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CANADIAN THESES ON MICROFICHE / THÈSES CANADIENNES SUR MICROFICHE

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TITLE OF THESIS / TITRE DE LA THÈSE The Composition of the Collagenous Matrices of Bovine Predentine and Dentine

UNIVERSITY / UNIVERSITÉ University of Alberta, Edmonton

DEGREE FOR WHICH THESIS WAS PRESENTED / GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE M Sc

YEAR THIS DEGREE CONFERRED / ANNÉE D'OBTENTION DE CE GRADE Fall 1975

NAME OF SUPERVISOR / NOM DU DIRECTEUR DE THÈSE Dr D J Carmichael

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THE UNIVERSITY OF ALBERTA

THE COMPOSITION OF THE COLLAGENOUS MATRICES OF BOVINE PREDENTINE AND CEMENTUM

by

© ANTOINETTE M. CHOVELON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

IN

ORAL BIOLOGY
(BIOCHEMISTRY)

FACULTY OF DENTISTRY

EDMONTON, ALBERTA

FALL, 1975

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read and recommended to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Composition of the Collagenous Matrices of Bovine Predentine and Cementum". Submitted by Antoinette M. Chovelon in partial fulfillment of the requirements for the degree of Master of Science.

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Supervisor

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Date .....

To my husband, Guy-Michel.

ABSTRACT

The existence of collagen in predentine and cementum can be regarded as firmly established. However as the amount of these tissues in teeth is very small, most studies have been histochemical in nature. The aim of this work was to extend the histochemical studies to a chemical basis.

Predentine was obtained from unerupted bovine teeth of animals one to two years old at slaughter. The translucent zone at the apex of the root was cut away and the predentine subsequently dissected from the dentine under a dissecting microscope. Cementum was collected from erupted bovine teeth. The roots of the teeth, from which the crowns had been sectioned off, were sliced longitudinally into thin lamellae of about 250 microns. Under the dissecting microscope, the cementum was carefully separated from the dentine.

Calcium and phosphorus determinations were carried out on both tissues. The EDTA decalcified insoluble collagenous matrices were then subjected to three major analyses: reducible cross-link, amino acid and neutral sugar determinations. An investigation was undertaken of the occurrence of phosphoprotein in these tissues.

Predentine:

Predentine was found to contain little calcium (.4%), an amount corresponding to 1% hydroxyapatite. The phosphorus content (.37%) was higher than could be accounted for as hydroxyapatite. The additional phosphorus was first thought to be part of the phosphoprotein Veis and coworkers[185,187,189] had isolated from decalcified bone and dentine and postulated to be the site for epitactic nucleation of mineralization for

the matrix. However upon analysis, predentine was found to be free of phosphoprotein, which is consistant with Weinstock and Leblond's[193] radioautographic study showing that a phosphoprotein traverses the predentine to the mineralization front where it is deposited in the dentine.

The reducible intermolecular cross-link content of predentine showed the same pattern as other mineralized tissues, that is, dihydroxylysinonorleucine and hydroxylysinonorleucine are the major cross-links with dihydroxylysinonorleucine being the predominant one.

The amino acid composition of predentine collagen generally resembled that of other collagens except that the glycine content was low and the proline content high indicating the sample was not completely pure. The neutral sugar results suggested that one contaminant was a glycoprotein.

The collagen hexose determination indicated that predentine is a high hexose collagen as the hydroxylysine was completely glycosylated. However, only a part appeared to exist as the disaccharide, glucosyl galactosyl hydroxylysine.

Cementum:

The Ca/P ratio of cementum was determined to be 2.3 (w/w). That of hydroxyapatite is 2.2 (w/w). Thus the mineral of cementum is hydroxyapatite.

Cementum was also found to be free of phosphoprotein. This result indicates that the role of phosphoprotein in mineralization must now be reconsidered.

The reducible intermolecular cross-link determination showed that dihydroxylysinonorleucine and hydroxylysinonorleucine were the major cross-links of cementum as in other calcified tissues. Of the two, di-

hydroxylysinonorleucine was the predominant one.

The amino acid composition of cementum resembled that of other collagens although the high proline and low glycine content indicated that the sample was contaminated. This observation is supported by the neutral sugar results which indicated one contaminant as a non-collagenous glycoprotein.

The neutral sugar determination on the collagen hexose showed it to contain 1.5g hexose/14g hydroxyproline. This value corresponds to 36.8% glycosylation of the hydroxylysine, of which only a part is the disaccharide.

Due to the limited amount of predentine and cementum samples, each determination was performed only once. Thus the results must be regarded as tentative.

ACKNOWLEDGEMENTS

I wish to express my sincerest thanks to Dr. D.J. Carmichael, Dr. C.H. Pearson and Dr. K.A. McMurchy for their guidance during the experimental phase and preparation of the thesis text. Their constructive criticism as well as many suggestions were greatly appreciated. Many thanks are due to Mr. D. Carmel and Mrs. P. Mudry for the endless patience during the dissection and collection of samples. My special appreciation is extended to Dr. M. Wohllebe for carrying out the amino acid and cross-link determinations as well as to Mrs. C. Dodd for her excellent technical assistance with the phosphoprotein determinations. Also, I would like to thank Mrs. R. Fuller for excellent typing services and her great care in the final typing.

Financial support received in the form of Graduate Teaching Assistantships during the period of laboratory work is gratefully acknowledged.

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INTRODUCTION

THE PURPOSE OF THE INVESTIGATION

Despite the extensive information available on the biochemical nature of collagen and other connective tissue elements, no definitive studies have been reported on the chemical character of the fibres found in cementum and predentine. Cementum is the thin layer of mineralized tissue which covers the whole root of the tooth. Its primary function is to attach the periodontal ligament to the tooth surface, thus relating the teeth to the jaws. It also plays a part in maintaining the width of the periodontal ligament and in repair of damage to the root of the tooth. Predentine, on the other hand, as the name implies, represents the precursor form of dentine. In contrast to dentine, however, our knowledge of predentine is meager. While the existence of collagen in this tissue has been firmly established, the identity of other components is less certain. Because of the very small amounts of tissue involved, most studies to date have been histochemical in nature. The aim of this work was to extend the histochemical studies to a chemical basis and so enlarge our knowledge of these two tissues.

REVIEW OF THE LITERATURE

I. SPECIFIC OBJECTIVES AND PRESENT STATE OF KNOWLEDGE

Connective tissues play many important roles in the body, serving portative, supportive, defensive, storage and reparative functions. They are composed of intercellular substances of which collagen is the principal component; and cells.

The fundamental unit of collagen is composed of three α chains. Each consists of about 1000 amino acids linked in an α-amino peptide linkage and has a molecular weight of about 100,000. The most common structure is $(\alpha 1)_2 \alpha 2$. Two of the chains, the $\alpha 1$, have identical primary structures, whereas the third, the $\alpha 2$, differs from the $\alpha 1$ in amino acid composition[1]. As a result, the $\alpha 2$ chain is more basic. Recently it has been found that the $\alpha 2$ is shorter than the $\alpha 1$[2]. At least four genetically-distinct collagen α chains designated $\alpha 1(I)$, $\alpha 2$, $\alpha 1(II)$ and $\alpha 1(III)$ are found in the major vertebrate connective tissues. These chains appear to occur normally in three types of molecules with the following chain compositions: $\alpha 1(I)_2 \alpha 2$, the Type I molecule which is the predominant collagen molecule in several tissues such as bone, tendon, dentine and mature dermis[3]; $\alpha 1(II)_3$, the Type II molecule which is the predominant species of collagen in hyaline cartilages[3]; $\alpha 1(III)_3$, the Type III molecule which coexists with Type I collagen in several tissues. Type III molecules are especially prevalent in young tissues[4] (discussed in greater detail on p. 25).

Each polypeptide within the collagen molecule chain forms a left-handed helix; each complete turn in the helix contains 3.3 amino acids which are separated by 2.9 Å. To form the molecule, each helical

polypeptide forms a right-handed triple helix in conjunction with two other α chains. The molecular dimensions are 3000 x 15 Å. However, the collagen molecule is not completely helical since the α chains have non-helical peptides at both the N-terminal and C-terminal ends.

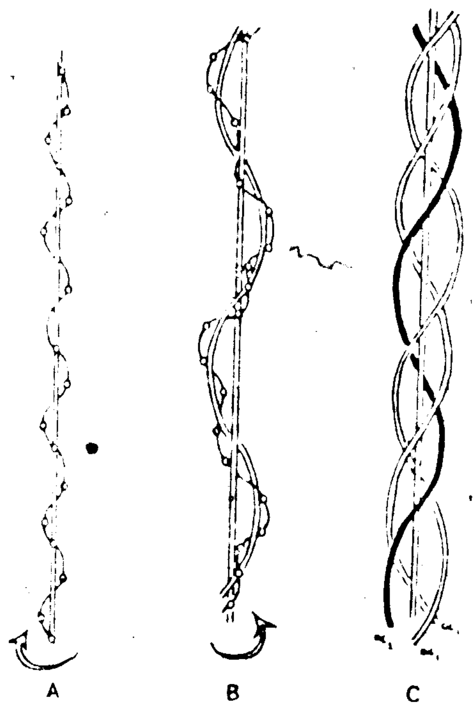


Fig. 1 Triple helical structure of collagen A. Single chain wound in single left-handed helix B. Axis of (A) wound in some right-handed helix so that the single chain itself forms a compound helix C. Arrangement of the axes of three chains in the collagen molecule. In (C) the chains themselves are omitted for simplicity 5

Fig. 2 Perspective view of the collagen structure for a height of about 30 Å[20]

The amino acid composition of collagen is unique in that about one-third of the residues are glycine, the simplest amino acid having a single hydrogen atom instead of a side chain. The two imino acids, proline and hydroxyproline, make up about two-ninths of the total amino

acids, and hydroxylysine makes up about 1 percent. See Table 1 for the amino acid composition of various mammalian tissues. From this table, one sees that collagen contains significant amounts of all the common amino acids except cystine and tryptophan.

Glycine, proline and hydroxyproline, the three most abundant amino acids in collagen, have an important bearing on its structure. It is surprising that a protein having an amino acid composition like collagen can form a helix. The ring structure of proline and hydroxyproline would normally prevent helix formation. However, the structure of the triple helical molecule is such that glycine in every third position lies inside the triple helix, while the ring structure of proline and hydroxyproline and the side chains of the other amino acids lie on the outside, thus allowing for close packing and α helix formation. Once formed, the presence of these rigid regions with bulky groups incorporated helps to stabilize it by preventing unwinding. The structure is also stabilized by hydrogen bonds between the peptide chains transverse to the axis of the molecule.

Several conformations for the triple helix have been proposed in the last two decades. Recently, an unambiguous structure determination appears to have been achieved. Traub and Yonath[7] studied the polytripeptide $(Gly\text{-}Pro\text{-}Pro)_n$ and found it to conform with the parameters of collagen, that is, this model conformed with the generally accepted bond lengths, bond angles, and minimum van der Waals contacts and is consistent with the x-ray pattern, infrared frequency, as well as other physical and chemical data relating to the structure. This conformation has only one hydrogen bond for three amino acids. A projection of this structure down the helix axis is shown in Fig. 3.

Until recently, hydroxyproline had been thought unessential for the structural integrity of the collagen molecule. Its function may now have been revealed. Several workers[9,10] have found that the amino acid

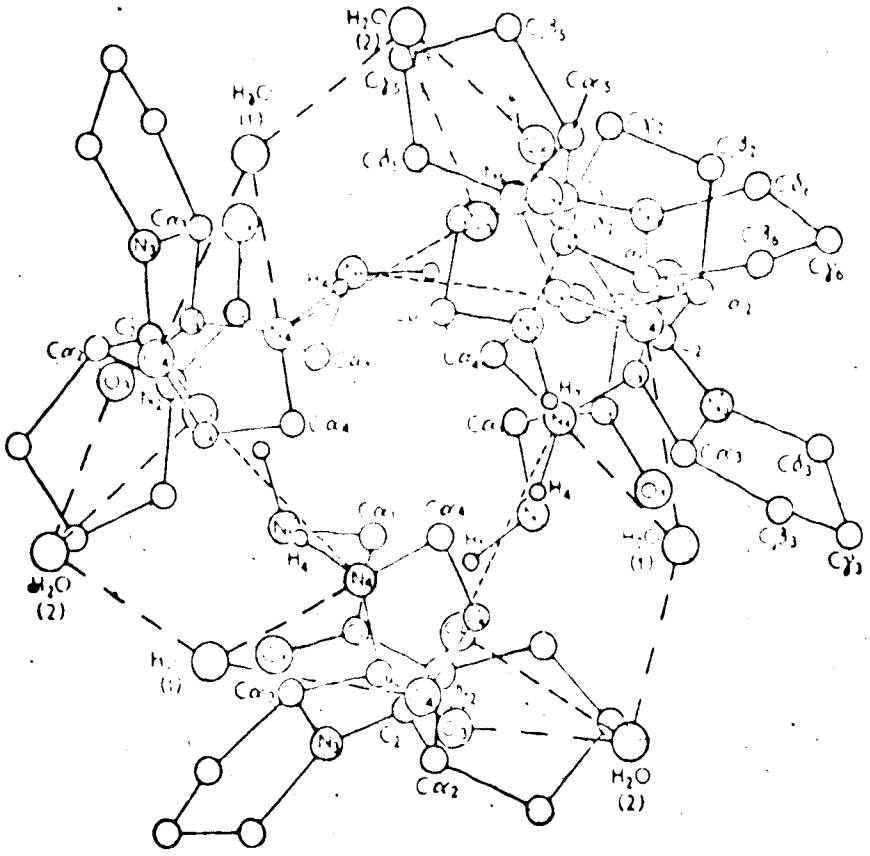


FIG. 3. Projection down the helix axis of (Gly-Pro-Pro)$_n$ structure, including two water molecules per tripeptide. The structure is viewed from the carboxyl end. Dashed lines indicate hydrogen bonds.

is important for the thermal stability of collagen. Jeminez et al[11] prepared chick tendon protocollagen samples which contained varying amounts of hydroxyproline. The thermal stability was monitored by susceptibility to complete digestion by pepsin, an enzyme which does not degrade helical molecules. They observed that the thermal stability varied directly with hydroxyproline content. The protocollagen samples which lacked hydroxyproline completely were found to have a denaturation temperature of 24°C. This implies that hydroxyproline is necessary for

the procollagen chains to assume the triple helix at body temperature.

Existing models for the collagen structure do not make allowances for the participation of the hydroxyl group of hydroxyproline in hydrogen bonding within the triple helix. Evidence for the participation of hydroxyprolyl residues in the stabilization of the collagen helix requires a re-evaluation of the atomic structures proposed for collagen.

Once the monomers are formed, these protofibrils bind extracellularly by electrostatic forces to form fibrils. Fibrils then combine to form fibers. When viewed under the electron microscope, the fibers have a distinctive and characteristic 640 Å banding. The primary structure of collagen has two special aspects which account for this. Firstly, there is a recurrence of certain similar sequences of amino acids. Glycine occurs in every third position with proline and hydroxyproline frequently following. This sequence is found in the non-polar or crystalline regions of the molecule. Secondly, this tripeptide alternates with a large polypeptide run which seems to have polar side chains of glutamic acid, aspartic acid and lysine. When collagen is stained with solutions of phosphotungstic acid or uranyl acetate, a characteristic band pattern is revealed which reflects the distribution of clusters of charged and uncharged amino acids along the molecule[12].

Recently, following the classical work of Kuhn and coworkers[12], Bruns and Gross[13] have established the number and position of reproducible bands in segment-long-spacing crystallites of calf skin collagen which will permit precise identification of individual bands or segments of the band pattern. They were able to collocate, on the basis of band numbers, published observations on positively stained segment-long-spacing crystallites which included cyanogen bromide and hydroxylamine

peptides, fragments produced by enzymatic cleavage, sites of scission by specific animal collagenases, amino acid residues identified by selective staining procedures, and clusters of specific residues where the amino acid sequence has been established.

The collagen becomes covalently cross-linked once the fibrils are formed. Two different types of cross-links form: intramolecular cross-links develop within the collagen monomer, whereas intermolecular cross-links form between the collagen molecules. The latter type of cross-link is physiologically important for strengthening the fibrils and rendering them insoluble. Evidence for cross-linking comes from denaturation studies on collagen. The products of such a study are α, β and γ components[14]. As a function of time, in vivo, cross-links form between chains to produce dimer components, the β components, which can be of two types when the cross-link is intramolecular, $\alpha 1 - \alpha 1$ (designated β_{11}) and $\alpha 1 - \alpha 2$ (designated β_{12}). When collagen is salt-extracted in the cold, predominantly $\alpha 1$ and $\alpha 2$ chains are obtained, making up 80-90 percent of the sample. On the other hand, when collagen is acid-extracted, 50-60 percent of the sample is β_{11} and β_{12}. This is because the acid-extractable collagen is more cross-linked than salt-extractable collagen. There also occurs a third component, the γ-component, which is a trimer composed of $\alpha 1 - \alpha 1 - \alpha 2$ (γ_{112}). These peptides account for the intramolecular cross-links. On the other hand, if other extraction methods are used, for example, 5M guanidine, pH 7.5, another type of β component can be extracted, the β_{22}, arising from intermolecular cross-linking between two $\alpha 2$ chains.

II. COLLAGEN BIOSYNTHESIS

The site of protein synthesis is the ribosome, which contains

RNA and proteins. The genetic information is brought to the ribosome by the single stranded RNA known as "messenger" RNA (mRNA) which has a primary structure complementary to a portion of one DNA strand. The nucleotide sequences of mRNA determines the amino acid sequence of the protein. Amino acids are attached to the soluble transfer RNA (tRNA) molecules which transfer amino acids to sites on ribosomes. The tRNA molecules are specific for a given amino acid but some amino acids are recognized by more than one tRNA. The mRNA is read in units of 3 nucleotides (triplet codon) for the incorporation of a single amino acid and the tRNA possesses a triplet anticodon that is complementary to the codon of the mRNA. It is through such interactions that the genetic message of the mRNA, originally derived from the active strand of DNA, is translated into a polypeptide sequence, incorporating sequentially one amino acid residue at a time beginning at the N-terminal end of the peptide chain.

Collagen biosynthesis follows the same general pattern as that for non-collagenous proteins. It is accomplished by a series of sequential steps. Firstly, the polypeptide precursor of collagen, procollagen, is assembled on ribosomal complexes[15-18]. Secondly, the appropriate proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine. This step differs from non-collagenous protein synthesis in that these amino acids are altered after incorporation into the protein. The third step involves glycosylation of some of the hydroxylysine with galactose or glucosylgalactose in o-glycosidic linkage.

Though synthesis of the protein chains of collagen resembles that of other non-collagenous proteins, there is still some controversy as to how they are assembled. It was first thought that, due to their

size, the α chains were synthesized in subunits which were released from the ribosome and subsequently joined in nonpeptidic covalent bonds [19-22]. Now evidence discredits this theory. In the analogous case of hemoglobin[24,25] the complete $\alpha 1$ and $\alpha 2$ chains have been shown to be synthesized simultaneously as single protein chains by sequential amino acid addition from the amino-terminal end[23]. It is still unknown whether the α chains combine to form the triple helix while still on the ribosomes. However, there is evidence which strongly indicates that both disulfide bond formation and triple helix assembly occur after release of pro α chains from the ribosome, probably within the cisternea of the endoplasmic reticulum[26-28].

In the past few years, researchers have discovered that the first polypeptide chains of collagen synthesized by cells are larger than the α chains[29-32]. All three chains of the procollagen molecules are found to have non-helical extensions at the NH_2-terminal end[33-36]. Each extension contains the same amount of cysteine, giving rise to disulfide bonds which link the three α chains[37]. These bonds stabilize the procollagen trimer and are thought to determine the correct longitudinal alignment of the three strands[38]. Perhaps these extensions, the registration peptides, initiate triple helix formation while the polypeptide is still on the ribosome. The tertiary structure of the complex, that is, the triple helical collagen plus the registration peptide, may keep the molecule from interacting and precipitating within the cell.

The first advances in knowledge of collagen biosynthesis came in the 1940's with Stetten and Schoenheimer[39]. These researchers found that when ^{15}N-proline was fed to rats, it was incorporated in collagen

in the hydroxyproline as well as in the proline. In subsequent experiments, Stetten[40] found that when he fed rats with ^{15}N-hydroxyproline, none of this was incorporated into the collagen. Other workers found a parallel situation for the incorporation of hydroxylysine[41-44]. It was therefore concluded that some of the proline and lysine residues were hydroxylated after completion of chain synthesis. On the other hand, it was later suggested that prolyl tRNA and lysyl tRNA were hydroxylated and as a result the hydroxyproline and hydroxylysine were then incorporated directly into the collagen polypeptide[45-51]. It has now been established that the correct view is the former of the two[51]. This was made possible with the discovery that it was atmospheric oxygen that was required for the hydroxylation step and not water[52,53]. Experiments were carried out under anaerobic conditions where ^{14}C-proline was incorporated into an unhydroxylated intermediate[54-58] of the same size as or larger than the α chains of collagen[57,59,60]. A substantial amount of the proline in the intermediate procollagen was hydroxylated to hydroxyproline when exposed to oxygen. When it was found that ferrous ion was required as a cofactor for the hydroxylation of proline[57,61], similar experiments were set up using metal chelators such as α,α'-dipyridyl. Early studies[62,63] showed that when hydroxylation was inhibited, secretion of collagen was also inhibited. There was a concomitant accumulation of the unhydroxylated material within the cells. When more ferrous ion was added to these cells, the preformed procollagen was hydroxylated and secreted as collagen[62]. It is thought the unhydroxylated procollagen cannot form the triple helix and as a result cannot be secreted. Thus hydroxylation of proline has an important physiological role directly related to the regulation of secretion of

collagen.

When the enzyme procollagen hydroxylase was isolated[64], it provided further proof that procollagen was an intermediate in collagen synthesis[65]. Secondly, it binds much more tightly to and hydroxylates much more readily a sequence of Gly-X-Pro in a large polypeptide than an identical sequence in a short polypeptide[65-69]. It would appear that hydroxylation occurs after the release of the nascent collagen chains from the ribosomal complex[70-72].

The hydroxylation of proline also requires α-ketoglutarate as well as ascorbate[73-76]. Ascorbate is the least specific in that it can be replaced by other reducing agents such as enediols. It stimulates the synthesis of peptidyl proline hydroxylase as well as activates it in vitro. However, the requirement for α-ketoglutarate is specific. It cannot be replaced by pyruvate nor oxaloacetic acid. The synthesis of one mole of hydroxyproline involves a stoichiometric conversion of α-ketoglutarate to succinate and CO_2[77], that is the reaction is substrate dependent and there occurs a stoichiometric decarboxylation of α-ketoglutarate coupled to the hydroxylation of the peptidyl proline residue[78]. The decarboxylation of α-ketoglutarate by the hydroxylase is not related to the decarboxylation of the same compound that occurs in the Krebs cycle in the mitochondria since it does not involve thiamine-pyrophosphate or require coenzyme A[79,80].

Hydroxylation of lysine follows the same mechanism as that of proline[81]. It requires oxygen, iron and α-ketoglutarate[65,82-84] but the enzyme, lysine hydroxylase, is different. Nor does ascorbic acid play the same role. When there is a deficiency of this vitamin, the hydroxylation of lysine is much less affected than the hydroxylation of

proline[75]. Peptidyl lysine hydroxylase will not further hydroxylate native collagen strongly suggests that hydroxylation occurs prior to triple helix formation[85-87]. There is no evidence that hydroxylysine has any effect on triple helix formation.

