uniquely primitive, embryonic-like mesenchymal tissue seen in the pulp. As the DHLNL/HLNL ratio decreases in the latter case, either from application of calcium hydroxide or normal physiologic ageing, the stage might be being set for the calcification that is known to occur.

Similar reasoning might account for the effect of calcium hydroxide in producing calcification in other circumstances such as secondary dentin formation, root end closure, and bony healing of periapical lesions. It has been suggested (92-94) that one of the most expedient methods of treating periapical lesions (often consisting of proliferating granulation tissue) associated with endodontically involved teeth is to fill the debrided canal with calcium hydroxide. In a study of acute and subacute granulomas, Bailey et.al. (95) found that the collagen of both types of inflammation had as their major cross-link DHLNL. During healing of the acute inflammation the tissue reverted to its normal cross-linking pattern with HLNL as the major cross-link. As long as the irritant persisted DHLNL continued as the major cross-link in the subacute inflammation. Once removed, a reversion to HLNL was seen. It would seem that in the case of healing granulation tissue a definite change in post-translation modifications of the protein had to occur before normalcy could be achieved. As in the case of pulpal calcification, the effect of calcium hydroxide placed within the root canal

might be to hasten this conversion of one cross-linking pattern to another by inhibiting the enzyme responsible for hydroxylation. The accelerated bony deposition within the periapical lesion which the author and others profess to see clinically might be a result of this accelerated conversion.