Glycosylation of the hydroxylysine is the final synthetic stage in completing the structure of the collagen molecule. It probably occurs while the nascent collagen peptides are still bound to the ribosomes[88]. To the appropriate hydroxylysine, galactose[195] is "tacked on" first in a β-glycosidic bond. To some of the galactosyl residues, glucose is then linked[195] in an α 1 $\longrightarrow$ 2-o-glycosidic linkage.

Once the collagen polypeptides are formed, hydroxylated, glycosylated and released from the ribosomal complexes, they probably migrate to the cisternae of the endoplasmic reticulum. Several studies have shown that the Golgi apparatus and its associated vesicles is implicated directly in the secretion of procollagen[91-94]. The Golgi vesicles were reported to contain filamentous threads while still associated with the Golgi and later as the vesicle traversed the cytoplasm, these threads appeared to aggregate into rod-like bundles of dimensions appropriate to aggregates of collagen molecules. According to Weinstock and Leblond[92], the newly synthesized procollagen is transported to the Golgi either by way of a transitional element or by fuzzy coated intermediate vesicles budding off from the rough endoplasmic reticulum. Experiments using microtubule disruptive drugs such as colchicine or vinblastine have implicated microtubules in procollagen secretion as these drugs impair collagen secretion[95-97]. Presumably microtubules play the role of a transport conduit along which the secretory vesicles move from their place of biogenesis within the Golgi apparatus to their eventual fusion

with the plasma membrane and excretion of their contents from the cell.

When the procollagen molecules are extruded into the extracellular space they aggregate to form microfibrils. However, it is extruded as the undegraded precursor which appear in electron micrographs as bundles which are 3000 or 6000 Å in length[91,98]. The packing arrangement in the larger bundles is such that the amino terminal regions are all oriented toward the center of the spindle and the carboxyl terminal regions point in opposite directions on either side of the central band. Recently[99-101], it has been demonstrated that the conversion of procollagen to collagen *in vivo* in rat skin, must be a multi-step process with at least two conversion steps. Intact procollagen and an intermediate form can be isolated from rat and bovine skin during the period of rapid growth of the animal. In odontoblasts[91,94], the first step in the formation of the extracellular fibre and fibril system is thus the dissolution of these antiparallel collagen molecule bundles and their conversion and reorganization into parallel alignment. This, perhaps, is the stage at which the enzyme procollagen peptidase makes its primary cleavage and serves in releasing individual molecules from the aggregate state. Electron micrographs[99] and pulse labeling studies[101] have shown that this intermediate material possesses an amino terminal extension and it is this extension which is thought to facilitate fibril formation. The final step in enzymic conversion to collagen is thought to occur after the microfibrils form[101].

In 1955, Schmitt et al[102] proposed the quarter-stagger theory whereby collagen molecules of protofibrils were displaced longitudinally with respect to one another by a distance equal to one-quarter of the length of the component molecules. However, more recently, it has been

found that the stagger is not one-quarter but such that there is a "hole" region of about 410 Å between each collagen molecule as well as an overlap zone of about 270 Å[103]. The length of the collagen molecule is 4.40 ± .02D where D is the period in the native fibril, that is, 690 Å in the wet state. See Fig. 4.

It seemed clear to Schmitt and his coworkers that the band pattern stained with phosphotungstic acid must represent a molecular map of the distribution of basic side chains along the length of the collagen macromolecule. In fact, collagen molecules have 5 charged regions 680 Å apart.

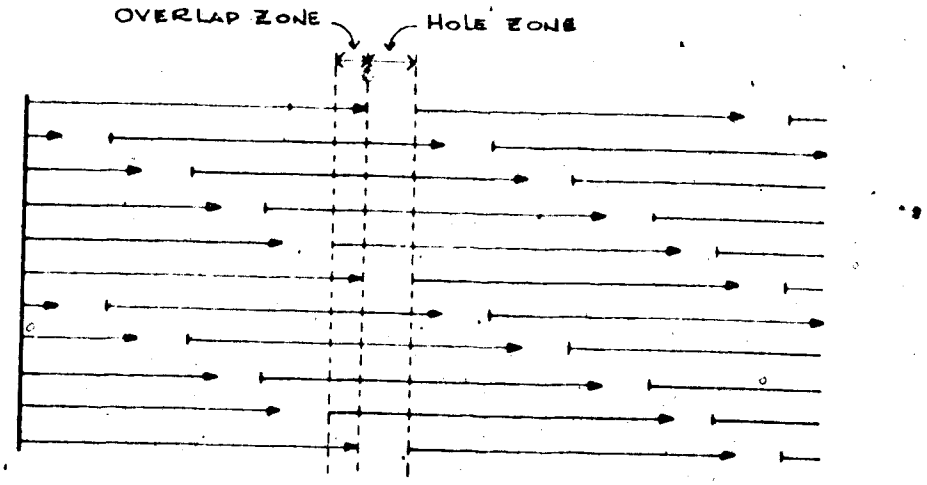


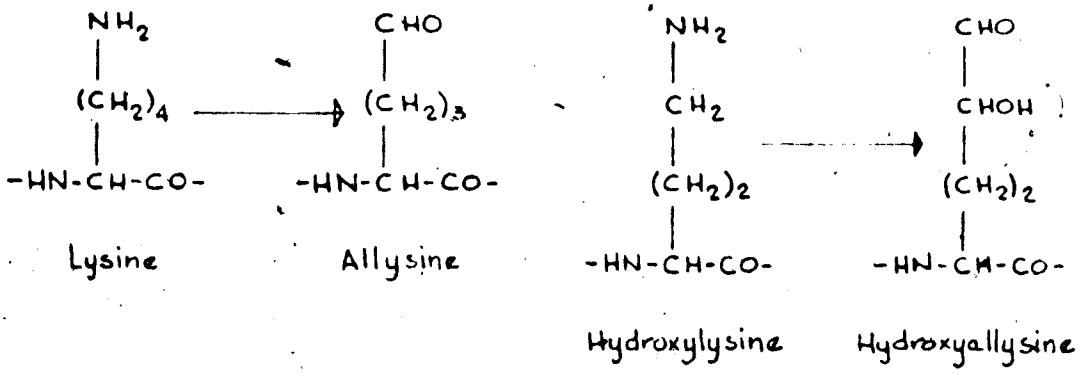
Fig. 4. Two dimensional representation of the packing arrangement of collagen macromolecules in the native-type fibril where overlap zone = .4D and hole zone = .6D

Much interest has been generated by the hole region of the collagen fibrils. It is thought that perhaps it provides a nidus for the deposition of hydroxyapatite in the process of calcification.

III. COLLAGEN CROSS-LINKING

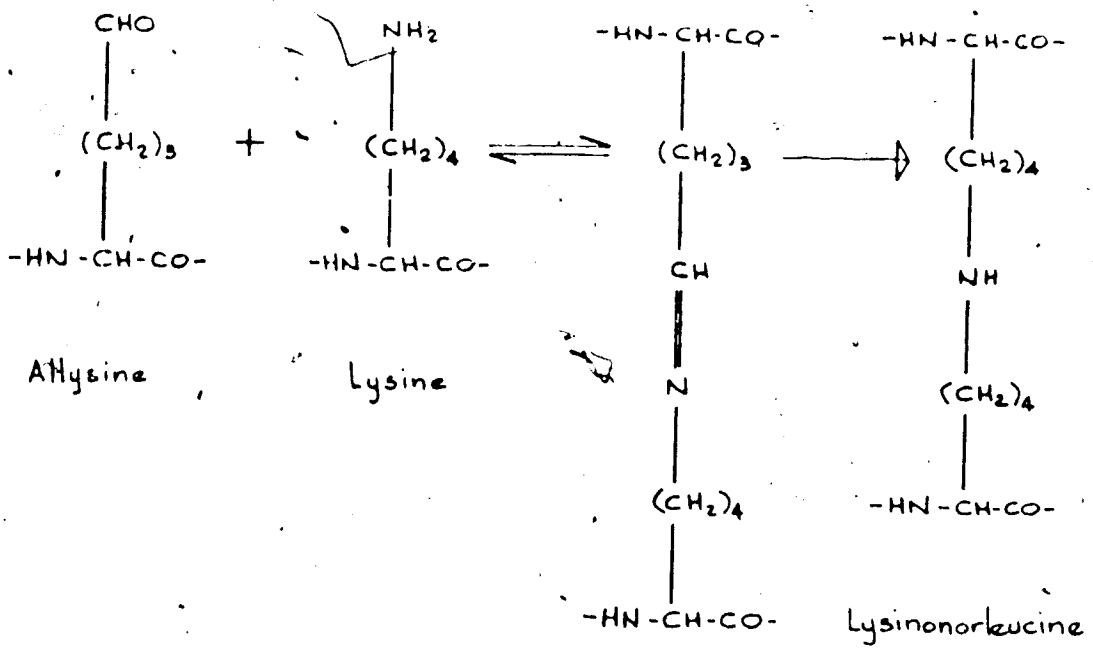
Cross-links form extracellularly once the molecules have aggregated into fibres. Two types of cross-links occur in collagen: intramolecular and intermolecular.

Both types of cross-links arise from lysyl or hydroxylysyl residues which are converted to the α-aminoadipic-γ-semialdehyde, more often called allysine and hydroxyallysine. In other words, the first step in cross-linking is the removal of the ϵ-amino group of a lysyl or hydroxylysyl residue and the formation of an aldehyde[104].



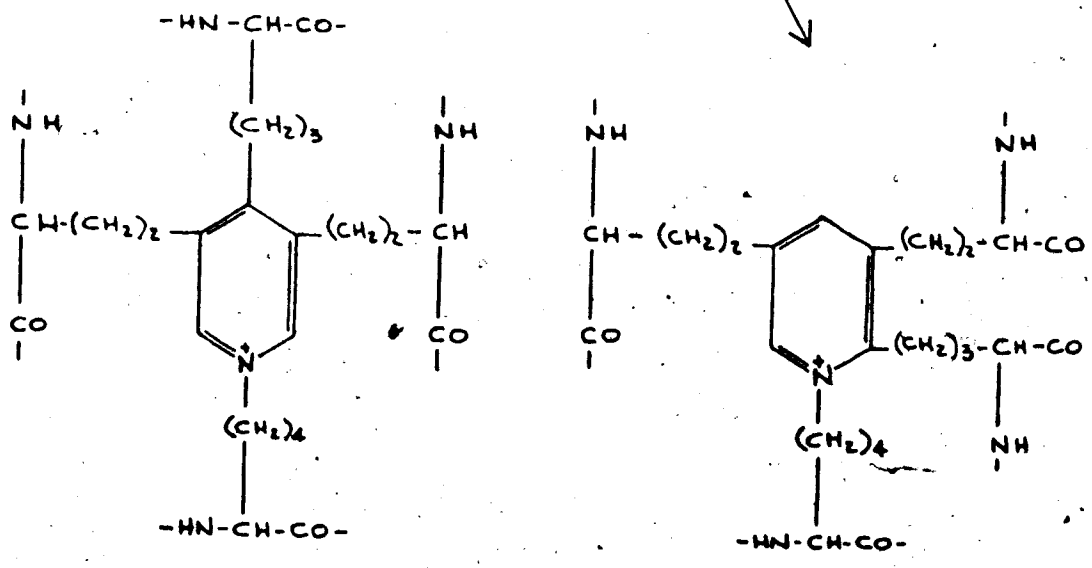
The formation of these precursor aldehydes is catalyzed by lysine oxidase[105] and are located most often in the nonhelical N-terminal region of the peptide although some have been found elsewhere in the chains[106,107].

From these aldehydes, crosslinks are formed via two types of reactions. The first is a Schiff base formation by a condensation of an allysine with the amino group of lysine or hydroxylysine on another α chain. This precursor of desmosine and isodesmosine[108,109] is not a stable cross-link and is considered not to be very important in collagen. However, Bailey[110] believes it is a precursor for the intermolecular cross-link lysinonorleucine.



Allysine Lysine Lysinonorleucine

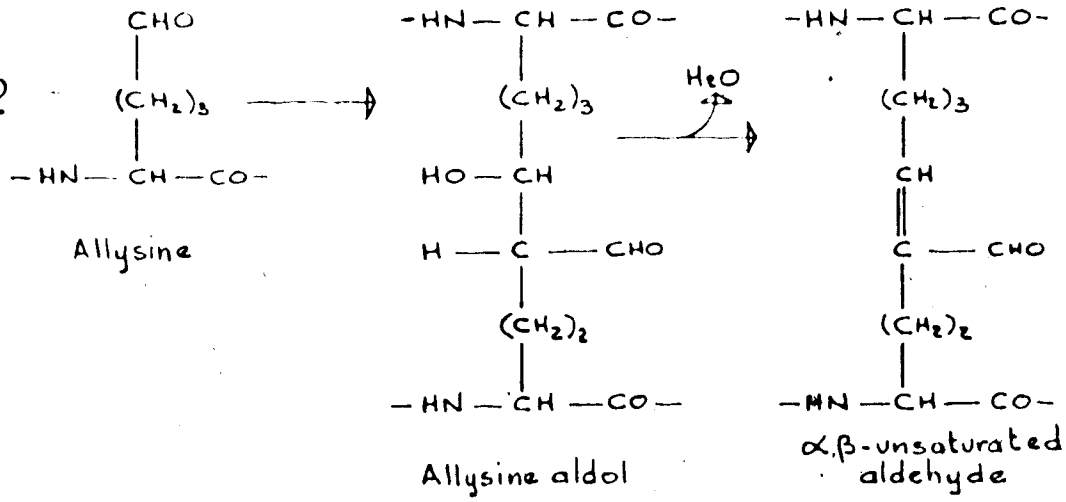
Dehydrolysinonorleucine



Desmosine
in elastin only

Isodesmosine
in elastin only

The second type of reaction involves an aldol condensation between two allysines on two adjacent α chains. The product is the intramolecular cross-link, allysine aldol, present only near the NH_2-terminal of collagen chains, dehydrates readily and is not stable to acid hydrolysis[108, 111]. It transforms during fibril formation and is thought to be a precursor of the intermolecular cross-link, hydroxylysinonorleucine[112] since it disappears as this cross-link appears.

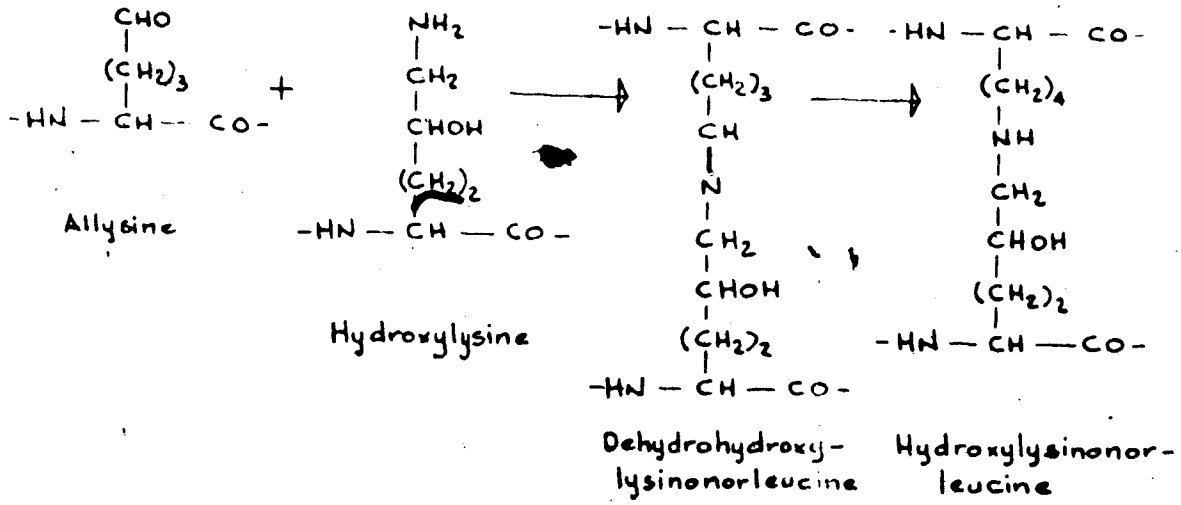


The role of intramolecular cross-links is in doubt since allysine aldol does not contribute significantly to molecular stability nor does it contribute to the stability of fibrils. Some researchers[110,113] believe that intramolecular cross-links are precursors of intermolecular cross-links. The strongest evidence supporting this thesis arises from studies on lathyrism. Lathyrogens inhibit the conversion of lysine to the aldehyde and since both intramolecular and intermolecular cross-linking is inhibited, perhaps the aldehyde is involved in both processes[108].

The role of intermolecular cross-links is better known. Collagen fibers have the unique ability to withstand stress due to the system of covalent cross-links between the individual collagen making up the fiber. Experimental lathyrism clearly demonstrates the need for these

cross-links, as it produces an extremely fragile fibre due to the slippage of adjacent molecules under tension[14]. It is possible to recognize a time-dependent process of maturation of the fibre by the changes in physical properties. An extensive system of these covalent intermolecular bonds between adjacent molecules would increase the tensile strength of the fibre by preventing slippage and would also be a logical explanation of the formation of insoluble collagen.

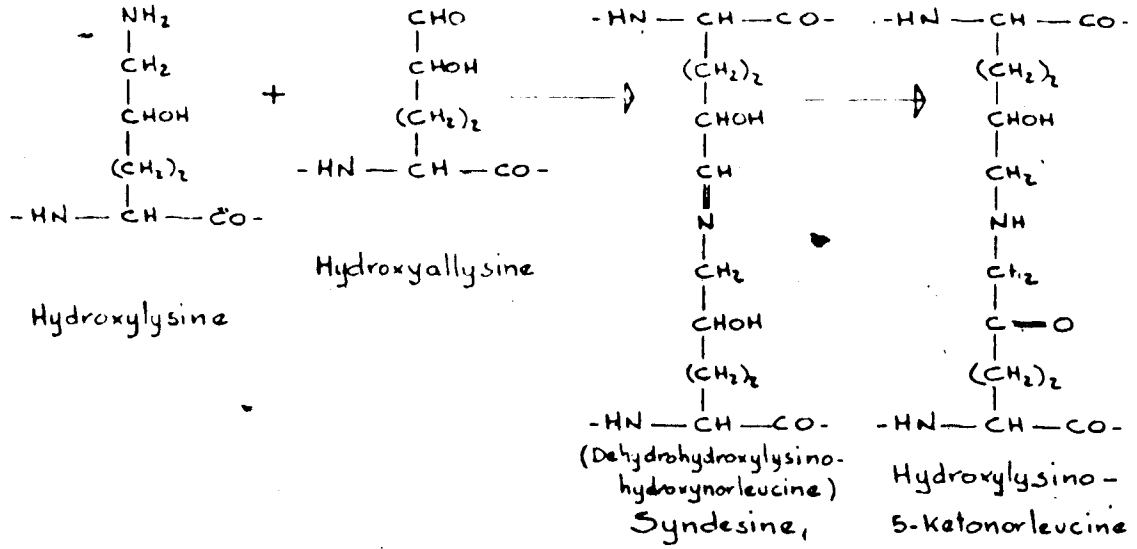
Chemical information on intermolecular cross-linking has been more difficult to obtain. Bailey and Peach[115,116] isolated a reduced product of a Schiff base condensation between a hydroxylysyl residue and an allysyl residue. The labile cross-link had the composition, hydroxylysinonorleucine.



It has been isolated in tendon as well as in reconstituted fibrils. This cross-link decreases with age and is thought to be, in part at least, an intermediate.

Bailey[115,117] isolated in insoluble collagen from chick bone and human and bovine teeth another cross-link which seems to be a major component. It is also present in chick and bovine tendon[118] from old animals and is a minor component of skin. This cross-link, syndesine, was first thought to be the product of an aldol condensation between an

allysyl residue and a hydroxyallysyl residue. It was later shown to be an aldimine derived from the condensation of hydroxyallysine and hydroxylysine[119,120].

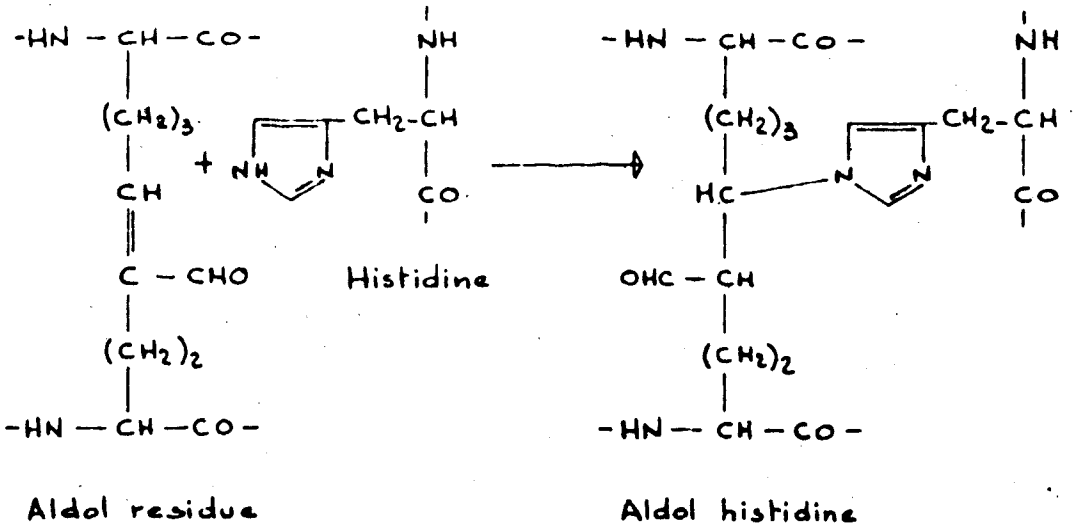


However, this is inconsistent with the unusual stability of dehydrohydroxylysinohydroxynorleucine to heat, dilute acids and D-penicillamine. Subsequently, it was found that there occurs a migration of the double bond to form the stable keto form by a spontaneous Amadori rearrangement[121]. The cross-link must therefore exist *in vivo* as hydroxylysino-5-ketonorleucine.