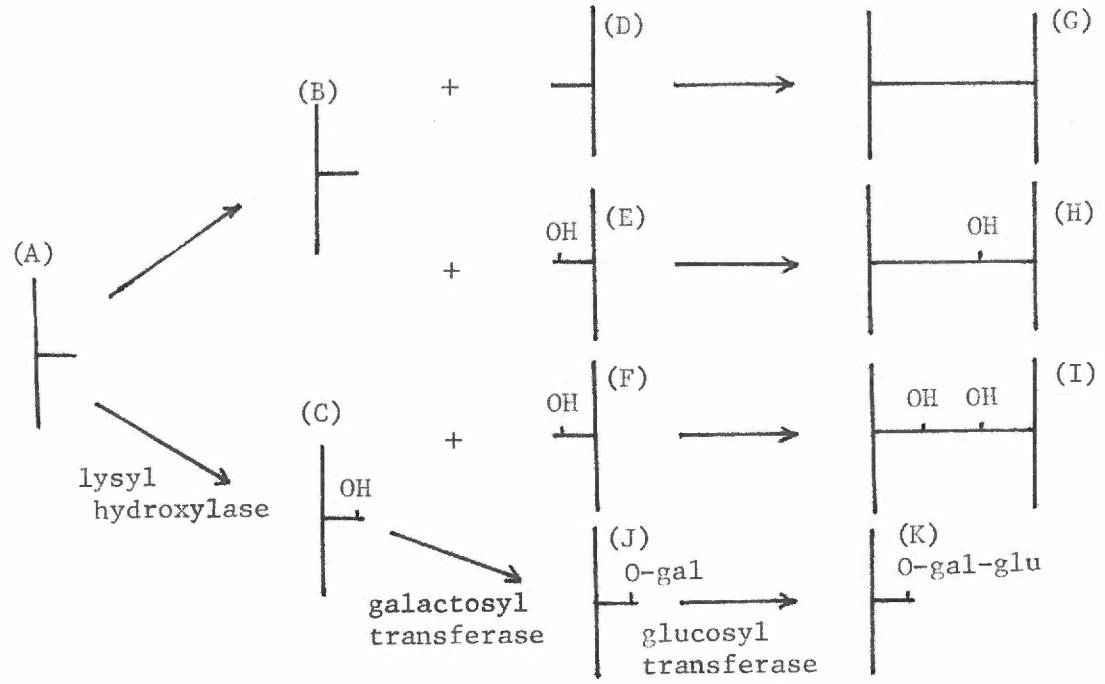


Figure 18. Proposed theory on calcium's effect on collagen hydroxylation and subsequent mineralization. The protocollagen molecule (A) has certain lysyl residues hydroxylated (C) with the aid of lysyl hydroxylase while others remain unhydroxylated (B). The unhydroxylated molecule (B) can undergo intermolecular bond formation with another unhydroxylated molecule (D) to form LNL (G) or with a hydroxylated molecule (E) to form HLNL (H). The hydroxylated form (C) can undergo bond formation with another hydroxylated molecule (F) to form DHLNL (I). Under the influence of two transferase enzymes the hydroxylated form (C) can also have two hexoses, galactose and glucose, sequentially added in a O-glycosidic linkage (J and K).

It is suggested that calcium inhibits lysyl hydroxylase thereby decreasing the number of hydroxylysine residues. The result of this would be decreased levels of DHLNL in relation to HLNL since the former requires twice as many hydroxylysine residues as the latter. If hydroxylysine-bound hexoses interfered with calcification, the ability of calcium to promote mineralization might be its inhibition of hydroxylation. A decrease in hydroxylation would decrease levels of hydroxylysine-bound hexoses and thereby facilitate calcification.

SUMMARY

It was the intent of this work to study collagen synthesis in the dental pulp of teeth from patients with as wide an age range as possible using the presence of reducible cross-links as a measure of active protein production. In the earlier years corresponding to the time of final root development, collagen synthesis was at a high level. This level decreased until, at an age during the mid-twenties, it was assumed that the rate of synthesis equaled the rate of degradation as it does in other mature connective tissues. This interpretation was based primarily on the gradual leveling off of the total amount of reducible cross-links present in the pulp and the percentage of collagen not increasing with age. An increase in collagen synthesis was definitely not seen during the time span investigated. These results led the author to conclude that changes in the collagenous matrix of the dental pulp are primarily those due to maturation during the time of final root development and that subsequent changes due to aging are probably minimal.

The significance of reducible cross-links within the pulp was discussed in light of other studies:

The fact that DHLNL is the major cross-link of pulpal connective tissue, predentin, and dentin correlates well with the belief that all three should be considered one and the same tissue.

No correlation between collagen type and cross-linking pattern was sought or found.

The presence of DHLNL and its ability to undergo a shift to the keto form was discussed with regard to pulp collagen extractability.

A proposal was made regarding a possible explanation of the mode of action of calcium hydroxide in promoting calcification of the dental pulp. Based on it's suggested ability to inhibit lysyl hydroxylase it was thought that the reagent might act by directly decreasing the DHLNL/HLNL ratio. While a satisfactory explanation as to why such a decrease would effect calcification has not been given, the idea correlated well with the observed results of this work insofar as the decrease in ratio seen during maturation of the pulp is accompanied by known pulpal calcification.

CONCLUSIONS

1. The isolation of the three reducible cross-links, DHLNL, HLNL, and LNL in human dental coronal pulp using the assay procedure described in this paper is possible.

2. The predominent cross-link in third molar coronal pulps of patients aged 16 to 40 years is DHLNL with the other two cross-links being present in minor amounts.

3. DHLNL showed a significant decrease in relation to both dry weight and weight of collagen during the time span investigated.

4. The rate of collagen synthesis in coronal pulp as measured by the presence of DHLNL in relation to collagen or dry weight of tissue showed a gradual decline over the age period studied.

5. The coronal pulp collagen concentration of human third molars was significantly higher in the age group of 16-17 years when compared to 23-25 and 28-30 years. The concentration in the latter two groups did not differ significantly.

6. The coronal pulp protein concentration decreased significantly over the age range studied and a tendency towards a diminishing collagen/protein ratio was also seen.

7. The percentage of water in coronal pulp decreases by approximately 7% between the ages of 16 and 40 years.

8. The coronal pulp showed a significant increase in calcium levels between the ages of 16 and 40 years.

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Appendix A. Pulp content (percentage) of different species.