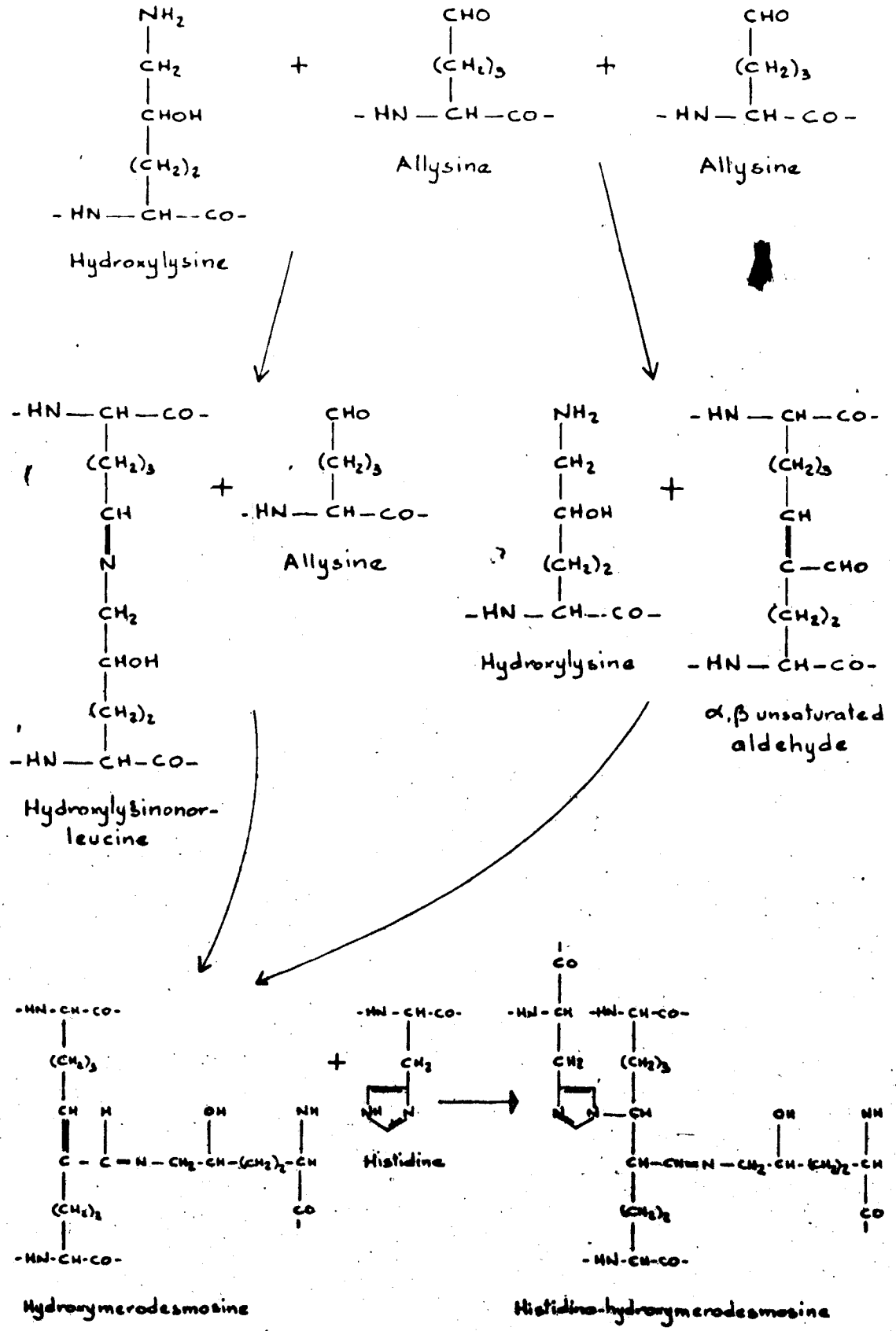
The above accounts for all products resulting from an aldol or Schiff base condensation between lysine, hydroxylysine and their aldol condensation of two hydroxyallysine residues which has not been reported. However, a higher molecular weight compound (or compounds) has been reported and may be a cross-link.

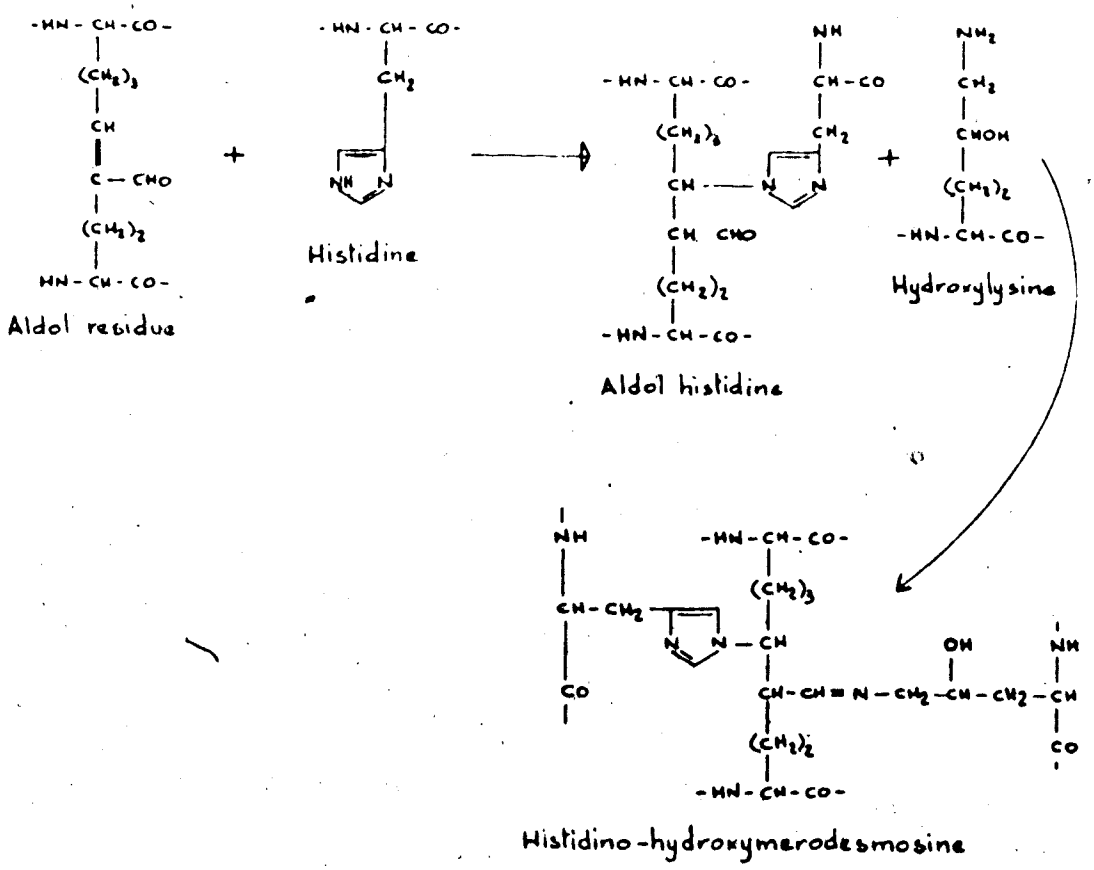
Cross-links arising from amino acids other than lysine residues and the result of reactions other than aldol and Schiff base condensations have been isolated. Disulfide bonds[122,123] are found in Ascaris cuticle collagen. There is also the aldol histidine which is found

predominant only in reconstituted cow skin collagen. It can potentially unite 3 polypeptide chains and is formed by a Michael addition of the imidazole of histidine, to the β-carbon of the α,β-unsaturated bond of an aldol condensation product[124,125]. Robins and Bailey[121] now believe this cross-link to be an artifact. The absence of the reduced form of this component, histidino-hydroxymerodesmosine on reduction under acid conditions and a concomitant increase in the amount of the aldol condensation product support this strongly. They believe that the Michael addition of the histidine residue to the aldol is base catalyzed by the borohydride.



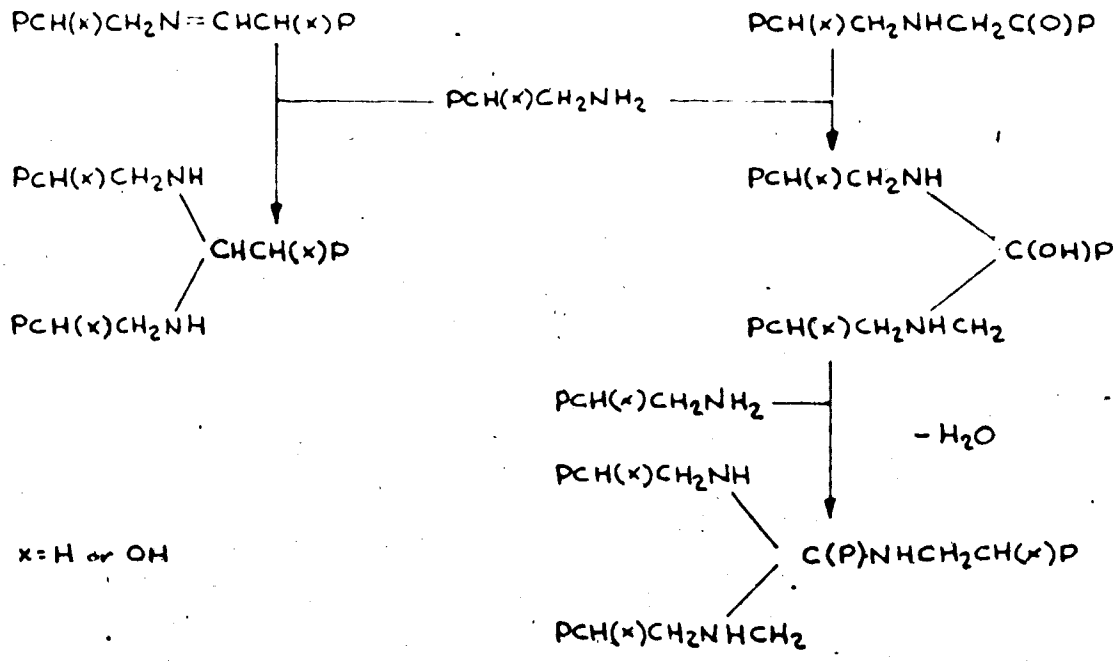
Merodesmosine, a trifunctional cross-link is found in collagen in small amounts in its monohydrated form. It is formed in reconstituted collagen and may arise via three alternate biosynthetic pathways. It may be an intermediate in cross-linking and it was proposed to be a precursor of histidino-hydroxymerodesmosine[125,126]. However, in view of the current findings, it is difficult to assess the role of this cross-link.


Hydroxylysine
Allysine
Allysine
Allysine
Hydroxylysinonor-leucine
Hydroxylysine
α,β unsaturated aldehyde
Hydroxymerodesmosine
Histidine
Histidino-hydroxymerodesmosine



Stable intermolecular cross-link formation is important in the maturation of collagen fibers. Bailey[127] proposed that these reducible electrophilic cross-links, which are progressively lost during aging[128], are transformed into more stable non-reducible cross-links whose structures have not yet been determined. Davis and Risen[129] tested this hypothesis and found that nucleophilic addition of lysine and/or hydroxylysine residues to the electrophilic double bond of the reducible cross-links transforms them into more stable, non-reducible cross-links. He also found that modification of lysine and hydroxylysine residues by destroying their ability to initiate nucleophilic attack on the electrophilic double bonds of the reducible cross-links completely blocks this transformation while the modification of histidine, arginine, glutamic

acid and aspartic acid is without effect. They proposed the following tentative structures.



These cross-links are capable of joining more than two collagen molecules. Davis and Risen hypothesized that the formation of these cross-links would explain the increasing resistance of maturing collagen fibers to dissolution in cold acid or alkali, to mechanical disruption, as well as to thermal disruption.

The location of intermolecular cross-links is difficult to ascertain. However, this information may soon be available as sequencing of the CNBr peptides of the α chains is well underway and cross-links have been isolated on various CNBr peptides[112,130].

IV. SEQUENCING OF CHAINS

The primary structure of collagen must carry information of higher levels of structure. It was with this thought in mind that researchers began concentrating on the sequencing of the α chains.

Sequencing of the α (I) chains is gradually being accomplished

as a result of CNBr cleavage. This method developed by Gross and Witkop[131,132], is based on the fact that CNBr cleaves the α chains at the methionine residues. The resulting peptides, 6 to 9 from the α 1 chain and 6 from the α 2 chain, are then separated by column chromatography and further cleaved if necessary by various enzymes, such as trypsin. Investigations of calf and rat skin collagen have provided the entire sequence of the amino acid residues of the α 1 chain. See Table 2 for the combined result as the differences between the two are few. Attempts to sequence the α 2 has not been quite as fruitful. Three of its 6 CNBr peptides are very long and as a result difficult to sequence. See Table 3 for α 2 CNBr peptides sequenced.

When the sequence of the α 1 chain is examined, one notices some striking features. Prior to sequencing, it was postulated that although glycine had to be in every third position of the helical portion, the other amino acids could occur at random, that is, in either position X or Y of the tripeptide Gly-X-Y. However, this does not appear to be so. There is a preponderance of some amino acids in a particular position of the tripeptide. Leucine, phenylalanine and proline are restricted to position X and glutamic acid occurs frequently in this position. On the other hand, threonine, arginine, methionine, lysine and hydroxylysine occur frequently in position Y and hydroxyproline occurs exclusively in position Y. Another interesting feature is that the hydroxylation of proline and lysine is often incomplete. The significance of these observations has yet to be elucidated.

An interesting paper recently published by Hulmes et al[133] examines the primary structure of collagen for the origin of molecular packing. They looked at the amino acid sequence of the triplet region

of the α 1 chain to see if there would be complementary relationships that would explain the stagger of multiples of 670 Å between the molecules of the fibril. They found that when the chains are staggered 0D, 1D, 2D, 3D and 4D (D = 234 ± 1 residues) the interactions are maximal between amino acids of opposite charge and between large hydrophobic amino acid residues. Unexpectedly they found that the hydrophobic interactions showed a fine structure in their pattern whereas the charged residues were less regular. Positively-charged residues tended to be near negatively-charged residues but did not show a distinct periodicity.

Fietzek et al[138] have recently sequenced the α 2-CB2 from calf, human, rabbit and pig skin collagen and compared them to that of rat and chick skin sequenced by Highberger et al. See Table 4. They observed 6 positions of high interspecies variability. However, the substitutions were conservative and in all cases the functionally important side chains were preserved in definite positions. The substitutions involved uncharged polar or apolar residues except in position 15 where a lysine-arginine substitution is found.

Much work is still required on the α 2 chain. However, once the α 2 chain is completely sequenced, a more detailed diagram of the interactions between the α chains and between collagen molecules will be possible.

V. TYPES OF COLLAGEN - CONTINUATION OF STRUCTURE: Special and Particular Cases

Until recently collagen was considered to have a chain composition $(\alpha 1)_2 \alpha 2$. Tissues of such a chain composition include rat skin[1], dogfish skin[139], human skin[140], chick skin[141] and chick bone[142]. Ex-

ceptions include codfish[143] where the three chains are different, and collagen from lower vertebrates and invertebrates where the three chains are identical[144-146]. However, several researchers have isolated different types of collagen which are genetically distinct. Miller and Matukas[147] observed a lack of stoichiometry when trying to isolate α 1 chains from α 2 chains in chick cartilage. Prior to this collagens examined had had α 1: α 2 ratios of 2:1. They found that although a proportion of cartilage collagen was identical to that of bone and skin of the chick, the cartilage contained another type of α 1 chain which they designated as Type II. They suggested that cartilage consists of 2 distinct collagen molecules, the $(\alpha 1)_2 \alpha 2$ and the $\alpha 1(II)_3$. Trelstad et al[148] supports this view and added that the α1 Type II had a much higher amount of carbohydrate, 5.5%, associated with it as compared to the .5% by weight of carbohydrate associated with Type I. Miller et al[4] have more recently isolated a third type of collagen, Type III, in newborn human skin. They suggest that human skin collagen is a mixture of molecules with the chain composition $\alpha 1(I)_2 \alpha 2$ and $\alpha 1(III)_3$. See Table 5 for a comparison of amino acid compositions of selected CNBr peptides from the 3 types, and Table 15.

Collagen from basement membranes[149] has not been formally numbered but by convention would normally be termed Type IV. It is also composed of 3 identical chains and has a much higher content of hydroxyproline, hydroxylysine and carbohydrate than interstitial collagens. The proline and hydroxyproline make up about 20% and the lysine and hydroxylysine make up about 5%. The 10-12% carbohydrate is virtually all glucosylgalactose linked to hydroxylysine; that is, glucose and galactose exist in almost equimolar amounts and account for all hexose.

Unlike other mammalian collagens it contains half-cystine. Another feature which distinguishes basement membrane is the low alanine content and its high phenylalanine content which is about twice that found in tendon collagen. See Table 6.

Recent developments indicate that vertebrate collagens are a heterogeneous population of molecules which are made up of five distinct genetic types: α 1(I), α 1(II), α 1(III), α 1(IV) and α 2. More types may be discovered. Cited earlier was codfish collagen[143] which had a molecule composed of 3 α chains, each of which appears to be unique.

As a result of these findings, structure and function can be more easily correlated. Several examples come to mind such as the cartilage transition to bone accompanied by a change from the cartilage species of collagen, $\alpha\,1(II)_3$ to the bone species, $\alpha\,1(I)_2\alpha 2$, and the change in collagen type occurring during the maturation of fetal skin $\alpha\,1(III)_3$ to the adult form $\alpha\,1(I)_2\alpha 2$. It can be seen, from these examples, that as function changes, so do the collagen types. Thus future investigations in particular to the role of collagen must now take into account the different molecular species.

VI. MINERALIZATION

Calcification or tissue mineralization is the deposition of inorganic crystals in or on an organic matrix. The inorganic crystals are composed of calcium and phosphate, bound in a form resembling hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. The organic components are the protein matrix, collagen: the mucopolysaccharide ground substance, thought to be chondroitin sulfate and acidic glycoproteins; and lipids.

The mechanism of calcification is still poorly understood. The fact that only teeth and bone normally calcify whereas other tissues do not even though the extracellular fluids are supersaturated with calcium phosphate remains a perplexing question. Several theories have been put forward to explain the phenomenon.

Role of Glycosaminoglycans

In the body, glycosaminoglycans are found in the form of hyaluronic acid, sialic acid, chondroitin sulfates and various other protein polysaccharides. They form the basis of the ground substance but their function is still unknown. Several workers[130-152] have found by progressive analysis of cartilage toward the ossifying front that chondroitin sulfate decreased abruptly as the calcium content increased. Glimcher[153] suggested that free anionic groups of acid mucopolysaccharides may inhibit calcification by binding calcium ions thus making them unavailable for crystallization. Perhaps in calcifying tissues the mucopolysac[redacted]es are removed destroying their inhibitory action. At the [redacted]ium is released, raising its local concentration and maki[redacted]e for calcification. The opposite view that the glycosam[redacted]romote calcification has also been proposed. Rubin and How[redacted] on the basis of a conformity between metachromatic staining and [redacted]uent mineral deposition, suggested that the property of calcifia[redacted] of epiphyseal cartilage and of tendon was associated with an [redacted]ased base-binding power of the matrix, the base-binding power be[redacted] chondroitin sulfate. On the other hand, Bowness[272] suggests the acid mucopolysaccharides, which he believes exist in 2 tissue compartments, play both roles, that of nucleation and inhibition. These compartments consist of 2 main fractions of chondroitin sulfate; one

fibrous and concerned with nucleation, the other being true ground substance and concerned with inhibition of calcification. However, other experiments have been carried out to further clarify the role of chondroitin sulfates using the uptake of ^{35}S as an indicator of mucopolysaccharide formation and ^{32}P as an indicator of calcification. It was found that the fall in S compounds did not occur in the same zone as the initiation of calcification. When glucose was added to the medium, ^{35}S uptake was increased whereas the enzyme inhibitor phloridzin reduced it. However, calcification was unaffected in both cases suggesting that mineralization is independent of mucopolysaccharide degradation[154]. Recent work on cathepsin D_1, which is thought to be one of the enzymes involved in the degradation, show it is not inhibited by most of the chemical agents which inhibit other proteinases.

Thus, the role of glycosaminoglycans in calcification still remains an open question.

Role of Lipids

In areas of teeth and bone where calcification is occurring, a substance which stains as a lipid has been detected. It exists in a form which is resistant to stain and must be treated with pyridine or alcohol to unmask it, that is, these solvents must remove a substance which protects it from the stain[155]. Wuthier[156] studied the distribution of this lipid component in the growth plate of bone and found its concentration increasing toward the active calcification site and subsequently decreasing dramatically when calcification was complete.

This lipid disappears in vitamin D deficiency and reappears when vitamin D is administered. Many workers have shown that the vitamin encourages the incorporation of phosphorus into phospholipids.

Cotmore et al[157] provided further evidence to the concept that phospholipids are involved in biological mineralization when they studied the calcium binding properties of phospholipids. Submicroscopic spherules were observed to form when Ca^{+2} and PO_4^{-2} ions combined with phosphatidyl serine, an acid phospholipid which Wuthier[156] isolated and characterized from calcifying cartilage.

Cellular Activity in Mineralization

Mitochondria: Isolated mitochondria were found, by Vasington and Murphy[158,159] and independently by De Luca and Engstrom[160] to accumulate large net amounts of Ca^{+2} from the suspending medium during electron transport. They observed that the mitochondria could accumulate the calcium up to several hundred times the initial Ca^{+2} content. Then in 1970, Lehninger[161] proposed that micro packets of amorphous calcium phosphate could be released from the cell and diffuse to specific calcifying sites.

Matthews[162] observed in the growth plate that ^{45}Ca was concentrated in the cells nearest the calcifying front. Subsequently, Martin and Matthews[163] found a gradient in ^{47}Ca concentration in the mitochondria in the chondrocyte. The gradient showed the concentration of ^{47}Ca gradually increasing in the mitochondria from the proliferative zone to that of provisional calcification at which point there was a decrease in mitochondrial calcium granules. In a later experiment, Matthews et al[164] examined and compared the mitochondrial granules in normal as well as rachitic rat epiphysis. They found that in rickets there were few granules in the mitochondria near the calcification front but when vitamin D was administered normal density and distribution of granules was reestablished. They suggested that both an apatite binding matrix and

inorganic phosphate were required by the mitochondria to form such granules. More recently Arsenis[165] found that the mitochondria of the columnar and hypertrophic zones of growth plate cells contained more enzymes which may be associated with calcification than did those of the resting zone.

Vesicles: Recently there has been another interesting development with the identification of vesicles containing crystallites between the hypertrophic chondrocytes in the growth plate as well as in osteoid and predentine.

Anderson et al[166,167] did an extensive study on the vesicles first described by Bonucci[168]. They found such vesicles, which contained or were lipid and had lipid membranes, in the longitudinal septa of the growth plate from the hypertrophic cells downward. These vesicles were closely associated with calcification, neighboring needle-like apatite structures. Their enzyme content was high in alkaline phosphatase, alkaline pyrophosphatase and ATPases; enzymes thought to be connected with calcification. However, they were very low in acid phosphatase and therefore not of lysozomal origin. These enzymes could raise the local content of orthophosphate, destroy phosphate inhibitors and lead to the formation of hydroxyapatite. The vesicles were found to possess a mechanism for ATP dependent transport of calcium and phosphorus into themselves. The crystals were initially closely associated with the inner surface of the vesicular membrane. Peress et al[169] speculate that the membrane serves as a nucleating center for the crystals. Kashiwa and Komorous[170] found mineralized spherules in cytoplasm of chondrocytes, in the matrix adjacent to the hypertrophic chondrocytes and also in the core of the spicules distal to the hypertrophic chon-

droevtes. They hypothesized that perhaps intracellular spherules might be the source of the extracellular ones.

Bernard and Pease[171] in studying intramembranous bone formation, described nucleating sites for apatite as being extrusions from osteoblasts within the osteoid. Crystals were seen to grow epitaxially into the surrounding collagen forming bone nodules which coalesced to form bone seams. Between coalescing nodules, fully formed collagen was observed.

Eisenmann and Glick[172] studied the calcification process in rat incisor teeth. They observed that the first crystalline material appeared in small round membrane-bound bodies interspersed among, but distinct from the collagen fibrils of predentine matrix. These crystal-containing bodies appeared to be cellular in origin and can be seen only in the very early stage of mineralization. Crystals were later observed to radiate beyond the crystal bodies and only then appear to be associated with collagen fibers.

Bernard[173] studied developing molars of mice and observed that the initial crystal formation occurred, in and adjacent to, cellular extensions of the odontoblasts into the predentine. When crystal growth was more extensive, the plasma membranes of these cellular extensions disappeared. Crystals growing from the calcification loci became spheroidal and coalesced to form mantle dentine. Later Bernard and Pease[171] observed that these vesicles budded off from osteoblasts and odontoblasts. Bernard believes that the involvement of structural protein is secondary to initial crystallization and that protein orients and structures the size of crystals rather than organizes their nucleation.

Role of Collagen

Since collagen fibrils and apatite crystallites have a close physical relationship, it is thought that collagen can act as a seed for the initiation of calcification. Termine et al[174] found that there is a direct physical binding between collagen fibrils and apatite crystallites.