Author	Animal	Collagen mg/mg dry weight x 100	Collagen mg/mg wet weight x 100	Collagen mg/mg protein x 100
Uitto et.al. (29)	Rabbit molar	-	0.69	12.0
	Rabbit incisor	-	0.52	10.3
Orlowski (26)	Pig	12.7	-	-
	Cow	16.8	-	-
Orlowski (30)	Rat incisor	3.3-4.7	-	-
Orlowski (14)	Rat molar	7.0	-	-
Van Amerongen et.al. (20)	Human bicuspid	25.0	-	-
Park (32)	Human bicuspid, apex open	-	-	18.5
	Human bicuspid, apex closed	-	-	36.6
Uitto et.al. (13)	Human bicuspid, apex open	-	1.44	30.0
	Human bicuspid, apex closed	-	2.08	37.1

Appendix B. Pulp composition

Age	Sample	Wet weight*	Dry weight*	Calcium content*	Percent water	Percent calcium
16	L44	21.89	3.66	0.0200	83.28	0.55
16	L59	19.40	3.48	0.0264	82.06	0.76
16	L72	17.63	3.17	0.0258	82.02	0.81
17	L60	14.46	2.21	0.0214	84.72	0.97
17	L73	4.49	2.81	0.0154	37.42	0.55
18	L53	20.98	1.83	0.0142	91.28	0.78
19	L63	16.57	2.68	0.0226	83.83	0.84
20	L55	11.10	1.63	0.0222	85.32	1.36
21	L61	10.08	1.43	0.0150	85.81	1.05
21	13-23	8.84	1.62	-	81.67	-
23	L62	6.04	1.36	0.0150	77.48	1.10
23	13-22	8.95	1.56	-	82.57	-
23	13-24	2.15	0.58	-	73.02	-
24	L43	13.01	2.05	0.0336	84.24	1.64
25	L64	5.03	0.82	0.0214	83.70	2.61
26	L57	7.62	1.39	0.0232	81.76	1.67
26	13-12	8.84	1.17	-	86.76	-
27	13-18	3.40	0.55	-	83.82	-
28	L65	9.03	1.28	0.0190	85.83	1.48
29	13-29	7.19	1.41	-	80.39	-
30	L66	5.62	1.69	0.0184	69.93	1.09
30	L49	5.06	1.08	-	78.66	-
32	12-9	-	1.40	0.0164	-	1.17
34	L54	5.68	0.80	0.0186	85.92	2.33
34	L67	2.97	0.43	0.0160	85.52	2.71
34	13-3	1.93	0.44	-	77.20	-
36	L68	1.09	0.66	0.0138	39.45	2.09
36	13-7	2.39	0.69	-	71.13	-
38	L69	1.23	0.76	0.0196	38.21	2.58
38	L48	4.98	1.00	-	79.92	-
40	L71	1.84	0.42	0.0196	77.17	4.67

*Wet weight, Dry weight, and Calcium content measured in milligrams.

Appendix C. Pulp composition.