Reconstituted collagen fibers act as a seed when placed in a solution supersaturated with respect to calcium and phosphate (epitaxy). Only native type fibrils having 640 Å banding are able to act as a nidus[175]. Due to the exceedingly minute size of the first crystals laid down and the tight packing of the collagen fibrils observed in most species, it is difficult to detect by electron microscopy whether the crystals are located on, between or within the fibrils when seeding begins. However, once the crystals are detectable, they are seen within the collagen fibrils, 640 Å apart mainly in the region of the interband thus showing a relationship to the bands, that is, to some structure having a period similar to the bands of collagen.

Collagen macromolecules are staggered by one quarter of their length giving rise to "holes" at regular intervals which correspond to the interband (see section on Collagen biosynthesis, p. 14). Glimcher and Krane[153] believe these holes permit the packing of collagen fibrils with calcium phosphate crystals. This ability collagen has to act as a seed may be due to the presence of specific sites. In other words, the initial crystallites have a specific spatial relationship to the collagen fibers. However, evidence of the exact physical relationship between the fibres and crystals at the initial crystallization phase is lacking. Hohling et al[176] found 6-7 sites for nucleation in the hole zone and half as

many in the overlapping zone of dentine and bone collagen. Davis and Walker[177] expound that the carboxyl group of glutamic acid and aspartic acid may be the nidus. They found that if glutamic acid and aspartic acid of decalcified bone and dentine were converted to glutamine and asparagine, recalcification was completely blocked whereas the modification of lysine, hydroxylysine, histidine and arginine residues had no effect. Glimcher[153], on the other hand, thought that certain ϵ-amino groups of lysine and hydroxylysine were nucleation centers. However, Davis (unpublished work) disagrees as he found that there was no effect on the rate or extent of calcification when the lysine and hydroxylysine were completely modified with ethoxyformic anhydride or acrylonitrile.

Katz and Li[178] have postulated that the molecular packing of collagen from different tissues may be a factor in calcification. They studied adult bovine dentine, adult rat bone, adult rat tail tendon and purified reconstituted steerskin collagen which they found to be compatible with the theory that collagen is packaged into hexagonal units containing 7 molecules (in cross section)[179]. Most importantly they observed that the intermolecular gap in bone and dentine was 6 Å whereas in tendon it was 3 Å. Phosphate ions, having a diameter of 4 Å, could therefore penetrate the bone and dentine by ionic diffusion but not the tendon.

Collagen Types: Recent studies on the chemistry of collagen have shown differences in its composition when taken from different sources (see section on Types of collagen, p. 25). Miller et al[4] have studied the composition of the different types of collagen monomers. They found that the α 1(I) and α 1(II) cyanogen bromide peptides differed in

amino acid content such as 4-hydroxyproline, aspartic acid and leucine. They also learned that the collagen of mature skin and bone was of the same genetic type, $\alpha 1(I)_2\alpha 2$[142,180] and that the collagen of cartilage was of the $\alpha 1(II)_3$ type[181]. It was observed that the type which calcifies is the $\alpha 1(I)_2\alpha 2$[182]. Toole et al[182] studied the collagen of rachitic osteoid (uncalcified layer between the osteogenetic fibers and bone) and found it to be of the $\alpha 1(I)_2\alpha 2$ type, like that of skin and bone but with one important difference. It had 50% more hydroxylysine than normal bone which they suggested could be glycosylated thus blocking calcification by steric interference with apatite crystal formation in the hole region of the collagen molecule.

Recently, Linsenmayer et al[183] studied the temporal and spatial transitions in collagen types during embryonic chick limb development. They perceived that at early stages of leg mesenchyme development, an $(\alpha 1)_2\alpha 2$ type collagen is produced. At a later stage, the limb cores began synthesizing $(\alpha 1)_3$ type collagen, while the outer portion of the limb still produces $(\alpha 1)_2\alpha 2$. At the stage of bone production, the $(\alpha 1)_2\alpha 2$ type collagen reappears. The $(\alpha 1)_2\alpha 2$ thereafter predominates in bone and $(\alpha 1)_3$ in cartilage. Although the reason for the different types of collagen is still unknown, it can be supposed that this may be related to specific functions and therefore perhaps to calcification.

Phosphoprotein: Soft tissue collagens have a similar amino acid and carbohydrate composition as that of calcified tissues. However, one major difference between the two is that calcified tissue collagens possess a phosphoprotein moiety which soft tissue collagens do not possess [184].

In 1964, Veis and Schleuter[185] isolated this phosphoprotein in

dentine and found it to be, at least partially, a phosphate bound to a serine as phosphoserine. They tentatively postulated that perhaps this phosphoprotein plays a role in the nucleation of mineralization as well as stabilizes the structure of collagen. Subsequently they further characterized the phosphoprotein finding it to be rich in aspartic acid, glutamic acid, depleted in hydroxyproline as well as possessing some hexose[186]. They also found it to be electrophoretically composed of a slow (S) and a fast (F) anionic component. They thought it to be localized in a few chain segments of the collagen molecule. Later Veis and Perry[187] characterized these S and F components. The S component, having a molecular weight of about 25,000, was found to be similar to the parent collagen and that the glycine, alanine, proline and hydroxyproline content made up 63% of the molecule. Also, it was rich in aspartic acid, glutamic acid and serine. The F component, found to be homogeneous by electrophoretic and ultracentrifugal techniques, was distinctly different, being rich in aspartic acid and serine but having small amounts of glycine, alanine and proline. It was of collagenous origin as hydroxyproline and hydroxylysine were present. The F component which was the phosphate rich component had a molecular weight of about 38,000 and contained almost all of the hydroxylysine which survived the periodate oxidation. In order for the hydroxylysine to have resisted periodate oxidation, its amino or hydroxyl group must have been involved in a covalent bond, otherwise it would have yielded ammonia and formaldehyde. Since the disaccharide glucosylgalactose is known to be linked to hydroxylysine[188], Veis and Perry proposed the F component to be composed of three parts: the first section comprised of proline, hydroxyproline and hydroxylysine among other amino acids, as

part of the peptide backbone of dentine collagen; the second portion, an oligosaccharide linked glycosidically to hydroxylysine to which the third part, the phosphoprotein moiety is covalently linked. They found the phosphoprotein to be present in an amount of less than 2% of the total protein or in a ratio of one molecule of phosphoprotein to four to six molecules of collagen. Therefore, this phosphoprotein could serve as the site for nucleation in the hole zone of the collagen fiber.

Carmichael et al[189] later isolated glucose and galactose from degradation products corresponding to the phosphoprotein and suggested that perhaps the anionic moiety is attached to the collagen disaccharide through a serine phosphate diester linkage.

Subsequently, Butler et al[190] found that the phosphoprotein could be extracted via a gentle non-hydrolytic extraction procedure. They decalcified rat incisors in acetic acid and followed this by extracting the decalcified tissue in Tris-NaCl. The Tris-NaCl solubilized most of the phosphoprotein thus providing evidence that the phosphoprotein could not be covalently bound. Davis and Walker[177] support Butler's view that phosphoprotein is not necessary for mineralization, as they were able to get dentine to calcify at the same rate and extent once the phosphoprotein was removed by Tris-NaCl extraction.

Carmichael and Dodd[191] proceeded to do a Tris-NaCl extraction followed by periodate oxidation on bovine dentine. They found that half the phosphoprotein was extracted in the Tris-NaCl but that the other half required periodate oxidation to release it. They also found that the carbohydrate content was greater in the Tris-NaCl extract material than in the periodate solubilized fraction. This they suggested may reflect the degree of degradation occurring during periodate oxidation.

Veis et al[192] characterized the Tris-NaCl soluble and the matrix bound phosphoprotein, finding an overall similarity between the two. They suggested that the soluble and the matrix bound phosphoprotein may serve a dual role in locating the deposition of mineral on the collagen matrix and inhibiting calcification of predentine. Recently, its possible role in locating the deposition of mineral has received support from Weinstock and Leblond's[193] elegant radioautographic studies on the deposition of phosphoprotein at the mineralization front of dentine. They showed the phosphoprotein crossed the predentine zone into the mineralizing front thus suggesting it may play a role in mineral deposition.

Up to now, phosphoprotein has been chemically shown to be present in bone and dentine. Although it has been seen radioautographically in a transitory state in predentine, the tissue has never been chemically analyzed for it and nor has cementum. If the theory, that phosphoprotein plays a role in mineralization is valid, then cementum as a calcified tissue should possess it. Predentine, being a tissue which will mineralize, should contain it only at the mineralization front. As a result, predentine and cementum were analyzed to see if phosphoprotein is a necessary component of calcified tissues.

With the current theories and experimental evidence, mineralization can be explained in general terms. Cellular activity is certainly important. However, the involvement of the mitochondria and its vesicles does not explain how the mineral is able to penetrate the collagen fibers. It must be remembered that 60-80% of the mineral is within the collagen and that the intermolecular gap in the collagen fiber of bone and dentine is just large enough to accomodate a phosphate ion. Perhaps

the mitochondria and/or vesicles bring high concentrations of calcium and phosphate to the collagen fiber and deperding which way the collagen fibers are packed, and what type it is, the phosphate is able to penetrate the intermolecular gap along with the calcium. Phosphoprotein and/or functional groups such as the carboxyl groups of glutamic acid and aspartic acid, act as the nidus for the initial crystal formation. The mitochondria and/or vesicles may then bring high concentrations of calcium and phosphate ions (or some compound containing these species) to the calcifying matrix allowing mineralization to continue on the exterior of the fibers.

Not much is known about how calcification is inhibited or controlled, what causes the local build up of calcium and phosphate nor why some tissues calcify more than others. From the above discussion, it is obvious that there is still much to be learned about the finer details of the process of mineralization.

VII. COLLAGEN: A GLYCOPROTEIN

Glycoproteins are proteins which have variable amounts of carbohydrate covalently linked to their peptide portion. They do not have characteristic amino acid compositions, although collagen is an obvious exception. On the other hand, their sugar components are distinctive and include D-galactose, D-mannose, D-glucose, L-fucose, D-xylose, L-arabinose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and the N- and O-acetyl and N-glycolyl derivatives of neuraminic acid.

Collagen's carbohydrate unit is an α-1,2-glucosylgalactose disaccharide[194-196]. As in almost all glycoproteins collagen contains its carbohydrate units in varying stages of completion or with minor

modifications (microheterogeneity). The type of glycopeptide bond in collagen is a β -glycosidic linkage between galactose and the hydroxyl group on the γ carbon of hydroxylysine[188,195,197]. It is an unusual linkage as it involves a glycosidic substitution onto a hydroxyl group vicinal to an unsubstituted amino group. In other glycoproteins, asparagine, serine and threonine are the residues to which the carbohydrate units are usually linked[198]. Collagen is unique in that only hydroxylysine is involved in the carbohydrate linkage. There is one exception. The carbohydrate units of annelid cuticle collagen are linked to serine and threonine and consist only of galactose mono-, di- and trisaccharides. This collagen contains no hydroxylysine but much larger amounts of serine and threonine than do other collagens[199,200].

Butler[201] has isolated the cyanogen bromide peptide which is rich in carbohydrate; the α 1-CB5. It is the fourth cyanogen bromide peptide from the amino terminal end and its probable sequence in the carbohydrate linkage region in guinea pig skin is -Gly-Met-Hyl(Glc,Gal)-Gly-His-Arg[188]. Morgan et al[202] more recently determined the sequence in carp swim bladder and human skin collagen and found it to be only slightly different from that of the guinea pig skin isolated by Butler.

Skin and dentine collagen contain less than 1% hexose whereas cartilage collagen contains up to 5% hexose. In skin, approximately half of the hexose is present in the disaccharide form[4].

The effect of the carbohydrate in collagen is still speculative. It is thought that the density of carbohydrate units may have an effect on the organization of collagen into fibrils. Tissues such as basement membranes having the largest number of hydroxylysine linked carbohydrate units show the least organization and no fibrillar structure. On

the other hand, tissues such as skin, tendon and sclera have the smallest number of hydroxylysine-linked carbohydrate units and display wide fibrils with distinct 640 Å banding. Tissues with intermediate amounts of hydroxylysine-linked carbohydrate units, such as corneal collagen, have narrow fibrils even though they possess 640 Å banding[194,202,203]. Morgan et al[202] feel that due to the size of the disaccharide prosthetic group, regular hexagonal packing of collagen in a quarter-stagger array could seriously be impaired.

The role of hydroxylysine linked carbohydrate units in collagen is as yet unapparent. Eylar[204] proposed some time ago that proteins are glycosylated so as to provide a passport for excretion from the synthesizing cell. Winterburn and Phelps[20] dispute this hypothesis and hypothesize that the carbohydrate tag determines the fate of the protein once it is extracellular. An example of this comes from Veis and Perry[187] who proposed that the phosphoprotein linked to the carbohydrate units in calcifying tissues may serve as a nucleation site for the initial epitactic nucleation of calcium ions (see section on Mineralization, p.35).

Spiro[207] has speculated that the sugars attached to hydroxylysine could possibly play a regulatory role indirectly, that is, the carbohydrate units may be attached to the hydroxylysine so that the residue could not participate in the types of cross-links occurring in collagens; this effect occurring due to steric hindrance. However, Eyre and Glimcher[208,209] and Robins and Bailey[206] have found evidence for a glycosylated cross-link in collagen. Therefore, steric hindrance of cross-link formation apparently does not occur.

As no work was undertaken on the glycosaminoglycans of predentine and cementum in this project, the topic will not be discussed here.

For a review on the subject see reference 210.

Proteoglycans and glycoproteins - Terminology: To understand the histochemical studies which are discussed below, a brief explanation of the terms glyc[redacted]an, proteoglycan and glycoprotein are given first. Protein-ca[redacted]mplexes in which the components are covalently link[redacted]ssues. The properties of the complex, depending on [redacted]portions of the protein and carbohydrate, will be more [redacted]e peptide or sugar component. Such influences can be seen f[redacted]ycans and glycoproteins.

[redacted]glycans are protein-carbohydrate complexes in which polysacchari[redacted]ke up as much as 90% or more of the complex. The protein component[redacted]on-collagenous and the carbohydrate, called glycosaminoglycans ([redacted]mes called mucopolysaccharide), are polysaccharides containing he[redacted]ne. These polysaccharides include hyaluronic acid, chondroitin-[redacted]ulfate, chondroitin-4-sulfate, keratan sulfate, dermatan sulfate, hepar[redacted]sulfate. The polysaccharide chains are unbranched except perha[redacted]arin sulfate and they also contain uronic acid except keratan[redacted]te. If present in the free form, the constituent polysacchari[redacted] chains show little affinity for collagen, apart from the ionic interaction which is fairly easily broken. However, when the polysaccharide chains are linked to the non-collagenous protein, the large molecules associate more firmly with the collagen.

Glycoproteins, of which collagen is a special case, are a covalent compound of non-collagenous protein and sugar chains. The sugar chains are sometimes branched and do not contain more than 20 residues. A large variety of sugars are found in glycoproteins. These include hexosamines and galactose (which is found in only one proteoglycan, that

containing keratan sulfate) as well as mannose, fucose and sialic acid, the latter two often occupying terminal positions on the oligosaccharide chains. Glycoproteins do not contain uronic acid but a few appear sulfated like most of the glycosaminoglycans and proteoglycans.

VIII. DENTINE, PREDENTINE AND CEMENTUM: The Present State of Knowledge

Teeth are composed of three calcified tissues: enamel, dentine and cementum. Dentine forms the bulk of the tooth and encloses the pulp cavity. Enamel envelops the dentine of the crown whereas cementum covers the root dentine. In calcification of dentine, a collagenous matrix must be laid down first. The collagen fibers, arranged in a trellis-like framework, are produced by the odontoblasts which line the pulpal surface of the dentine. Since mineralization lags behind matrix formation, there exists a zone of uncalcified tissue called predentine especially noticeable at the apex of the immature tooth[211].

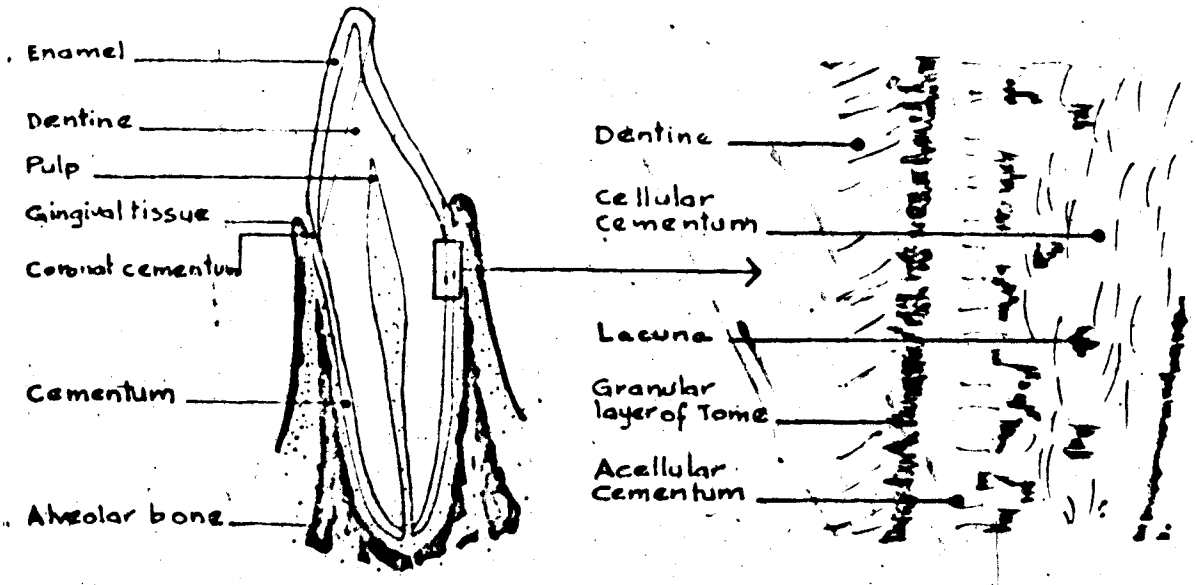


Fig. 5. Relation of the main dental tissues

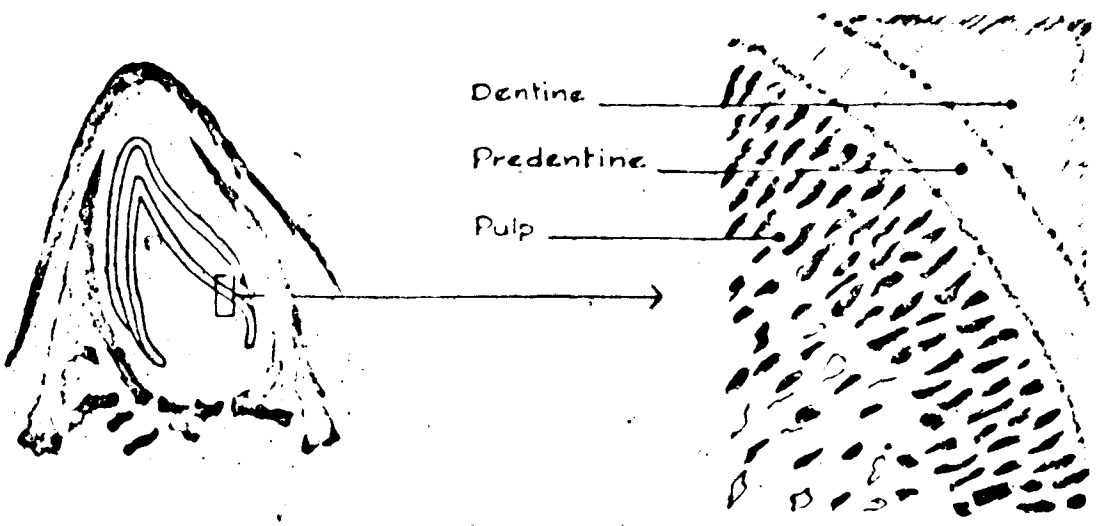


Fig.6. Diagrammatic illustration of an unerupted tooth

Dentine and Predentine: Little is known about predentine due to the small amount of tissue in teeth. As a result most studies on predentine have been histochemical in nature.

Considerable work has been done on dentine. Wislocki et al[212] were the first to observe histologically the presence of mucopolysaccharides in dentine using the periodic acid-Schiff (PAS) technique. Subsequently, using the same technique, Matthiessen[213] expanded these results to include predentine which he found to be PAS positive. Other histochemical investigations have shown significant amounts of acid mucopolysaccharide in predentine. Bevelander and Johnson[214] as well as Weill[215] found predentine intensely metachromatic. However, Greulich and Leblond[216] found that predentine stained weakly compared to dentine,

indicating a relative absence of glycoprotein in predentine. This has been the observation of several other workers[217-219]. Fullmer and Alpher[220] were unable to detect any glycoprotein in predentine.

Recently Weinstock[221], using the acidified phosphotungstic acid technique[222] for glycoprotein, observed that the stained material in dentine occurred between and associated with the collagen fibers. In predentine the interfibrillar material was unreactive although the collagen fibers did stain slightly. Weinstock suggests that glycoprotein may play a role in the initiation of calcification as predentine acquires a carbohydrate rich material, probably glycoprotein, as it is transformed into dentine.

The proteoglycans of dentine have been the subject of several recent studies. The limiting factor in these studies has been their low concentration in dentine. Clark et al[223] found that the major acid glycosaminoglycan of human dentine-cementum was chondroitin-6-sulfate. They also reported a small amount of hyaluronic acid (2% of the total hexosamine) and were unable to detect keratan sulfate and dermatan sulfate. Engfeldt and Hjerpe[224] have more recently characterized the glycosaminoglycans of both canine predentine and dentine. The major polysaccharides in both these tissues were galactosaminoglycans tentatively identified as chondroitin sulfate. They could not identify a small amount of material which corresponded to the amount of hyaluronic acid Clark et al had isolated from human dentine-cementum. Thus Engfeldt and Hjerpe could not conclude that hyaluronic acid was excluded from predentine and dentine. These authors reported that the glycosaminoglycan hexosamine content of dentine was 0.06% of the organic dry weight and that of predentine was 0.12%. Their results indicate that there is more

chondroitin-6-sulfate in predentine than dentine. Jones and Leaver[225] attempted to clarify the conflicting reports concerning the nature and amounts of the constituent glycosaminoglycans of dentine. They demonstrated that the major glycosaminoglycan of human dentine is chondroitin-4-sulfate but it was suggested that the chondroitin-4-sulfate:chondroitin-6-sulfate ratio may vary with age since chondroitin-6-sulfate may be lost during maturation. Jones and Leaver also found 2-6% of hyaluronic acid, 2-3% of dermatan sulfate and a definite but small amount of keratan sulfate in human dentine.

Linde[226] has recently isolated the glycosaminoglycans of the odontoblast predentine layer of porcine teeth. The total amount of hexosamine was found to be 0.29% of the dry weight. This figure includes hexosamine from the glycoprotein and glycosaminoglycans. He obtained five hexosamine containing fractions when the glycosaminoglycans were separated on CPC cellulose micro columns, each of which was predominantly composed of one of the following components: chondroitin-4-sulfate (30% of the total), chondroitin-6-sulfate (16%), hyaluronic acid (14%), dermatan sulfate (7%), and keratan sulfate and glycoproteins (32%). The keratan sulfate was found to account for about 4% of the total hexosamine.

Veis and coworkers[185,187,192,227] have also done considerable work on dentine. They found that, although dentine collagen resisted acid swelling and had no neutral salt soluble or acid soluble collagen, there were few differences in composition from corium (dermis) collagens except for the presence of phosphoprotein in dentine (see section on Mineralization, p. 35). They reported that the two collagens contained a comparable quantity of hexose (2.2 residues/1000 amino acid residues).

Recently Volpin and Veis[228] have studied the CNBr peptides of skin and dentine collagens. Except for variations in hydroxylation of proline and lysine, they found few differences of significance between the CNBr peptides derived from the α 1(I) and α 2 chains of insoluble bovine corium, dentine collagen and the corresponding CNBr peptides from the soluble bovine collagens. However, in the insoluble bovine corium and dentine collagens, three distinct new classes of CNBr peptides were detected. Both collagens were found to contain an acid stable intermolecular cross-link component, α 1-CB6' [α 1-CB6 + α 1-CB(0, 1)]. Both also contained two additional peptides attributed to the presence of an α 1(III) chain, the α 1(III)-CB3 and α 1(III)-CB (4,5). Evidence was also obtained indicating the presence of a peptide α 2-CB4' consisting of α 2-CB4 and a noncollagenous polypeptide attachment. Both corium collagen and dentine collagens were analyzed for hexose and bound phosphate groups. In dentine collagen, only α (I)-CB5 contained the glucosegalactosyl hydroxylysine, while α 1(I)-CB3 contained only the galactosyl hydroxylysine which was also present in α 1(I)-CB5. The α 2-CB4 and α 2-CB5 contained mixtures of mono- and disaccharide attachments. In addition, Volpin and Veis were able to isolate from the dentine collagen α 1(I)-CB6, α 2-CB4 and α 2-CB5 containing covalently bound phosphate groups which were not found in corium collagen. It seems likely that the hexose is present as a hexose phosphate rather than the phosphate attachment being via a serine hydroxyl. The acid-insoluble CNBr digestion residues were found to contain portions of collagen with uncleaved methionyl residues in close association with a highly acidic polypeptide. This was particularly true in dentine collagen where this polypeptide was rich in phosphoserine groups.

As mentioned above, Veis and Schleuter[185] have identified in dentine an acidic peptide containing a high content of organically bound phosphorus. Weinstock and Leblond[193] found that this phosphoprotein passed through the predentine to reach the mineralization front possibly playing a role in calcification at the dentine-predentine junction.

Cementum: Cementum, the root covering of the tooth, is the calcified dental tissue which most resembles alveolar bone in its composition, structure and behaviour. It provides a medium for the attachment of the collagen fibers of the periodontal ligament that bind the tooth to the surrounding structures as well as playing a role in preserving the width of the periodontal ligament. It assists in maintaining the length of the root available for support of the tooth and effects repair of damage of the root[229].

Cementum is formed in much the same way as dentine. Cementoblasts lay down an uncalcified collagenous tissue called cementoid or precementum which is subsequently mineralized to form cementum. However, since calcification is not as rapid as matrix formation, a thin layer of cementoid tissue is seen on the surface of the cementum.

Two types of cementum can be differentiated morphologically depending on the presence or absence of cells and they are therefore known as acellular (primary) cementum and cellular (secondary) cementum. See Fig. 5. Acellular cementum may cover the root dentine from the cementoenamel junction of the apex, but is often missing on the apical third of the root. The more predominant cellular cementum usually covers the apical root dentine and acellular cementum and includes cementocytes representing cementoblasts which have been left behind and surrounded during cementum formation.

Collagen fibrils of cementum are orientated two ways[230,231]. Sharpey's fibers, the group of fibers which run at right angles to the cementum surface, originate from the periodontal ligament and are incorporated in the cementum as it mineralizes. The second group of fibers are thought to be produced by the cementoblasts. These fibers are not directly attached to the bone but are fundamental to the inherent structure of cementum. The two groups of fibers tend to run at right angles to one another.

Cementum is less calcified than dentine. Selvig and Selvig[232] found that the combined calcium plus magnesium content of human cementum was in the range of 25.7 to 26.6% whereas that of dentine was 26.8 to 27.6%. The phosphorus content of cementum ranged from 11.8 to 12.5% compared to 12.2 to 13.2% in dentine. Neider et al[233] report similar findings. Selvig also observed differences in the distribution of mineral in the matrix fibers of cellular cementum as well as in those of acellular cementum. The acellular cementum, he noted to be more calcified than the cellular form, the latter also containing uncalcified cores of Sharpey's fibers.

Glimcher et al[234] have determined the amino acid composition of coronal cementum (see Fig. 5) which was found to be characteristic of collagen, that is, the organic matrix contains at least 90% collagen. Cementum matrix collagen resembles bone and dentine collagens in that it is virtually insoluble in neutral and acidic buffers.

The non-collagen constituents of cementum have not been characterized nor has its collagen been studied in great detail. For example, it has yet to be determined whether the phosphoprotein moiety found in

bone and dentine, which is thought to serve as the nucleation site for mineralization, occurs in cementum[185,190,191,184].

From the above it is apparent that further chemical analyses are required to gain additional insights on predentine and cementum.

I. MATERIALS

Fresh bovine jaws were obtained from animals between 1 and 2 years old at slaughter. Unerupted and erupted incisors and canines were extracted as well as unerupted molars. The unerupted teeth were freed of their dental sacs, and the pulps were removed. These as well as the erupted teeth were then washed by soaking in distilled water at 4° overnight.

Predentine was obtained from the unerupted teeth by cutting away the translucent zone at the apex of the root. The fragments were then rewashed in distilled water and lyophilized. The predentine was subsequently dissected under a dissecting microscope to remove the adhering odontoblasts and the opaque dentine.

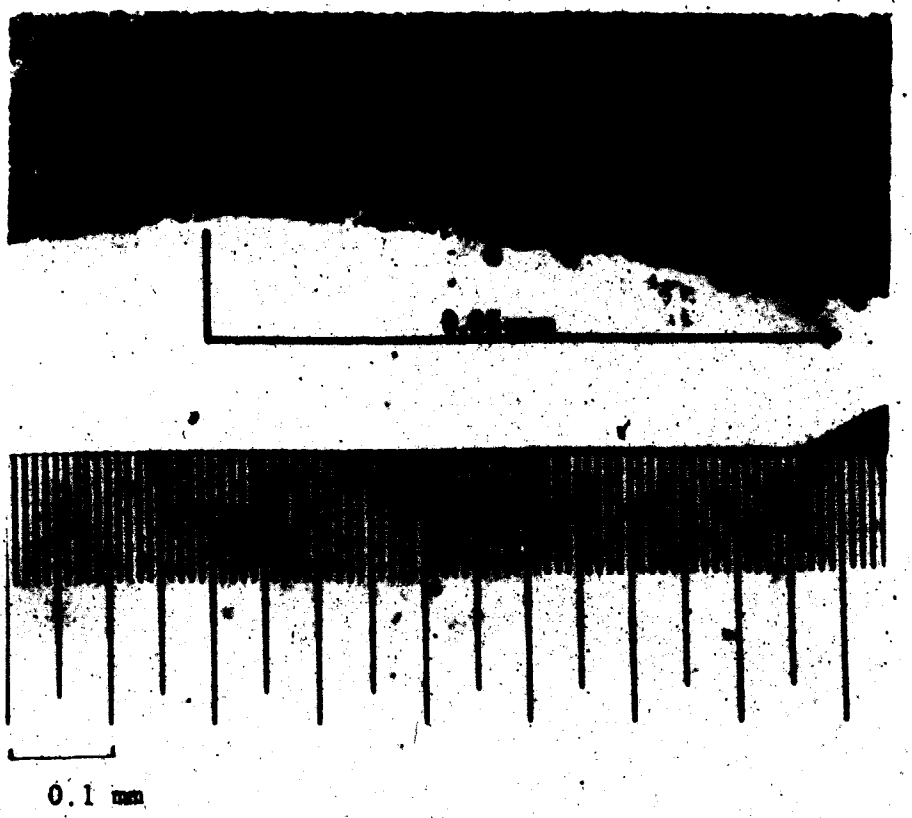


Fig. 7. Hemotoxylin-eosin stained predentine showing mineralization front. The length of the predentine region is indicated. (By courtesy of Dr. K.A. McMurchy)

The erupted teeth were scraped clean of any adhering soft tissue. The crowns were sectioned below the cervical line with a Gillings Hamco Fine Sectioning Machine. The root was then sliced longitudinally into thin lamellae of about 250 microns. Under the dissecting microscope, the somewhat more translucent cementum, which appears as a thin layer next to the dentine, was carefully separated from the dentine and then lyophilized.

II. METHODS

Density Gradient Isolation of Cementum

To ensure that the cementum was not significantly contaminated with dentine, the Manly and Hodge[235] flotation method was performed on a sample of cementum. The sample was ground dry in a Heidelberg Colloidal Mill, and the resulting powder passed through a 60 μ sieve. The powder was then centrifuged in a mixture of bromoform and acetone having a specific gravity of 2.11. The sample separated into a light (cementum) and heavy (dentine) fraction. As an additional check for purity, the heavy fraction was examined for dentinal tubules under the light microscope.

Calcium

In order that any organically bound calcium might be freed, the calcium content of predentine and cementum was determined following acid hydrolysis in 10N H_2SO_4. The calcium was determined by EDTA titration according to the method of Ericsson[236]. This method involves the firm binding of calcium in an un-ionized complex with EDTA. A small amount of $MgCl_2$ (.4 mls of 1mM solution) was added so that the indicator, Eriochrome Black T, would shift from red to blue when all Mg ions had

been bound by the EDTA titration solution as with calcium alone the color shift is too gradual and indistinct.

Phosphorus

The phosphorus content was determined using the modifications by Eastoe[237] of the Fiske and Subbarow[238] method with the exception that the amino-napthol sulfonic reagent was prepared according to the procedure of Bartlett[239]. This method is based on the reduction of phosphomolybdic acid by 1-amino-2-napthol-4-sulfonic acid which is blue in the presence of sulfite.

Decalcification

The predentine and cementum fragments were demineralized by repeated extraction at 4° in 0.5M EDTA adjusted to pH 7.4 with NaOH. Extraction of cementum continued for 17 days and predentine, which contained little calcium, for 4 days, at which time calcium could no longer be detected in the residual matrix by atomic absorption spectrometry. The method employed here was a modification of the procedure of Willis[240]. Lanthanum was used in the analysis to prevent interference by phosphorus. With this procedure, calcium content cannot be detected below 0.15 parts per million. The residual cementum and predentine were washed with distilled water until EDTA could no longer be detected. The EDTA test involves the Ericsson[236] method where adding Mg to a 5 mls portion of the water wash indicates the presence of EDTA using Eriochrome Black T as the indicator.

Tris-NaCl Extraction

Butler et al[190] showed that phosphoprotein could be removed by a gentle non-hydrolytic extraction with Tris-NaCl. Thus, the decalci-

fied predentine and cementum residues were extracted for 3 periods, each of 24 hours, in 500 volumes of 0.5M Tris-NaCl (pH 7.4) containing 1.0M NaCl. The remaining residues were washed in distilled water and lyophilized. The extracts were dialyzed against distilled water and lyophilized.

Phosphoprotein Determination

Carmichael and Dodd[191] found that not all the phosphoprotein was Tris-NaCl soluble and that some required periodate oxidation to remove it. As a result, the EDTA and Tris-NaCl extracted predentine and cementum matrices were subjected to oxidative degradation for 44 hours with sodium metaperiodate following the procedure of Carmichael et al[189]. The 54 mg of predentine and 71 mg of cementum which were solubilized in 10 mls of sodium metaperiodate (0.025M $NaIO_4$ + 0.025M $NaHCO_3$, pH 7.75) were dialyzed at 4°C against 4 liters of 0.01M sodium bicarbonate for 3 days, changing the sodium bicarbonate twice daily. The retentate was subsequently dialyzed exhaustively against distilled water and then lyophilized. The dried residue of each weighed about 45 mg, and each was analyzed for calcium and phosphorus. Using the procedure of Clark and Veis[241], electrophoresis of 50 µl and 100 µl of predentine (270 µg lyophilized periodate soluble material/ml Tris-Glycine buffer) and cementum (310 µg lyophilized periodate soluble material/ml Tris-Glycine buffer) in a polyacrylamide gel at pH 8.4 and run at 4mA/tube, separated the non-diffusible components. Following electrophoretic separation, the gels were sliced transversely and analyzed for phosphorus.

Determination of Lysine-derived Cross-links

Suspensions of 12 mg each of predentine preparation and cementum

preparation in 1.8 mls of 0.15M saline (pH 7.4) were reduced with 0.2 mls of tritiated sodium borohydride (18.4 mCi/mmole) dissolved in 0.01M NaOH (30:1 w/w ratio of predentine and cementum preparations to $NaBH_4$). The tritiated sodium borohydride was standardized, according to Paz et al[242], by reduction of 4-(p-nitrobenzamido)-butyraldehyde to 4-(p-nitrobenzamido)-butanol which had a specific activity of 5.45 mCi/mmole. The pH was adjusted to 9.0 and the reaction mixture stirred at room temperature for 1 hour. After acidification to pH 4.0 with 50% acetic acid, the reaction mixture was exhaustively dialyzed against distilled water at 4° and then lyophilized.

Acid hydrolysis in 6N HCl under N_2 in sealed tubes at 108° for 22 hours or alkaline hydrolysis in 2M KOH under N_2 in teflon bottles at 108° for 20 hours was carried out on portions of the reduced cementum and predentine preparations. Reduced aldimine cross-links were determined in the acid hydrolysates using the Jeol 5AH amino acid analyzer (0.8 x 45 cm column, LCR-2 resin, 60°) eluting with 0.35M (Na^+) sodium citrate buffer, pH 5.28 at a flow rate of 0.62 ml/min. Reduced cross-link precursors ϵ-hydroxynorleucine and δ, ϵ-dihydroxynorleucine were determined in alkaline hydrolysates using the amino acid analyzer (0.8 x 45 cm column, LCR-2 resin, 54°) eluting with 0.2M (Na^+) citrate buffer, pH 2.93 containing 3% propanol, 0.2M (Na^+) sodium citrate buffer, pH 3.35, and 0.2M (Na^+) sodium citrate, pH 4.10, at a flow rate of 0.80 ml/min. For both the reduced cross-links and the cross-link precursors, fractions were collected using a split stream device, and each fraction was mixed with 10 ml Aquasol (New England Nuclear) and counted in a Nuclear Chicago Mark I Liquid Scintillation System.

Amino Acid Analysis

Samples of predentine and cementum matrices were hydrolyzed in 6N HCl at 110° in sealed tubes under N_2 for 22 hours. Amino acid analyses were carried out using the two-column system on a Jeol 5AH analyzer. No corrections were made for hydrolysis losses.

Separation of Collagen and Non-collagen Glycopeptides[243]

EDTA and Tris-NaCl extracted predentine and cementum matrices were each digested 16 hours at 65° with 0.03 ml of previously activated papain (Sigma, 2x recrystallized) in 4 mls of 0.1M acetic acid sodium acetate buffer (pH 6.0) containing 88 mg of cysteine hydrochloride and 186 mg of EDTA/100 mls. 3.0 mls of the digest were added to a Sephadex G-25 column (fine grade, 33 x 1.5 cm) and eluted with water at room temperature. The flow rate was 45 mls/hr and 2.5 - 3.0 mls fractions were collected. A monitor for non-amino sugars was performed on 0.1 ml of each fraction. This was done by modifying the orcinol-H_2SO_4 method for increased sensitivity; that is, 1.5 mls of 80% H_2SO_4-1% orcinol solution was added to the 0.1 mls portion of each fraction previously diluted to 0.5 mls. Two orcinol positive peaks were detected; peak 1 at the void volume containing the higher molecular weight, non-collagen glycopeptides and the retarded peak 2, containing the collagen glycopeptides[243].

Neutral Sugar Analysis

The fractions containing peak 1 were pooled as well as those for peak 2. The pooled fractions were then lyophilized and hydrolyzed in 2N HCl for 4 hours at 105°. However, before hydrolysis, 25 µg of L-Rhamnose was added to each to act as an internal standard. The hydro-

lysates were neutralized with the bicarbonate form of Dowex 1 x 8 (20-50 mesh beads). The slurries were then poured into small chromatography columns collecting the effluent and several water washes. These were lyophilized and analyzed for neutral sugars on a Technicon Carbohydrate Autoanalyzer. The dry residue was dissolved in 1.0 ml of borate buffer 1 and added to the carbohydrate column (type S chromobeads). A similar buffer gradient as the standard method was used except that the volume per chamber was reduced from 50 ml to 30 mls. The temperature was as in the standard method.

In the standard method, the column was 68 cm x 0.6 cm long which corresponds to an 8 hour run. As a more rapid separation was desired and this column length separates a rather large number of sugars (including disaccharides) some of which were not expected in our analyses, the column length was shortened to 33.5 cm. The sugars of interest, mannose, fucose, galactose, xylose and glucose, were well separated.

Elution was achieved at 45 mls/hr at 53.5° C with the following gradient in a nine chambered autograd: chambers 1 and 2, 30 mls of buffer 1 (0.1M H_3BO_3, pH 8.00); chamber 3, 15 mls of buffer 1 and 15 mls of buffer 2 (0.1M H_3BO_3 + 0.05M NaCl, pH 8.00); chamber 4, 12 mls of buffer 3 (0.1M H_3BO_3 + 0.1M NaCl, pH 8.00) and 18 mls of buffer 4 (0.2M H_3BO_3, pH 8.00); chambers 5, 6 and 7, 15 mls of buffer 4 and 15 mls of buffer 5 (0.2M H_3BO_3 + 0.2M NaCl, pH 9.50); chambers 8 and 9, 30 mls of buffer 5. The effluent was analyzed for neutral sugars with orcinol-H_2SO_4 (70% H_2SO_4-0.1% orcinol, Sigma). This colorimetric system was as in the standard Technicon system for carbohydrates (Technicon Development Bulletin 124). The individual monosaccharides were quantitated by comparing their areas with that of Rhamnose. See Table 8 for a diagrammatic illustration

of typical chromatograms of G-2 Peak 1 and Peak 2. It can be seen that the sugars of interest were very well separated but one or two unknown orcinol positive compounds were detected especially from the G-25 Peak 1 hydrolysate. The final unknown peak immediately following glucose appeared in all chromatograms including those from unhydrolyzed standard mixtures of pure sugars and therefore did not appear to be a hydrolysis artifact. This unknown could possibly be due to degradation products of more than one sugar, formed on the column under the alkaline conditions and elevated temperature.

The color yields in the orcinol method are different for each sugar. The acid hydrolyses losses were different for each sugar. There was also a possibility of mechanical losses particularly during the lyophilization of the resin neutralized hydrolysate. The addition of a known quantity of L-Rhamnose before acid hydrolyses eliminates some of these problems. However, it was necessary to carry out analysis of hydrolyzed standard mixtures of sugars, including L-Rhamnose, in order to obtain reliable colour yields relative to L-Rhamnose.[5] The colour yields relative to Rhamnose also varied to some extent with the total quantity of sugar in a given peak; in other words, the standard curve was not linear. This had to be taken into account when applying the colour factors.

Hydroxyproline

Hydroxyproline content was determined by the method of Stegemann and Stalder[244] on a 0.1 ml portion of the papain digest which was hydrolyzed in 6N HCl at 103° for 23 hours. The hydrolysates were evaporated to dryness in vacuo over P_2O_5 and NaOH. The small amount of acid remaining in the residues were neutralized before the analyses were performed.

The range of this method is from 1 µg to 8 µg hydroxyproline per 2 mls of sample.

1. PREDENTINE

Prior to EDTA extraction, the calcium content of predentine was determined to be 0.4% (Table 7). This value corresponds to a hydroxyapatite content of 1% which in turn corresponds to a phosphorus content of 0.18%. However, the phosphorus content was found to be 0.37%. The predentine fraction was 99% organic material.

The EDTA extract was dialyzed against distilled water and then lyophilized. The dry residue was found to have a phosphorus content of 0.07%. The phosphorus content of the pooled Tris-NaCl extracts was 1.7%, this being considerably lower than the comparable Tris-NaCl extract of dentine.

In the EDTA-Tris-NaCl extracted predentine residue neither phosphorus nor calcium (by the method of Ericsson[236]) could be detected.

When subjected to oxidative degradation with sodium metaperiodate, the EDTA-Tris-NaCl extracted predentine was completely solubilized. The resulting extract, which had been dialyzed first against sodium bicarbonate and then against distilled water, was lyophilized and analyzed for phosphorus. The phosphorus content was found to be 0.02%. Several anionic components were revealed by electrophoresis of this fraction, including a lightly staining band with a mobility corresponding to the phosphoprotein component previously demonstrated in dentine[185]. See Table 9 for gel results. However, no phosphorus could be detected in this band. Total absence of phosphorus in this fraction cannot be regarded as established in view of the small sample size, low phosphorus content of the sample, and low staining intensity of this band. However, the use of a comparable sample size of dentine produced a signifi-

cant amount of phosphoprotein at this position.

The amino acid composition of the EDTA-Tris-NaCl extracted predentine (Table 10) generally resembled that of dentine matrix collagen. The glycine content was reduced from a content of about 320 residues/1000 amino acids to 281 residues/1000. The hydroxyproline content of 90 residues/1000 was also low compared to the normal value of about 100 residues/1000. The proline (174 residues/1000) and the hydroxylysine (12.4 residues/1000) were high as compared to the normal values for proline (about 115 residues/1000) and hydroxylysine (about 4-8 residues/1000) as in skin or dentine collagen.

Chemically reduced aldimine cross-link content as well as cross-link precursor content are recorded in Table 11 accompanied with similar data for dentine matrix collagen. The hydroxylysinohydroxynorleucine content of predentine was 4.8×10^{-1} lysine derived residues/1000, while the hydroxylysinonorleucine content was 3.5×10^{-2}. Thus, the major cross-link of predentine is hydroxylysinohydroxynorleucine. The cross-link precursors, dihydroxynorleucine and hydroxynorleucine, were present in concentrations of 1.2×10^{-2} lysine derived residues/1000.

The neutral sugar results are given in Table 12. The total neutral sugar content of the Sephadex G-25 peaks was considered more accurate when obtained from the sum of the results derived from the auto-analyzer than from the modified orcinol results. The total in each case was expressed as g neutral sugar/14g hydroxyproline. (The hydroxyproline content of the papain digest was used in this calculation as this avoids errors due to the possibility of incomplete digestion of the collagen by papain.) This value gives quantities which are close to a percentage on the dry weight of a pure collagen. The G-25 peak 2 re-

sults (collagen hexose) for predentine collagen corresponded to 4.9g hexose/14g hydroxyproline, of which 52% was galactose and 41% glucose. The hexose of the non-collagen glycoprotein associated with the predentine collagen (G-25 peak 1) corresponded to 2.2g hexose/14g hydroxyproline. This hexose was made up of 20% glucose, 33% galactose, 23% mannose, and 10% fucose.

II. CEMENTUM

Undecalcified cementum, examined by the flotation procedure, contained less than 1% of the heavier "dentine" fraction. However, when scrutinized under the microscope, this heavier fraction was also found to be mainly cementum.

Cementum was shown to have a calcium content of 26.3% by weight (Table 7). This percentage of calcium corresponds to a hydroxyapatite content of 66% and an organic content of 34%. Phosphorus content was determined to be 11.4%, and hence the Ca/P ratio is 2.3 (w/w). The Ca/P ratio of hydroxyapatite is 2.2 (w/w). Thus, the mineral of cementum is hydroxyapatite, like that of other calcified tissues.

The phosphorus content of the EDTA extract was 0.14%, whereas that of the pooled Tris-NaCl extracts was 1.76%. No phosphorus or calcium could be detected in the decalcified Tris-NaCl extracted cementum.