Age	Sample	Collagen mg/mg dry weight	Protein mg/mg dry weight	Collagen / Protein x 100
17	14-1	0.2306	0.7544	30.57
17	14-2	0.2384	0.8381	28.45
17	14-3	0.2258	0.7631	29.59
16	14-4	0.2000	0.7606	26.30
16	14-5	0.3450	0.7575	45.54
16	14-6	0.4154	0.8213	50.58
16	14-7	0.1811	0.7006	25.85
17	14-8	0.3242	0.8256	39.27
17	14-9	0.3247	0.8825	36.79
17	14-10	0.3027	0.8519	35.53
17	14-11	0.2838	0.8106	35.01
17	14-12	0.2665	0.8063	33.05
17	14-13	0.2744	0.7706	35.61
17	14-14	0.3269	0.8275	39.50
16	14-15	0.3054	0.8694	35.13
16	14-16	0.2673	0.8006	33.39
16	14-17	0.3141	0.9056	34.68
17	14-18	0.3529	0.7788	45.31
17	14-19	0.3795	0.8050	47.14
17	14-20	0.4158	0.7888	52.71
24	14-21	0.1761	0.7550	23.32
23	14-22	0.1285	0.8263	15.55
24	14-23	0.2500	0.7788	32.10
24	14-24	0.2329	0.8431	27.62
24	14-25	0.2333	0.7344	31.77
23	14-26	0.2551	0.7819	32.63
24	14-27	0.1934	0.9700	19.94
23	14-28	0.2285	0.6650	34.36
23	14-29	0.2269	0.6425	35.32
23	14-30	0.2267	0.6938	32.68
23	14-31	0.2513	0.8025	31.31
23	14-32	0.2220	0.6438	34.48
24	14-34	0.2336	0.7375	31.67
25	14-35	0.1882	0.7594	24.78
25	14-36	0.1626	0.6163	26.38
25	14-37	0.2012	0.8150	24.69
25	14-38	0.1737	0.7263	23.92
23	14-39	0.1800	0.7881	22.84
23	14-40	0.2275	0.6919	32.88
29	14-41	0.2609	0.7900	33.03
29	14-43	0.2602	0.7394	35.19
30	14-45	0.1763	0.7825	22.53
30	14-47	0.1226	0.5925	20.69
29	14-48	0.2299	0.7300	31.49
29	14-49	0.2150	0.6500	33.08
29	14-50	0.2970	0.7056	42.09
28	14-53	0.1993	0.7244	27.51
29	14-56	0.2513	0.7813	32.61

Appendix D. Analysis of variance with regard to collagen concentration of three different age groups.

(A) 16-17 years

(B) 23-25 years

(C) 28-30 years

Source	SS	df	MS	F
Total	2080.7	47		
Between	840.0	2	420.00	15.23*
Within	1240.7	45	27.57	

*Significant at $p < 0.01$, table value for F = 5.13.

Scheffe $F_{A,B} = 27.80$

Scheffe $F_{A,C} = 12.70$

Scheffe $F_{B,C} = 0.41$

Appendix E. Analysis of variance with regard to protein concentration of three different age groups.

(A) 16-17 years

(B) 23-25 years

(C) 28-30 years

Source	SS	df	MS	F
Total	0.2627	47		
Between	0.0534	2	0.0267	5.68*
Within	0.2093	45	0.0047	

*Significant at $p < 0.05$, table value for F = 3.21.

Scheffe $F_{A,B} = 6.22$

Scheffe $F_{A,C} = 9.34$

Scheffe $F_{B,C} = 1.12$

Appendix F. Analysis of variance with regard to collagen/protein ratios of three different age groups.

(A) 16-17 years

(B) 23-25 years

(C) 28-30 years

Source	SS	df	MS	F
Total	0.2809	47		
Between	0.0759	2	0.0380	8.25*
Within	0.2050	45	0.0046	

*Significant at $p < 0.01$, table value of F = 3.21.

Scheffe $F_{A,B} = 15.96$

Scheffe $F_{A,C} = 5.14$

Scheffe $F_{B,C} = 0.85$

Appendix G. Reagents, materials, and equipment.

Anti-Foam A Concentrate	Sigma Chemical Company Box 14508 St. Louis, Missouri 14508
Tritiated sodium borohydride	Amersham 2636 S. Clearbrook Drive Arlington Heights, Illinois 60005
Spherical cation exchange resin	Mark Instruments Company, Inc. Box 271 Villanova, Pennsylvania 19085
Packard Insta-Gel	Packard Instrument Company, Inc. 2200 Warrenville Road Downes Grove, Illinois 60515
Mark Instruments pump	Mark Instruments Company, Inc. Box 271 Villanova, Pennsylvania 19085
Technicon gradient mixer	Technicon Industrial Systems 511 Benedict Avenue Tarrytown, New York 10591
Gilson Micro-Fractionator fraction collector	Gilson Medical Electronics, Inc. Box 27 Middleton, Wisconsin 53562
Bausch and Lomb colorimeter	Bausch and Lomb Analytical Systems Division 14 Inverness Drive E. Denver, Colorado 80200
Glyoxal bis(2-hydroxy-anil)	Aldrich Chemical Company,Inc Milwaukee, Wisconsin 53200