Decalcified Tris-NaCl extracted cementum was completely solubilized when subjected to oxidative degradation with sodium metaperiodate. The resulting extract was dialyzed against 4 liters of 0.01M sodium bicarbonate, and then exhaustively dialyzed against distilled water. The retentate was lyophilized and the calcium content was determined to be 0.3% and the phosphorus content to be 0.03%. Upon electrophoretic examination, a large diffuse anionic component and a discrete band with a

mobility approximately that of the phosphoprotein found in dentine were revealed. See Table 9. The gels were analyzed for phosphorus and found to be free of this element. Since the sample size was small and the consequent component concentrations were low, a control experiment was set up with a comparable weight of decalcified Tris-NaCl extracted dentine. The periodate extract was analyzed just as that of the cementum, and then lyophilized. The dried residue was then subjected to polyacrylamide gel electrophoresis, and the phosphoprotein fraction was analyzed for phosphorus. The presence of phosphorus was clearly established in this fraction.

Amino acid analysis of the decalcified Tris-NaCl extracted cementum matrix revealed its composition to be characteristic of collagen (Table 13). The arginine (57.6 residues/1000 amino acids) and the proline (156 residues/1000) are higher than in bone and dentine.

Table 11 shows the contents of reduced aldimine cross-links and cross-link precursors, jointly with similar results for dentine matrix collagen. The cross-link, hydroxylysinohydroxynorleucine, was present in the concentration of 2.6×10^{-1} lysine derived residues/1000 whereas hydroxylysinonorleucine was detected to be 5.2×10^{-2}. Thus, the major cross-link of cementum is hydroxylysinohydroxynorleucine. The cross-link precursor, dihydroxynorleucine, was found to occur in concentrations of 3.2×10^{-2} lysine derived residues/1000 and hydroxynorleucine content was 1.1×10^{-2}.

The neutral sugar results are given in Table 12. The G-25 peak 2 result for cementum collagen was 1.5g hexose/14g hydroxyproline of which 62% was galactose and 38% glucose. The hexose of the non-collagen glycoprotein associated with the cementum collagen (G-25 Peak 1)

corresponded to 0.9μ hexose/14μ hydroxyproline. This hexose was composed of 40% galactose, 26% mannose, 7% fucose, 16% glucose, and 11% of an unknown.

DISCUSSION

Few chemical analysis have been carried out on predentine. Most studies have been histological in nature apart from the recent studies on glycosaminoglycans. Cementum has not been studied chemically in great detail either. Selvig[245] has studied its genesis and fine structure histologically as well as with the electron microscope and determined its mineral content. Glimcher[234] has reported the amino acid composition of the less typical coronal cementum whereas Birkedal-Hansen et al[246] have reported the amino acid composition of bovine cementum. As a result further chemical analysis were carried out on these tissues in order to acquire more information about their nature. These included four major analyses: reducible cross-link, amino acid and neutral sugar determinations and an investigation of the occurrence of phosphoprotein in predentine and cementum.

I. PHOSPHOPROTEIN

Predentine: The inorganic crystals of teeth closely resemble hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. From its molecular weight of 1000, the calcium content of hydroxyapatite is calculated to be 40%. The calcium content of predentine (0.4%) therefore corresponds to 1% hydroxyapatite. Based on this figure of 1% hydroxyapatite one can now estimate the phosphorus content of predentine to be 0.18%. However, upon analysis, predentine was found to contain 0.37% phosphorus. This additional phosphorus may be organically bound and/or inorganic.

Veis and coworkers[185,187,189] have isolated, from decalcified bone and dentine matrix, a highly anionic phosphoprotein which is covalently linked to the collagen. They suggested it may provide the

sites for the epitactic nucleation of mineralization for the matrix. This phosphoprotein was also found in Tris-NaCl extracted decalcified dentine matrix[191]. Thus it was logical first to extract this soluble phosphoprotein from predentine. However, once the predentine had been extracted with EDTA and Tris-NaCl, phosphorus could no longer be detected in the residue. This would tend to indicate that the collagen bound phosphoprotein was absent in predentine but the possibility that phosphorus was present below the limits of detection (0.3 μg phosphorus/aliquot analyzed) could not be excluded. Thus the insoluble residue was examined for the presence of small quantities of phosphoprotein. Oxidative degradation, carried out on a sample of predentine, led to the solubilization of a fraction containing a low concentration of phosphorus (0.02%). This fraction, upon electrophoretic examination, was shown to be composed of several anionic components, one of which displayed an electrophoretic mobility closely similar to that of dentine phosphoprotein. No phosphorus could be detected in this band. However, calculation showed that even if all the phosphorus known to be present in the fraction obtained after periodate treatment was in one electrophoretic band, the concentration in the final analytical solution was below the limit of detection (0.3 μg phosphorus/aliquot analyzed). Therefore, there was no proof that the phosphoprotein was absent. However, the predentine component isolated by electrophoresis could not be the same as the dentine phosphoprotein as the absence or virtual absence of phosphate from the protein would result in it having a significantly different mobility. Hence, it is probable the two components differ in primary composition, but this could only be determined by an amino acid determination and there was insufficient sample for this.

The above results suggest the absence of a bound phosphoprotein in predentine and thus support Weinstock and Leblond's[193] radioautographic study on the deposition of a phosphoprotein in dentine. These researchers, using ^{33}P and ^{3}H proline labels, were able to demonstrate that a phosphoprotein, secreted by the odontoblasts, traverses the predentine to the mineralization front where it is deposited in the dentine.

EDTA extracts[192] as well as Tris-NaCl extracts[191] have been found to contain soluble phosphoprotein. Both the EDTA extract and the Tris-NaCl extract of predentine contained non-diffusible fractions containing phosphorus. However, the EDTA extract and the Tris-NaCl extract of dentine contain much more phosphorus than those of predentine. The Tris-NaCl soluble non-dialysable fractions of both dentine and predentine matrices form about 5% of the organic matrix[191]. Hence, if a soluble phosphoprotein is present in predentine, it is present in a much lower concentration than in dentine. One should expect to find a very small transitory fraction.

Cementum: The values obtained in this study for the calcium (26.3%) and phosphorus (11.4%) contents of bovine cementum are in agreement with the results given below for human tissue. The calcium and phosphorus contents of human cementum range from 25.7 to 26.6% and 11.8 to 12.5% dry weight respectively[232]. The organic content of bovine cementum was found to be 34%.

Phosphoprotein is thought to be the site of nucleation for mineralization[185]. The content of this component found in cementum closely resembles that of predentine. The Tris-NaCl extraction solubilized about 5% of the decalcified cementum matrix. The non-diffusible components of this extract contained 1.76% phosphorus.

When the decalcified Tris-NaCl extracted cementum matrix was analysed for phosphorus and calcium, neither of these two elements were detected. Oxidative degradation solubilized a non-diffusible material which contained 0.03% phosphorus and 0.3% calcium. Electrophoretic examination of this material revealed a large diffuse anionic component and a discrete band which had a mobility similar to that of dentine phosphoprotein. However, no phosphorus could be detected in the gel although phosphorus was clearly discernible in a control experiment utilizing an equal weight of dentine. As in the case of the predentine, the level of phosphorus in the fraction obtained after periodate treatment was so low that when the resulting electrophoretic band was analyzed, the concentration of phosphorus would have been below the limits of detection. Therefore, the complete absence of the phosphoprotein was not proved.

The lack of phosphoprotein in cementum is very surprising. Veis and coworkers[140,185,187,189] isolated the phosphoprotein in the mineralized tissues, bone and dentine[184], but were unable to detect it in skin. As a result they hypothesized that it may play a role in mineralization. As this fraction is imperceptible in cementum, the function of phosphoprotein in calcified tissue matrices must be reviewed.

II. REDUCIBLE CROSS-LINKS:

Because of the fundamental role of cross-links in preserving tissue integrity, it was important to establish the cross-link pattern in these tissues. It is important to remember that with the techniques presently available, only reducible cross-links can be investigated. Perhaps other cross-links, which are not reducible, are present in collagen in addition to the reducible ones.

Predentine: The most abundant reducible intermolecular cross-link in mineralized[117,120] and embryonic tissues[247] has been reported to be dehydrodihydroxylysinonorleucine. During aging this cross-link is replaced by other polyfunctional cross-links[247,128]. It is therefore not surprising that the major reducible cross-link of predentine (4.8×10^{-1} lysine derived residues/1000 amino acid residues) and dentine (5.8×10^{-1} residues/1000) from unerupted teeth is dehydrodihydroxylysinonorleucine, detected as the reduced aldimine. There is a slight increase of the cross-link in dentine which may be accounted for by taking into consideration the age of the tissue. The source of the dentine was unerupted teeth, that is, a young organ in which growth and cross-link formation is at a peak. Davis[248] found dehydrodihydroxylysinonorleucine to be reduced to 1.6×10^{-1} residues/1000 in erupted bovine molar dentine collagen. As predentine matures, the cross-links peak in unerupted dentine and subsequently drop significantly in the erupted dentine.

Dehydrohydroxylysinonorleucine also decreases in concentration with age after having peaked during the period of maximum rate of growth[128]. Comparing the results obtained for predentine and dentine, one observes a considerable drop in the concentration of this reducible cross-link in dentine. Hence, the decrease of dehydrohydroxylysinonorleucine occurs much more rapidly than is the case for dehydrodihydroxylysinonorleucine.

Bailey[127] proposed that the reducible cross-links were intermediates which are transformed into more stable non-reducible cross-links. Davis and coworkers[129,249] have recently shown that lysine and/or hydroxylysine residues can be added to the electrophilic double bond of the reducible cross-links, transforming these into stable non-reducible

cross-links which in turn can join more than two collagen molecules.

Thus the above observations that dehydrodihydroxylysinonorleucine and dehydrohydroxylysinonorleucine decrease, that is, are transformed with age in predentine, are in accord with the current theories on the fate of collagen cross-links in maturing tissues.

Although less marked, the cross-link precursors show a similar relationship to development as the cross-links. Dihydroxynorleucine is slightly increased in the dentine matrix collagen while hydroxynorleucine is decreased, because there is more hydroxylysine in regions susceptible to lysine oxidase.

The equilibrium between reducible cross-links and their precursors is much more favourable in the case of hydroxyallysine derived reducible cross-links than allysine derived reducible cross-links. This is a result of isomerization of the hydroxyallysine derived cross-links to the more stable α-keto-amine cross-links. Therefore, one equivalent of hydroxyallysine will be in equilibrium with more of its derived cross-links than will one equivalent of allysine.

Cementum: As in other calcified tissue collagens, as well as predentine, the major reducible cross-link of cementum is dehydrodihydroxylysinonorleucine. This reducible cross-link decreases in concentration with maturation of the tissue probably brought about by its modification to a more stable non-reducible cross-link. Therefore, it is to be expected that this cross-link is considerably lower in concentration in cementum matrix than in dentine matrix as the cementum was collected from erupted teeth and the dentine from younger unerupted teeth. On the other hand, the minor cross-link of cementum, dehydrohydroxylysinonorleucine, occurs in greater concentration than in dentine.

Cementum is the calcified tissue which most resembles alveolar bone in its composition, structure and behavior. Comparison of the cementum cross-link results with those obtained by Davis[248] on bone (Table 14) shows the cross-link content of the two matrices to be very similar. In this comparison, it will also be noted that less calcified tissues, cementum included, seem to contain more dehydrohydroxylysinonorleucine than the more calcified tissues.

The cross-link precursor results are similar to those of dentine, that is, the dihydroxynorleucine in cementum is more plentiful than hydroxynorleucine. Mechanic[120] found this to be also the case in bone. As discussed in section on predentine cross-links, this indicates that there is more hydroxylysine in regions susceptible to lysine oxidase.

The implications of the cementum cross-link and precursor results are the same as that for the predentine results, that is, the equilibrium between reducible cross-links and their precursors is more favorable in the case of hydroxyallysine derived reducible cross-links than allysine derived reducible cross-links.

III. AMINO ACID COMPOSITION

Predentine: The EDTA-Tris-NaCl extracted predentine matrix proved to have an amino acid composition generally similar to that of dentine matrix collagen. Comparison of these results reveal that the lysine (23 residues/1000) and the hydroxylysine (12.4 residues/1000) of predentine are higher than the lysine (18.9 residues/1000) and hydroxylysine (9.2 residues/1000) of dentine. The sum of hydroxylysine and lysine (35.4 residues/1000) is slightly higher than the normal range for tissue collagens (29-33 residues/1000) and significantly higher than the sum in den-

tine (28.1 residues/1000). The hydroxylysine content of collagen is known to vary widely from tissue to tissue and with the age of the animal in the same tissue[250,142] even though the total of lysine and hydroxylysine remains constant. A progressive loss of hydroxylysine and an equivalent increase in lysine content has been observed in maturing rat and chick skin[250] as well as in mineralizing turkey tendon collagen[251], that is, the sum of these two amino acids remained constant. This constancy was not observed in the transformation of predentine to dentine. It must be remembered that only one amino acid analysis was performed on the predentine matrix and that the above observation may be due to experimental error. However, if the experimental errors are not significant, this finding may be significant and may indicate that maturation is not the only process occurring in the transformation of predentine to dentine.

Cartilage collagens[253], are known to contain higher levels of hydroxylysine than the Type I collagen of skin, tendon and bone. This is due to the genetically different types of collagen known to occur in this type of tissue. The amino acid composition of these different types of collagen are being determined. Type III collagen which was first isolated from fetal skin[4] has been recently isolated from dentine[228] and gingival tissues[254]. Its amino acid composition does not exhibit higher levels of hydroxylysine than the Type I (skin, bone, tendon) and as a result it is difficult to compare predentine with Type III collagen[253,255,256]. See Table 15. However, Type II (cartilage) does have an increased hydroxylysine content[257-260]. Recently Linsenmayer[260] compared the α1(II) and α1(I) chains of embryonic chick cartilage. The sum of hydroxylysine and lysine of the α1(II) chain (36 residues/1000) was

higher than that of the α1(I) (32.2 residues/1000). There was also considerably more hydroxylysine in the Type II collagen (19 residues/1000) than in the Type I collagen (7.2 residues/1000). In both predentine and Type II collagen (see Table 15), the alanine content is low while the tyrosine is high. Thus from the point of view of amino acid composition, predentine approaches the Type II values more closely than the Type I. Hence, the transformation of predentine to dentine may be analogous to bone formation, that is, Type II collagen is replaced by Type I collagen.

The proline and hydroxyproline results are more difficult to account for. The sum of proline (174 residues/1000) and hydroxyproline (90 residues/1000) in predentine is 264 residues/1000, while in dentine it is 216.5 residues/1000. The normal range is from 213 to 218 residues/1000. Bornstein[261,262] had observed incomplete hydroxylation of individual prolyl residues in early sequence studies of rat skin and tail tendon collagen. He hypothesized that age as well as dietary factors may have a possible effect on hydroxylation of proline. Juva and Prockop[69] have shown that the reduced affinity of prolyl hydroxylase for partially hydroxylated chains accounts for the phenomenon of incomplete hydroxylation of individual prolyl residues observed by Bornstein. Berg and Prockop[86] as well as Murphy and Rosenbloom[85] have reached the conclusion that the triple helical conformation prevents its further hydroxylation. Several laboratories[142,263] have reported that there is no change in the overall extent of proline hydroxylation with age. A comparison of the sum of hydroxyproline and proline contents of the α1(I), α1(II) and α1(III) shows no significant differences. See Table 15. The glycine and hydroxyproline contents of predentine proved to be low

whereas the proline content was high. As animal non-collagenous proteins contain no hydroxyproline and are relatively low in glycine but fairly rich in proline[89,264,265], the above discrepancies in the analysis of predentine collagen are understandable. The reduced glycine and hydroxyproline content and the elevated proline value are probably due to the predentine collagen being contaminated with non-collagenous protein. The neutral sugar results suggest that the contaminant is glycoprotein.

Cementum: The amino acid composition of the EDTA - Tris-NaCl extracted cementum matrix is in general agreement with that of other calcified tissues. The hydroxylysine content of cementum (10.6 residues/1000) was similar to that of dentine (9.2 residues/1000) but much higher than that of bone (6.4 residues/1000). See Table 13. This trend is also observed in the less typical coronal cementum[234] as well as in the bovine cementum Birkedal-Hansen et al[246] analyzed. See Table 16. The per cent hydroxylation of lysine in cementum collagen (35%), dentine (32%) and bone (19.5%) also showed the same pattern. This difference between the dental tissues and bone may prove to be significant. The hydroxylysine content of cementum collagen is very similar to that obtained from periodonal ligament collagen from which the Sharpey's fibres originate[269]. The sum of the lysine and hydroxylysine in cementum (30.6 residues/1000) was intmediate between that of bone (32.8 residues/1000) and dentine (28.1 residues/1000); the normal range for mammalian tissues being between 29 and 33 residues/1000.

From the above experimental data, cementum collagen appears to resemble dentine collagen rather than that of bone. This is surprising as cementum has always been thought to resemble bone more than any other tissue. These results are difficult to explain in terms of the genetic

types of collagen as bone and dentine have the same genetic type, the $\alpha 1(I)_2 \alpha 2$. Very recently Butler et al[252] have shown that bovine cementum collagen is predominantly Type I, with less than 5% of Type III present. The effect of the latter on the average amino acid composition as obtained in the present work would be negligible. However, the difference observed between cementum and bone may be due to the presence of some Type II collagen in cementum as this type of collagen has elevated levels of hydroxylysine[257-260]. There are no reports of Type II collagen occurring in bone.

Cementum proved to have a low hydroxyproline content and a high proline content as compared to bone and dentine. The sum of proline (156 residues/1000) and hydroxyproline (81.2 residues/1000) was 237.2 residues/1000 which is higher than the normal range of 211 to 218 residues/1000. The glycine content was somewhat low indicating the cementum collagen was not pure. This is also reflected in the low hydroxyproline and high proline content. One contaminant was an insoluble non-collagenous glycoprotein associated with the collagen.

The differences in amino acid composition observed between cementum, bone and dentine may partly be due to the different purification methods utilized, such as different periods of extraction in EDTA and whether or not the tissue was extracted with Tris-NaCl. It may also be that the same purification methods were more effective on one tissue than another, but if so, this may indicate some fundamental differences such as non-collagenous components more closely associated with one collagen than with another.

Some reservations should be made about comparing average amino acid values obtained here for predentine, dentine and cementum with values

for individual purified α 1 chains of Types I, II and III. For example when looking at the individual α 1 chains of the Type I collagen, the α 2 is not taken into consideration. Also the fact that these chains are highly purified does not show the repercussions of even a very small amount of contaminant on the average amino acid composition.

IV. NEUTRAL SUGARS

Predentine: The separation of collagen and non-collagen glycopeptides released from insoluble tissue fractions by papain is similar to that obtained when collagenase is employed followed by trypsin or pronase[207, 266]

Only one analysis for neutral sugars was carried out on the separated glycopeptides and hexosamines and other sugars were not determined due to the small amount of predentine available. Accordingly, the quantitation of collagen hexoses (peak 2) requires confirmation. Although the modified manual orcinol method yielded a lower result than the Technicon carbohydrate method, both values were still much higher than has been found for skin[148,243] and dentine[185] collagens, which contain less than 1% hexose. Predentine collagen seems to resemble cartilagenous tissue collagens[148,243] in its apparent high hexose content. It has yet to be proven that the sugar is linked to the hydroxylysine because there was not enough of the G-25 peak 2, the collagen glycopeptide containing peak, to determine the glycosylated amino acids by the methods of Spiro[194,270].

The galactose content corresponds to 13.5 residues of galactose/1000 amino acids. This indicates, with an 8% discrepancy, complete glycosylation of the hydroxylysine.

The calculation for predentine is as follows:

let y = number of grams of galactose/14 grams of hydroxyproline (4.9g neutral sugar/14g hydroxyproline of which 59% is galactose = 2.9g galactose/14g hydroxyproline)

131 = molecular weight of hydroxyproline (M.W. hypro)

180 = molecular weight of galactose (M.W. gal.)

90 = number of residues hydroxyproline/1000 amino acid residues determined by amino acid analysis (res. hypro/1000 res.)

residues of galactose/1000 amino acids residues

$$= \frac{y}{14} \times \text{M.W. hypro} \times \text{res hypro/1000 res.} \times \frac{1}{\text{M.W. gal.}}$$

$$= \frac{y \times \text{M.W. hypro} \times \text{res hypro/1000 res.}}{14 \times \text{M.W. gal.}}$$

$$= \frac{2.9 \times 131 \times 90}{14 \times 180}$$

= 13.5 moles galactose/1000 amino acid residues

The excess of galactose over glucose indicates that only a part of the hexose is in the disaccharide form. Presumably galactosylhydroxylysine is also present as in other collagens[194-196] such as dentine[228] and skin collagen[4,228].

Peak 1 was found to contain significant amounts of mannose, fucose and galactose. These hexoses are the major sugar components of non-collagenous glycoproteins[89,243,264,265,267]. This strongly suggests that, as in other connective tissues, glycoproteins were important components of the non-collagenous material associated with the insoluble collagen. Glucose, which is frequently found in tissue glycoproteins[243,265,266,267] but not in serum glycoproteins occurred in relatively high concentration. The significance of insoluble glycoproteins has yet to be elucidated. Their wide distribution in connective tissues, some-

times in quite large amounts[243], has become increasingly evident in recent years.

Perhaps the limited PAS staining of predentine, mentioned earlier, may be due to the insoluble glycoprotein. Further work is required on the proteoglycans and soluble glycoproteins of predentine.

Cementum: The soluble glycoproteins and proteoglycans of cementum were not studied in the present work as the sample was small and the EDTA and Tris-NaCl extracts were utilized for the calcium and phosphorus determinations. As a result we concentrated on the insoluble glycoprotein fraction. This non-collagenous fraction appeared in peak 1 of the Sephadex gel chromatography of the cementum papain digest. It was found to contain glucose and generally resemble glycoproteins from other connective tissues in containing significant amounts of galactose, mannose and fucose[243,264,265,268]. This fraction is probably heterogenous like the similar fraction of other connective tissues[243,264,265,268]. Its composition may vary with age and from tissue to tissue[243].

As opposed to skin collagen which contains about 0.5g hexose/14g hydroxyproline[148,243], (0.46g hexose/14g hydroxyproline in our lab - unpublished results) cementum collagen was found by a similar technique to contain 1.5g hexose/14g hydroxyproline, of which 62% was galactose. This corresponds to 3.9 residues of galactose/1000 amino residues. See calculation for predentine on p. 76. Comparing this value with the number of hydroxylysine residues (10.6/1000) indicates 36.8% glycosylation, of which only a part is the disaccharide. However, as with predentine, there was insufficient tissue for proof to be obtained that the hexoses were linked to hydroxylysine.

From the above hexose results on predentine and cementum a

tentative prediction can now be made concerning collagen types. Trelstad et al[148] have found that there is a relatively high amount of carbohydrate, 5.5% by weight, covalently bound to the cartilage Type II collagen as compared to the 0.5% hexose in skin Type I collagen. We, therefore, have indirect evidence that predentine, by its carbohydrate content, may be of the cartilage Type II collagen. The rather high per cent of hydroxylated lysine residues is consistent with the increased sugar content. However, the increase in sugar content (4.9g hexose/14g hydroxyproline in predentine as compared to 0.46g/14g hydroxyproline in skin) was much larger than the increase in hydroxylated lysine (12.4 residues/1000 amino acid residues in predentine as compared to an average value of 6.8 residues/1000 for Type I collagens).

Cementum has a sugar content of 1.5% which is intermediate between dentine and skin, and cartilage. This may be an indication that cementum collagen, which contains two types of fibers, the Sharpey's fibers and the intrinsic, is a mixture of two types of collagen, that of skin and dentine: the $\alpha 1(I)_2\alpha 2$ and that of cartilage: the $\alpha 1(II)_3$, although the hydroxylysine results show no indication of this. The hydroxylysine content is not significantly higher than that of the periodontal ligament[269]. See Table 16.

The hexose content of the collagen of dental tissues is of interest as it is thought to have a relationship with the morphology of the collagen fibers[203,207,243] (see section on Collagen: a glycoprotein, p. 40) as well as with calcification.

It is thought that the seeding site for apatite crystals occurs in the hole region of the collagen fibrils (see sections on Collagen biosynthesis, p. 14 and Mineralization, p. 33), and that modification of the

ϵ-amino groups of lysine and hydroxylysine in this vicinity inhibits nucleation[154]. Since the glucose-galactose moiety attached to the hydroxylysine is thought to project in the hole region[201], calcification may be hindered by steric interference. Transformation of a non-calcified tissue to a calcified one, such as predentine to dentine, might then involve an enzymic modification of these strategically located residues, although there is no evidence for such a change in fully formed collagen fibers. There are other possibilities. The transformation of predentine may involve the resorption of collagen which is Type I, but nevertheless has a high hexose content, with a concurrent replacement with Type I collagen having a low hexose content. On the other hand, the transformation may involve a Type II collagen replacement with Type I. There is no evidence for the above statements, however. Collagen is not thought to be resorbed in the predentine to dentine transformation.

In the section on the role of glycosaminoglycans in mineralization (p. 28), it was suggested that acid mucopolysaccharides can either initiate or inhibit calcification[153,271]. Bowness[272], on the other hand, suggested that they could do both if a two compartment system was considered; one compartment being fibrous and concerned with nucleation and the other being the true ground substance and concerned with the inhibition of calcification. The two compartment theory may also be applicable to glycoproteins. However, as opposed to Bowness's theory, the insoluble glycoprotein, which may be presumed to occur in the fibrous compartment, may act as an inhibitor of calcification. These glycoproteins have high contents of glutamic and aspartic acid which are capable of binding calcium. One would therefore expect that it would be necessary for the insoluble glycoprotein to be removed before

calcification can occur. It is interesting to note that, using similar methods, the non-collagenous neutral sugar content of dentine (Pearson unpublished results) was determined to be one-tenth that of predentine.

V. SUMMARY:

Predentine was found to be virtually phosphoprotein free. As the basic premise was that phosphoprotein provided a nidus for calcification and therefore should not occur in uncalcified tissues and if so, only in very small quantities as in a transitory state, this result was expected. The lack of this component in cementum, however, did come as a surprise and now the role of phosphoprotein in calcified tissues must be reconsidered.

The major cross-links of the calcified tissues bone and dentine are hydroxylysinohydroxynorleucine and hydroxylysinonorleucine. These were found to be the major cross-links in predentine and cementum also. Of the two cross-links, hydroxylysinohydroxynorleucine was the predominant one in both tissues. The cross-link precursors dihydroxynorleucine and hydroxynorleucine showed the same trend as that of the cross-links.

The amino acid compositions of predentine and cementum generally resembled that of other collagens. There is some indication that predentine may possess Type II collagen, this being also reflected in its apparently high collagen hexose content. Cementum is probably mostly Type I collagen judging by its amino acid composition. However, its collagen hexose content indicates a possible mixture of Type I and II collagen. The non-collagenous neutral sugar results indicate that the predentine and cementum collagens are associated with an insoluble glycoprotein as in other connective tissues.

VI. SUGGESTIONS FOR FURTHER WORK

From the discussion above it is obvious that there were many quantitation problems as the amount of sample for each analysis was very limited especially in the case of predentine.

Engfeldt and Hjerpe[224] chose to use dentine and predentine from rachitic beagle puppies for studying the glycosaminoglycans of these tissues, as this provides a large predentine zone and therefore high yield of predentine. This method of obtaining predentine was not satisfactory in this project as it is not known what effects richets may have on the collagen or on the predentine tissue as a whole. Linde[226] chose, on the other hand, to obtain the odontoblast-predentine layer by scraping the pulp cavity of unerupted porcine teeth. This method was not chosen either as it invites contamination of the predentine sample with odontoblasts as well as with dentine. Perhaps in future, whole teeth could be decalcified, although the decalcification process would take much longer than the usual way where the teeth are broken into splinters. The decalcified tooth could then be sectioned longitudinally and the predentine dissected from both the apex of the tooth and the pulp cavity. This method was recently used very efficaciously on erupted bovine teeth by Birkedal-Hansen et al[246,252] to obtain cementum and thus could also be used in future to collect predentine as well.

Having thus obtained sufficient sample of both predentine and cementum, the phosphoprotein determinations should be repeated with enough sample such that the EDTA and Tris-NaCl soluble phosphoprotein (if any) as well as the periodate soluble phosphoprotein (if any) can be isolated and their amino acid and hexose composition characterized. The amino acid composition, cross-link and cross-link precursor content of

both the predentine and cementum matrix should be verified as well as their neutral sugar composition. The glycosylated amino acids could then be identified and quantitated by ion exchange column chromatography [194,270]. The non-collagenous contaminants referred to earlier in the neutral sugar section (p. 76) could be isolated by first digesting the insoluble residue with collagenase so as to rid the sample of collagen. The remaining residue could be treated with reducing agents so as to solubilize the insoluble proteoglycans and glycoproteins thus enabling their separation characterization by usual methods[223-226,243,264,265]. The soluble proteoglycans and glycoproteins of both tissues could also be investigated. It would also be very interesting to see a collagen type determination on predentine.

PUBLICATIONS

The following publications will appear based on the present work: -

1. Carmichael, D.J., Chovelon, A. and Pearson, C.H.: The composition of the insoluble collagenous matrix of bovine predentine. Calc. Tiss. Res.: in press, 1975.

2. Chovelon, A., Carmichael, D.J. and Pearson, C.H.: The composition of the organic matrix of bovine cementum. Arch. Oral Biol.: in press, 1975.

TABLE 1

AMINO ACID COMPOSITION OF SEVERAL MAMMALIAN TISSUE COLLAGENS[1]

	Tendon	Skin	Uterus	Renal Reticulum	Bone
Alanine	110.7	114.5	95.9	96.5	113.5
Glycine	324	324.4	337.1	309	319
Valine	25.4	24.5	21.6	26.8	23.6
Leucine	26.0	24.8	24.5	35.8	25.5
Isoleucine	11.1	10.4	10.8	18.0	13.3
Proline	126.4	125.1	108.2	97.2	123.4
Phenylalanine	14.2	12.6	12.3	18.1	13.9
Tyrosine	3.6	3.5	2.2	3.0	4.5
Serine	36.9	36.9	41.9	42.8	35.9
Threonine	18.5	18.3	20.6	21.9	18.4
Cystine	--	trace	trace	--	--
Methionine	5.7	7.0	5.8	8.6	5.3
Arginine	49.0	49.0	49.0	45.3	47.1
Histidine	5.4	5.4	5.4	5.3	5.8
Lysine	21.6	26.6	24.9	21.6	28.0
Aspartic acid	48.4	47.2	52.5	52.9	47.0
Glutamic acid	72.3	77.7	73.6	76.7	72.2
Hydroxyproline	92.1	90.9	108.6	107.7	100.2
Hydroxylysine	8.9	5.9	5.1	12.2	3.5

Values expressed in amino acid residues per 1000 total amino acid residues.

1. Values obtained from Eastoe, J.E.[6]

TABLE 2

THE AMINO ACID SEQUENCE OF THE α1 CHAIN[1]

pGlu-Met-Ser-Tyr-Gly-Tyr-Asp-Glu-Lys-Ser-Ala-Gly-Val-Ser-Val-Pro-

1	Gly-Pro-Met-Gly-Pro-Ser-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Pro-Gln-Gly-Phe-Gln-Gly-Pro-Hyp-
28	Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser-Gly-Pro-Met-Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Lys-Asn-Gly-Asp-Asp-
55	Gly-Glu-Ala-Gly-Lys-Pro-Gly-Arg-Hyp-Gly-Gln-Arg-Gly-Pro-Hyp-Gly-Pro-Gln-Gly-Ala-Arg-Gly-Leu-Hyp-Gly-Thr-Ala-
82	Gly-Leu-Hyp-Gly-Met-Hyl-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu-Asp-Gly-Ala-Lys-Gly-Asn-Thr-Gly-Pro-Ala-Gly-Pro-Lys-
109	Gly-Glu-Hyp-Gly-Ser-Hyp-Gly-Glx-Asx-Gly-Ala-Hyp-Gly-Gln-Met-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Glu-Arg-Gly-Arg-Hyp-
136	Gly-Pro-Hyp-Gly-Ser-Ala-Gly-Ala-Arg-Gly-Asp-Asp-Gly-Ala-Val-Gly-Ala-Ala-Gly-Pro-Hyp-Gly-Pro-Thr-Gly-Pro-Thr-
163	Gly-Pro-Hyp-Gly-Phe-Hyp-Gly-Ala-Ala-Gly-Ala-Lys-Gly-Glu-Ala-Gly-Pro-Gln-Gly-Ala-Arg-Gly-Ser-Glu-Gly-Pro-Gln-
190	Gly-Val-Arg-Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Ala-Ala-Gly-Pro-Ala-Gly-Asn-Hyp-Gly-Ala-Asp-Gly-Gln-Hyp-
217	Gly-Ala-Lys-Gly-Ala-Asn-Gly-Ala-Hyp-Gly-Ile-Ala-Gly-Ala-Hyp-Gly-Phe-Hyp-Gly-Ala-Arg-Gly-Pro-Ser-Gly-Pro-Gln-
244	Gly-Pro-Ser-Gly-Ala-Hyp-Gly-Pro-Lys-Gly-Asn-Ser-Gly-Glu-Hyp-Gly-Ala-Hyp-Gly-Asn-Lys-Gly-Asp-Thr-Gly-Ala-Lys-
271	Gly-Glu-Hyp-Gly-Pro-Ala-Gly-Val-Gln-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Glu-Glu-Gly-Lys-Arg-Gly-Ala-Arg-Gly-Glu-Hyp-
298	Gly-Pro-Ser-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Glu-Arg-Gly-Gly-Hyp-Gly-Ser-Arg-Gly-Phe-Hyp-Gly-Ala-Asp-Gly-Val-Ala-
325	Gly-Pro-Lys-Gly-Pro-Ala-Gly-Glu-Arg-Gly-Ser-Hyp-Gly-Pro-Ala-Gly-Pro-Lys-Gly-Ser-Hyp-Gly-Glu-Ala-Gly-Arg-Hyp-
352	Gly-Glu-Ala-Gly-Leu-Hyp-Gly-Ala-Lys-Gly-Leu-Thr-Gly-Ser-Hyp-Gly-Ser-Hyp-Gly-Pro-Asp-Gly-Lys-Thr-Gly-Pro-Hyp-
379	Gly-Pro-Ala-Gly-Gln-Asp-Gly-Arg-Hyp-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Ala-Arg-Gly-Gln-Ala-Gly-Val-Met-Gly-Phe-Hyp-
406	Gly-Pro-Lys-Gly-Ala-Ala-Gly-Glu-Hyp-Gly-Lys-Ala-Gly-Glu-Arg-Gly-Val-Hyp-Gly-Pro-Hyp-Gly-Ala-Val-Gly-Pro-Ala-
433	Gly-Lys-Asp-Gly-Glu-Ala-Gly-Ala-Gln-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Pro-Ala-Gly-Glu-Arg-Gly-Glu-Gln-Gly-Pro-Ala-
460	Gly-Ser-Hyp-Gly-Phe-Gln-Gly-Leu-Hyp-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Glu-Ala-Gly-Lys-Hyp-Gly-Glu-Gln-Gly-Val-Hyp-
487	Gly-Asp-Leu-Gly-Ala-Hyp-Gly-Pro-Ser-Gly-Ala-Arg-Gly-Glu-Arg-Gly-Phe-Hyp-Gly-Glu-Arg-Gly-Val-Glu-Gly-Pro-Hyp-
514	Gly-Pro-Ala-Gly-Pro-Arg-Gly-Ala-Asn-Gly-Ala-Hyp-Gly-Asn-Asp-Gly-Ala-Lys-Gly-Asp-Ala-Gly-Ala-Hyp-Gly-Ala-Hyp-
541	Gly-Ser-Gln-Gly-Ala-Hyp-Gly-Leu-Gln-Gly-Met-Hyp-Gly-Glu-Arg-Gly-Ala-Ala-Gly-Leu-Hyp-Gly-Pro-Lys-Gly-Asp-Arg-
568	Gly-Asp-Ala-Gly-Pro-Lys-Gly-Ala-Asp-Gly-Ala-Pro-Gly-Lys-Asp-Gly-Val-Arg-Gly-Leu-Thr-Gly-Pro-Ile-Gly-Pro-Hyp-
595	Gly-Pro-Ala-Gly-Ala-Hyp-Gly-Asp-Lys-Gly-Glu-Ala-Gly-Pro-Ser-Gly-Pro-Ala-Gly-Thr-Arg-Gly-Ala-Hyp-Gly-Asp-Arg-
622	Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Phe-Ala-Gly-Pro-Hyp-Gly-Ala-Asp-Gly-Gln-Hyp-Gly-Ala-Lys-Gly-Glu-Hyp-
649	Gly-Asp-Ala-Gly-Ala-Lys-Gly-Asp-Ala-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Pro-Ile-Gly-Asn-Val-
676	Gly-Ala-Hyp-Gly-Pro-Hyl-Gly-Ala-Arg-Gly-Ser-Ala-Gly-Pro-Hyp-Gly-Ala-Thr-Gly-Phe-Hyp-Gly-Ala-Ala-Gly-Arg-Val-
703	Gly-Pro-Hyp-Gly-Pro-Ser-Gly-Asn-Ala-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Lys-Glu-Gly-Ser-Lys-Gly-Pro-Arg-
730	Gly-Glu-Thr-Gly-Pro-Ala-Gly-Arg-Hyp-Gly-Glu-Val-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Glu-Lys-Gly-Ala-Hyp-
757	Gly-Ala-Asp-Gly-Pro-Ala-Gly-Ala-Hyp-Gly-Thr-Pro-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp-
784	Gly-Gln-Arg-Gly-Glu-Arg-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-Pro-Ser-Gly-Glu-Hyp-Gly-Lys-Gln-Gly-Pro-Ser-Gly-Ala-Ser-
811	Gly-Glu-Arg-Gly-Pro-Hyp-Gly-Pro-Met-Gly-Pro-Hyp-Gly-Leu-Ala-Gly-Pro-Hyp-Gly-Glu-Ser-Gly-Arg-Glu-Gly-Ala-Hyp-
838	Gly-Ala-Glu-Gly-Ser-Hyp-Gly-Arg-Asp-Gly-Ser-Hyp-Gly-Ala-Lys-Gly-Asp-Arg-Gly-Glu-Thr-Gly-Pro-Ala-Gly-Ala-Hyp-
865	Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Ala-Hyp-Gly-Pro-Val-Gly-Pro-Ala-Gly-Lys-Ser-Gly-Asp-Arg-Gly-Glu-Thr-Gly-Pro-Ala-
892	Gly-Pro-Ile-Gly-Pro-Val-Gly-Pro-Ala-Gly-Ala-Arg-Gly-Pro-Ala-Gly-Pro-Gln-Gly-Pro-Arg-Gly-Asx-Hyl-Gly-Glx-Thr-
919	Gly-Glx-Glx-Gly-Asx-Arg-Gly-Ile-Hyl-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu-Gln-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Ser-Hyp-
946	Gly-Glu-Gln-Gly-Pro-Ser-Gly-Ala-Ser-Gly-Pro-Ala-Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Ser-Ala-Gly-Ser-Hyp-Gly-Lys-Asp-
973	Gly-Leu-Asn-Gly-Leu-Hyp-Gly-Pro-Ile-Gly-Hyp-Hyp-Gly-Pro-Arg-Gly-Arg-Thr-Gly-Asp-Ala-Gly-Pro-Ala-Gly-Pro-Hyp-
1000	Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Pro-

Ser-Gly-Gly-Tyr-Asp-Leu-Ser-Phe-Leu-Pro-Gln-Pro-Pro-Gln-Gln-Glx-Lys-Ala-His-Asp-Gly-Gly-Arg-Tyr-Tyr

The amino acid sequence of the α1 chain from rat (residues 1-402) and calf (residues 403-1011) skin collagen. The N terminal (from rat) and the C-terminal (from calf) non helical regions are separated from the triplet region and are not numbered.

1. Table is taken from Hulmes et al[133]

TABLE 3

AMINO ACID SEQUENCE OF α2CNBr PEPTIDES SEQUENCED TO DATE FROM CHICK, RAT AND CALF SKIN COLLAGEN

α2CNBr peptides

α2-CB1[1]

Chick Pca-Tyr-Asp-Pro-Ser-Lys-Ala-Ala-Asp-Phe-Gly-Pro-Gly-Pro-Met

Rat Pca-Tyr-Ser-Asp-Lys-Gly-Val-Ser-Ala-Gly-Pro-Gly-Pro-Met

α2-CB0[2]

Rat Gly-Leu-Met

α2-CB4[2] 42 N-terminal amino acids of 330 residues

Rat Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Ala-Val-Gly-Ala-Hyp-Gly-Pro-Gln

Calf Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Ala-Ser-Gly-Ala-Hyp-Gly-Pro-Gln

Rat Gly-Phe-Gln-Gly-Pro-Ala-Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Gln-Hyp

Calf Gly-Phe-Gln-Gly-Pro-Hyp-Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Gln-Hyp

Rat Gly-Pro-Ala-Gly-Pro-Arg-Gly-Pro-Ala-Gly-Pro-Hyp

Calf Gly-Pro-Ala-Gly-Ala-Arg-Gly-Pro-Hyp-Gly-Pro-Hyp

α2-CB2[3]

Chick Gly-Pro-Ala-Gly-Asn-Arg-Gly-Ala-Ser-Gly-Pro-Ala-Gly-Val-Lys

Rat Gly-Pro-Hyp-Gly-Asn-Arg-Gly-Thr-Ser-Gly-Pro-Ala-Gly-Val-Arg

Chick Gly-Pro-Asn-Gly-Asp-Ala-Gly-Arg-Hyp-Gly-Glu-Hyp-Gly-Leu-Hse

Rat Gly,Pro,Asp,Gly,Asp,Ala,Gly,Arg,Hyp,Gly,Gln,Hyp,Gly,Leu,Hse

α2-CB3

α2-CB5[4] 45 N-terminal residues of 320 residues

Rat Thr-Gly-Phe-Hyp-Gly-Ala-Ala-Gly-Arg-Thr-Gly-[illegible]-Gly-Pro-
Ser-Gly-Ile-Thr-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-[illegible]-Ala-Gly-Lys-
Glu-Gly-Ile-Lys-Gly-Pro-Arg-Gly-Asp-Gln-Gly-Pro-Val-Gly-Arg

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NAME OF AUTHOR / NOM DE L'AUTEUR Valerie M CHEVELDA

TITLE OF THESIS / TITRE DE LA THÈSE The Composition of the Collagenous Matrices of Bovine Periodontium and Dentition

UNIVERSITY / UNIVERSITÉ University of Alberta, Edmonton

DEGREE FOR WHICH THESIS WAS PRESENTED / GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE M Sc

YEAR THIS DEGREE CONFERRED / ANNÉE D'OBTENTION DE CE GRADE Fall 1975

NAME OF SUPERVISOR / NOM DU DIRECTEUR DE THÈSE Dr D J Carmichael

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THE UNIVERSITY OF ALBERTA

THE COMPOSITION OF THE COLLAGENOUS MATRICES OF BOVINE PREDENTINE AND CEMENTUM

by

© ANTOINETTE M. CHOVELON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

IN

ORAL BIOLOGY
(BIOCHEMISTRY)

FACULTY OF DENTISTRY

EDMONTON, ALBERTA

FALL, 1975

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read and recommended to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Composition of the Collagenous Matrices of Bovine Predentine and Cementum". Submitted by Antoinette M. Chovelon in partial fulfillment of the requirements for the degree of Master of Science.

.....

Supervisor

.....

.....

.....

Date .....

To my husband, Guy-Michel.

ABSTRACT

The existence of collagen in predentine and cementum can be regarded as firmly established. However as the amount of these tissues in teeth is very small, most studies have been histochemical in nature. The aim of this work was to extend the histochemical studies to a chemical basis.

Predentine was obtained from unerupted bovine teeth of animals one to two years old at slaughter. The translucent zone at the apex of the root was cut away and the predentine subsequently dissected from the dentine under a dissecting microscope. Cementum was collected from erupted bovine teeth. The roots of the teeth, from which the crowns had been sectioned off, were sliced longitudinally into thin lamellae of about 250 microns. Under the dissecting microscope, the cementum was carefully separated from the dentine.

Calcium and phosphorus determinations were carried out on both tissues. The EDTA decalcified insoluble collagenous matrices were then subjected to three major analyses: reducible cross-link, amino acid and neutral sugar determinations. An investigation was undertaken of the occurrence of phosphoprotein in these tissues.

Predentine:

Predentine was found to contain little calcium (.4%), an amount corresponding to 1% hydroxyapatite. The phosphorus content (.37%) was higher than could be accounted for as hydroxyapatite. The additional phosphorus was first thought to be part of the phosphoprotein Veis and coworkers[185,187,189] had isolated from decalcified bone and dentine and postulated to be the site for epitactic nucleation of mineralization for

the matrix. However upon analysis, predentine was found to be free of phosphoprotein, which is consistant with Weinstock and Leblond's[193] radioautographic study showing that a phosphoprotein traverses the predentine to the mineralization front where it is deposited in the dentine.

The reducible intermolecular cross-link content of predentine showed the same pattern as other mineralized tissues, that is, dihydroxylysinonorleucine and hydroxylysinonorleucine are the major cross-links with dihydroxylysinonorleucine being the predominant one.

The amino acid composition of predentine collagen generally resembled that of other collagens except that the glycine content was low and the proline content high indicating the sample was not completely pure. The neutral sugar results suggested that one contaminant was a glycoprotein.

The collagen hexose determination indicated that predentine is a high hexose collagen as the hydroxylysine was completely glycosylated. However, only a part appeared to exist as the disaccharide, glucosyl galactosyl hydroxylysine.

Cementum:

The Ca/P ratio of cementum was determined to be 2.3 (w/w). That of hydroxyapatite is 2.2 (w/w). Thus the mineral of cementum is hydroxyapatite.

Cementum was also found to be free of phosphoprotein. This result indicates that the role of phosphoprotein in mineralization must now be reconsidered.

The reducible intermolecular cross-link determination showed that dihydroxylysinonorleucine and hydroxylysinonorleucine were the major cross-links of cementum as in other calcified tissues. Of the two, di-

hydroxylysinonorleucine was the predominant one.

The amino acid composition of cementum resembled that of other collagens although the high proline and low glycine content indicated that the sample was contaminated. This observation is supported by the neutral sugar results which indicated one contaminant as a non-collagenous glycoprotein.

The neutral sugar determination on the collagen hexose showed it to contain 1.5g hexose/14g hydroxyproline. This value corresponds to 36.8% glycosylation of the hydroxylysine, of which only a part is the disaccharide.

Due to the limited amount of predentine and cementum samples, each determination was performed only once. Thus the results must be regarded as tentative.

ACKNOWLEDGEMENTS

I wish to express my sincerest thanks to Dr. D.J. Carmichael, Dr. C.H. Pearson and Dr. K.A. McMurchy for their guidance during the experimental phase and preparation of the thesis text. Their constructive criticism as well as many suggestions were greatly appreciated. Many thanks are due to Mr. D. Carmel and Mrs. P. Mudry for the endless patience during the dissection and collection of sample. My special appreciation is extended to Dr. M. Wohllebe for carrying out the amino acid and cross-link determinations as well as to Mrs. C. Dodd for her excellent technical assistance with the phosphoprotein determinations. Also, I would like to thank Mrs. R. Fuller for excellent typing services and her great care in the final typing.

Financial support received in the form of Graduate Teaching Assistantships during the period of laboratory work is gratefully acknowledged.

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INTRODUCTION

THE PURPOSE OF THE INVESTIGATION

Despite the extensive information available on the biochemical nature of collagen and other connective tissue elements, no definitive studies have been reported on the chemical character of the fibres found in cementum and predentine. Cementum is the thin layer of mineralized tissue which covers the whole root of the tooth. Its primary function is to attach the periodontal ligament to the tooth surface, thus relating the teeth to the jaws. It also plays a part in maintaining the width of the periodontal ligament and in repair of damage to the root of the tooth. Predentine, on the other hand, as the name implies, represents the precursor form of dentine. In contrast to dentine, however, our knowledge of predentine is meager. While the existence of collagen in this tissue has been firmly established, the identity of other components is less certain. Because of the very small amounts of tissue involved, most studies to date have been histochemical in nature. The aim of this work was to extend the histochemical studies to a chemical basis and so enlarge our knowledge of these two tissues.

REVIEW OF THE LITERATURE

I. SPECIFIC OBJECTIVES AND PRESENT STATE OF KNOWLEDGE

Connective tissues play many important roles in the body, serving portative, supportive, defensive, storage and reparative functions. They are composed of intercellular substances of which collagen is the principal component; and cells.

The fundamental unit of collagen is composed of three α chains. Each consists of about 1000 amino acids linked in an α-amino peptide linkage and has a molecular weight of about 100,000. The most common structure is $(\alpha 1)_2\alpha 2$. Two of the chains, the $\alpha 1$, have identical primary structures, whereas the third, the $\alpha 2$, differs from the $\alpha 1$ in amino acid composition[1]. As a result, the $\alpha 2$ chain is more basic. Recently it has been found that the $\alpha 2$ is shorter than the $\alpha 1$[2]. At least four genetically-distinct collagen α chains designated $\alpha 1(I)$, $\alpha 2$, $\alpha 1(II)$ and $\alpha 1(III)$ are found in the major vertebrate connective tissues. These chains appear to occur normally in three types of molecules with the following chain compositions: $\alpha 1(I)_2\alpha 2$, the Type I molecule which is the predominant collagen molecule in several tissues such as bone, tendon, dentine and mature dermis[3]; $\alpha 1(II)_3$, the Type II molecule which is the predominant species of collagen in hyaline cartilages[3]; $\alpha 1(III)_3$, the Type III molecule which coexists with Type I collagen in several tissues. Type III molecules are especially prevalent in young tissues[4] (discussed in greater detail on p. 25).

Each polypeptide within the collagen molecule chain forms a left-handed helix; each complete turn in the helix contains 3.3 amino acids which are separated by 2.9 Å. To form the molecule, each helical

polypeptide forms a right-handed triple helix in conjunction with two other α chains. The molecular dimensions are 3000 x 15 Å. However, the collagen molecule is not completely helical since the α chains have non-helical peptides at both the N-terminal and C-terminal ends.

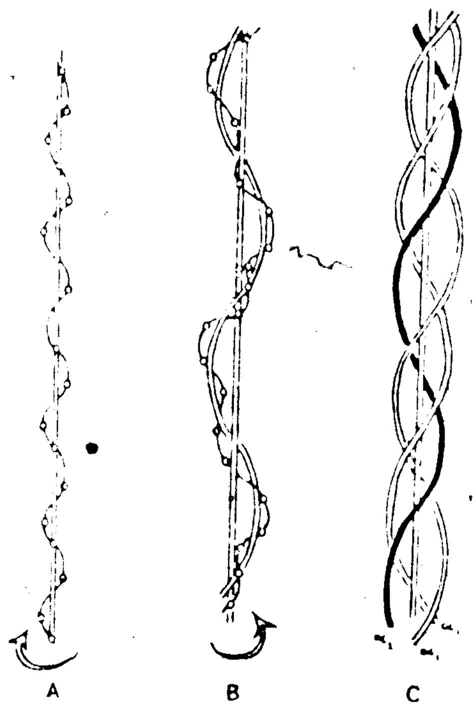


Fig. 1 Triple helical structure of collagen. A. Single chain wound in single left-handed helix. B. Axis of (A) wound in somewhat right-handed helix so that the single chain itself forms a compound helix. C. Arrangement of the axes of three chains in the collagen molecule. In (C) the chains themselves are omitted for simplicity 5

Fig. 2 Perspective view of the collagen structure for a height of about 30 Å 90

The amino acid composition of collagen is unique in that about one-third of the residues are glycine, the simplest amino acid having a single hydrogen atom instead of a side chain. The two imino acids, proline and hydroxyproline, make up about two-ninths of the total amino

acids, and hydroxylysine makes up about 1 percent. See Table 1 for the amino acid composition of various mammalian tissues. From this table, one sees that collagen contains significant amounts of all the common amino acids except cystine and tryptophan.

Glycine, proline and hydroxyproline, the three most abundant amino acids in collagen, have an important bearing on its structure. It is surprising that a protein having an amino acid composition like collagen can form a helix. The ring structure of proline and hydroxyproline would normally prevent helix formation. However, the structure of the triple helical molecule is such that glycine in every third position lies inside the triple helix, while the ring structure of proline and hydroxyproline and the side chains of the other amino acids lie on the outside, thus allowing for close packing and α helix formation. Once formed, the presence of these rigid regions with bulky groups incorporated helps to stabilize it by preventing unwinding. The structure is also stabilized by hydrogen bonds between the peptide chains transverse to the axis of the molecule.

Several conformations for the triple helix have been proposed in the last two decades. Recently, an unambiguous structure determination appears to have been achieved. Traub and Yonath[7] studied the polytripeptide $(Gly\text{-}Pro\text{-}Pro)_n$ and found it to conform with the parameters of collagen, that is, this model conformed with the generally accepted bond lengths, bond angles, and minimum van der Waals contacts and is consistent with the x-ray pattern, infrared frequency, as well as other physical and chemical data relating to the structure. This conformation has only one hydrogen bond for three amino acids. A projection of this structure down the helix axis is shown in Fig. 3.

Until recently, hydroxyproline had been thought unessential for the structural integrity of the collagen molecule. Its function may now have been revealed. Several workers[9,10] have found that the amino acid

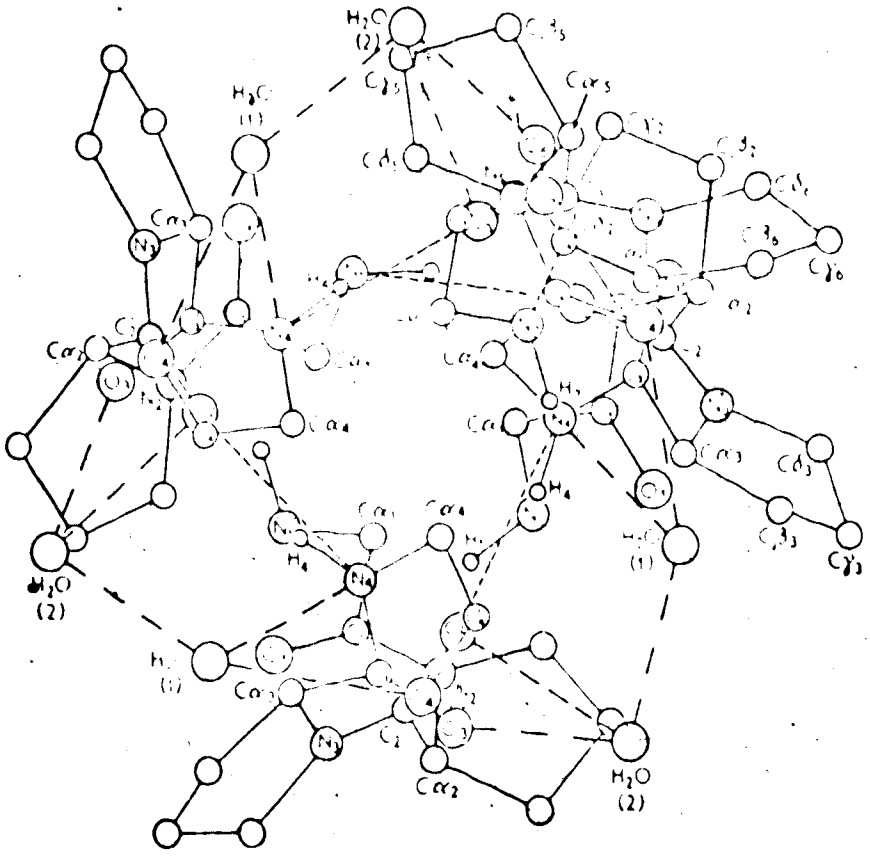


FIG. 3. Projection down the helix axis of (Gly-Pro-Pro)$_n$ structure, including two water molecules per tripeptide. The structure is viewed from the carboxyl end. Dashed lines indicate hydrogen bonds.

is important for the thermal stability of collagen. Jeminez et al[11] prepared chick tendon protocollagen samples which contained varying amounts of hydroxyproline. The thermal stability was monitored by susceptibility to complete digestion by pepsin, an enzyme which does not degrade helical molecules. They observed that the thermal stability varied directly with hydroxyproline content. The protocollagen samples which lacked hydroxyproline completely were found to have a denaturation temperature of 24°C. This implies that hydroxyproline is necessary for

the procollagen chains to assume the triple helix at body temperature.

Existing models for the collagen structure do not make allowances for the participation of the hydroxyl group of hydroxyproline in hydrogen bonding within the triple helix. Evidence for the participation of hydroxyprolyl residues in the stabilization of the collagen helix requires a re-evaluation of the atomic structures proposed for collagen.

Once the monomers are formed, these protofibrils bind extracellularly by electrostatic forces to form fibrils. Fibrils then combine to form fibers. When viewed under the electron microscope, the fibers have a distinctive and characteristic 640 Å banding. The primary structure of collagen has two special aspects which account for this. Firstly, there is a recurrence of certain similar sequences of amino acids. Glycine occurs in every third position with proline and hydroxyproline frequently following. This sequence is found in the non-polar or crystalline regions of the molecule. Secondly, this tripeptide alternates with a large polypeptide run which seems to have polar side chains of glutamic acid, aspartic acid and lysine. When collagen is stained with solutions of phosphotungstic acid or uranyl acetate, a characteristic band pattern is revealed which reflects the distribution of clusters of charged and uncharged amino acids along the molecule[12].

Recently, following the classical work of Kuhn and coworkers[12], Bruns and Gross[13] have established the number and position of reproducible bands in segment-long-spacing crystallites of calf skin collagen which will permit precise identification of individual bands or segments of the band pattern. They were able to collocate, on the basis of band numbers, published observations on positively stained segment-long-spacing crystallites which included cyanogen bromide and hydroxylamine

peptides, fragments produced by enzymatic cleavage, sites of scission by specific animal collagenases, amino acid residues identified by selective staining procedures, and clusters of specific residues where the amino acid sequence has been established.

The collagen becomes covalently cross-linked once the fibrils are formed. Two different types of cross-links form: intramolecular cross-links develop within the collagen monomer, whereas intermolecular cross-links form between the collagen molecules. The latter type of cross-link is physiologically important for strengthening the fibrils and rendering them insoluble. Evidence for cross-linking comes from denaturation studies on collagen. The products of such a study are α, β and γ components[14]. As a function of time, in vivo, cross-links form between chains to produce dimer components, the β components, which can be of two types when the cross-link is intramolecular, $\alpha 1 - \alpha 1$ (designated β_{11}) and $\alpha 1 - \alpha 2$ (designated β_{12}). When collagen is salt-extracted in the cold, predominantly $\alpha 1$ and $\alpha 2$ chains are obtained, making up 80-90 percent of the sample. On the other hand, when collagen is acid-extracted, 50-60 percent of the sample is β_{11} and β_{12}. This is because the acid-extractable collagen is more cross-linked than salt-extractable collagen. There also occurs a third component, the γ-component, which is a trimer composed of $\alpha 1 - \alpha 1 - \alpha 2$ (γ_{112}). These peptides account for the intramolecular cross-links. On the other hand, if other extraction methods are used, for example, 5M guanidine, pH 7.5, another type of β component can be extracted, the β_{22}, arising from intermolecular cross-linking between two $\alpha 2$ chains.

II. COLLAGEN BIOSYNTHESIS

The site of protein synthesis is the ribosome, which contains

RNA and proteins. The genetic information is brought to the ribosome by the single stranded RNA known as "messenger" RNA (mRNA) which has a primary structure complementary to a portion of one DNA strand. The nucleotide sequences of mRNA determines the amino acid sequence of the protein. Amino acids are attached to the soluble transfer RNA (tRNA) molecules which transfer amino acids to sites on ribosomes. The tRNA molecules are specific for a given amino acid but some amino acids are recognized by more than one tRNA. The mRNA is read in units of 3 nucleotides (triplet codon) for the incorporation of a single amino acid and the tRNA possesses a triplet anticodon that is complementary to the codon of the mRNA. It is through such interactions that the genetic message of the mRNA, originally derived from the active strand of DNA, is translated into a polypeptide sequence, incorporating sequentially one amino acid residue at a time beginning at the N-terminal end of the peptide chain.

Collagen biosynthesis follows the same general pattern as that for non-collagenous proteins. It is accomplished by a series of sequential steps. Firstly, the polypeptide precursor of collagen, procollagen, is assembled on ribosomal complexes[15-18]. Secondly, the appropriate proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine. This step differs from non-collagenous protein synthesis in that these amino acids are altered after incorporation into the protein. The third step involves glycosylation of some of the hydroxylysine with galactose or glucosylgalactose in o-glycosidic linkage.

Though synthesis of the protein chains of collagen resembles that of other non-collagenous proteins, there is still some controversy as to how they are assembled. It was first thought that, due to their

size, the α chains were synthesized in subunits which were released from the ribosome and subsequently joined in nonpeptidic covalent bonds [19-22]. Now evidence discredits this theory. In the analogous case of hemoglobin[24,25] the complete $\alpha 1$ and $\alpha 2$ chains have been shown to be synthesized simultaneously as single protein chains by sequential amino acid addition from the amino-terminal end[23]. It is still unknown whether the α chains combine to form the triple helix while still on the ribosomes. However, there is evidence which strongly indicates that both disulfide bond formation and triple helix assembly occur after release of pro α chains from the ribosome, probably within the cisternea of the endoplasmic reticulum[26-28].

In the past few years, researchers have discovered that the first polypeptide chains of collagen synthesized by cells are larger than the α chains[29-32]. All three chains of the procollagen molecules are found to have non-helical extensions at the NH_2-terminal end[33-36]. Each extension contains the same amount of cysteine, giving rise to disulfide bonds which link the three α chains[37]. These bonds stabilize the procollagen trimer and are thought to determine the correct longitudinal alignment of the three strands[38]. Perhaps these extensions, the registration peptides, initiate triple helix formation while the polypeptide is still on the ribosome. The tertiary structure of the complex, that is, the triple helical collagen plus the registration peptide, may keep the molecule from interacting and precipitating within the cell.

The first advances in knowledge of collagen biosynthesis came in the 1940's with Stetten and Schoenheimer[39]. These researchers found that when ^{15}N-proline was fed to rats, it was incorporated in collagen

in the hydroxyproline as well as in the proline. In subsequent experiments, Stetten[40] found that when he fed rats with ^{15}N-hydroxyproline, none of this was incorporated into the collagen. Other workers found a parallel situation for the incorporation of hydroxylysine[41-44]. It was therefore concluded that some of the proline and lysine residues were hydroxylated after completion of chain synthesis. On the other hand, it was later suggested that prolyl tRNA and lysyl tRNA were hydroxylated and as a result the hydroxyproline and hydroxylysine were then incorporated directly into the collagen polypeptide[45-51]. It has now been established that the correct view is the former of the two[51]. This was made possible with the discovery that it was atmospheric oxygen that was required for the hydroxylation step and not water[52,53]. Experiments were carried out under anaerobic conditions where ^{14}C-proline was incorporated into an unhydroxylated intermediate[54-58] of the same size as or larger than the α chains of collagen[57,59,60]. A substantial amount of the proline in the intermediate procollagen was hydroxylated to hydroxyproline when exposed to oxygen. When it was found that ferrous ion was required as a cofactor for the hydroxylation of proline[57,61], similar experiments were set up using metal chelators such as α,α'-dipyridyl. Early studies[62,63] showed that when hydroxylation was inhibited, secretion of collagen was also inhibited. There was a concomitant accumulation of the unhydroxylated material within the cells. When more ferrous ion was added to these cells, the preformed procollagen was hydroxylated and secreted as collagen[62]. It is thought the unhydroxylated procollagen cannot form the triple helix and as a result cannot be secreted. Thus hydroxylation of proline has an important physiological role directly related to the regulation of secretion of

collagen.

When the enzyme procollagen hydroxylase was isolated[64], it provided further proof that procollagen was an intermediate in collagen synthesis[65]. Secondly, it binds much more tightly to and hydroxylates much more readily a sequence of Gly-X-Pro in a large polypeptide than an identical sequence in a short polypeptide[65-69]. It would appear that hydroxylation occurs after the release of the nascent collagen chains from the ribosomal complex[70-72].

The hydroxylation of proline also requires α-ketoglutarate as well as ascorbate[73-76]. Ascorbate is the least specific in that it can be replaced by other reducing agents such as enediols. It stimulates the synthesis of peptidyl proline hydroxylase as well as activates it in vitro. However, the requirement for α-ketoglutarate is specific. It cannot be replaced by pyruvate nor oxaloacetic acid. The synthesis of one mole of hydroxyproline involves a stoichiometric conversion of α-ketoglutarate to succinate and CO_2[77], that is the reaction is substrate dependent and there occurs a stoichiometric decarboxylation of α-ketoglutarate coupled to the hydroxylation of the peptidyl proline residue[78]. The decarboxylation of α-ketoglutarate by the hydroxylase is not related to the decarboxylation of the same compound that occurs in the Krebs cycle in the mitochondria since it does not involve thiamine-pyrophosphate or require coenzyme A[79,80].

Hydroxylation of lysine follows the same mechanism as that of proline[81]. It requires oxygen, iron and α-ketoglutarate[65,82-84] but the enzyme, lysine hydroxylase, is different. Nor does ascorbic acid play the same role. When there is a deficiency of this vitamin, the hydroxylation of lysine is much less affected than the hydroxylation of

proline[75]. Peptidyl lysine hydroxylase will not further hydroxylate native collagen strongly suggests that hydroxylation occurs prior to triple helix formation[85-87]. There is no evidence that hydroxylysine has any effect on triple helix formation.

Glycosylation of the hydroxylysine is the final synthetic stage in completing the structure of the collagen molecule. It probably occurs while the nascent collagen peptides are still bound to the ribosomes[88]. To the appropriate hydroxylysine, galactose[195] is "tacked on" first in a β-glycosidic bond. To some of the galactosyl residues, glucose is then linked[195] in an α 1 $\longrightarrow$ 2-o-glycosidic linkage.

Once the collagen polypeptides are formed, hydroxylated, glycosylated and released from the ribosomal complexes, they probably migrate to the cisternae of the endoplasmic reticulum. Several studies have shown that the Golgi apparatus and its associated vesicles is implicated directly in the secretion of procollagen[91-94]. The Golgi vesicles were reported to contain filamentous threads while still associated with the Golgi and later as the vesicle traversed the cytoplasm, these threads appeared to aggregate into rod-like bundles of dimensions appropriate to aggregates of collagen molecules. According to Weinstock and Leblond[92], the newly synthesized procollagen is transported to the Golgi either by way of a transitional element or by fuzzy coated intermediate vesicles budding off from the rough endoplasmic reticulum. Experiments using microtubule disruptive drugs such as colchicine or vinblastine have implicated microtubules in procollagen secretion as these drugs impair collagen secretion[95-97]. Presumably microtubules play the role of a transport conduit along which the secretory vesicles move from their place of biogenesis within the Golgi apparatus to their eventual fusion

with the plasma membrane and excretion of their contents from the cell.

When the procollagen molecules are extruded into the extracellular space they aggregate to form microfibrils. However, it is extruded as the undegraded precursor which appear in electron micrographs as bundles which are 3000 or 6000 Å in length[91,98]. The packing arrangement in the larger bundles is such that the amino terminal regions are all oriented toward the center of the spindle and the carboxyl terminal regions point in opposite directions on either side of the central band. Recently[99-101], it has been demonstrated that the conversion of procollagen to collagen *in vivo* in rat skin, must be a multi-step process with at least two conversion steps. Intact procollagen and an intermediate form can be isolated from rat and bovine skin during the period of rapid growth of the animal. In odontoblasts[91,94], the first step in the formation of the extracellular fibre and fibril system is thus the dissolution of these antiparallel collagen molecule bundles and their conversion and reorganization into parallel alignment. This, perhaps, is the stage at which the enzyme procollagen peptidase makes its primary cleavage and serves in releasing individual molecules from the aggregate state. Electron micrographs[99] and pulse labeling studies[101] have shown that this intermediate material possesses an amino terminal extension and it is this extension which is thought to facilitate fibril formation. The final step in enzymic conversion to collagen is thought to occur after the microfibrils form[101].

In 1955, Schmitt et al[102] proposed the quarter-stagger theory whereby collagen molecules of protofibrils were displaced longitudinally with respect to one another by a distance equal to one-quarter of the length of the component molecules. However, more recently, it has been

found that the stagger is not one-quarter but such that there is a "hole" region of about 410 Å between each collagen molecule as well as an overlap zone of about 270 Å[0103]. The length of the collagen molecule is 4.40 ± .02D where D is the period in the native fibril, that is, 690 Å in the wet state. See Fig. 4.

It seemed clear to Schmitt and his coworkers that the band pattern stained with phosphotungstic acid must represent a molecular map of the distribution of basic side chains along the length of the collagen macromolecule. In fact, collagen molecules have 5 charged regions 680 Å apart.

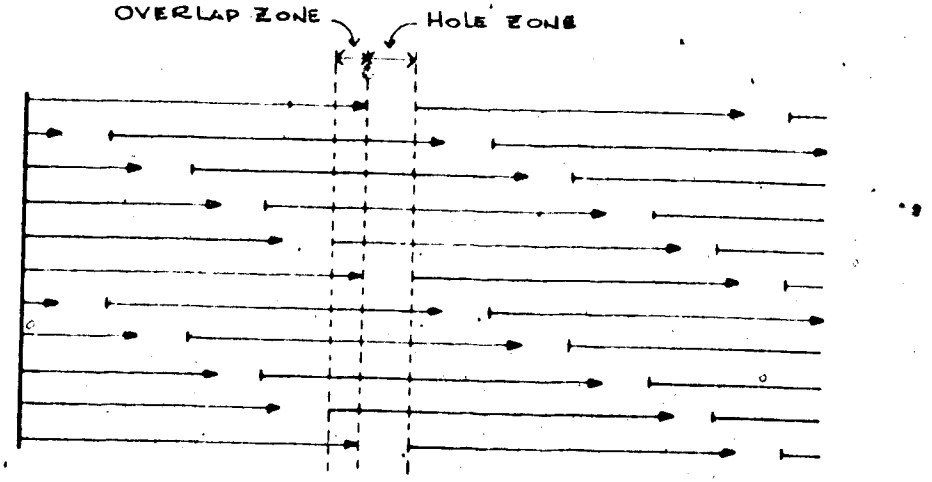


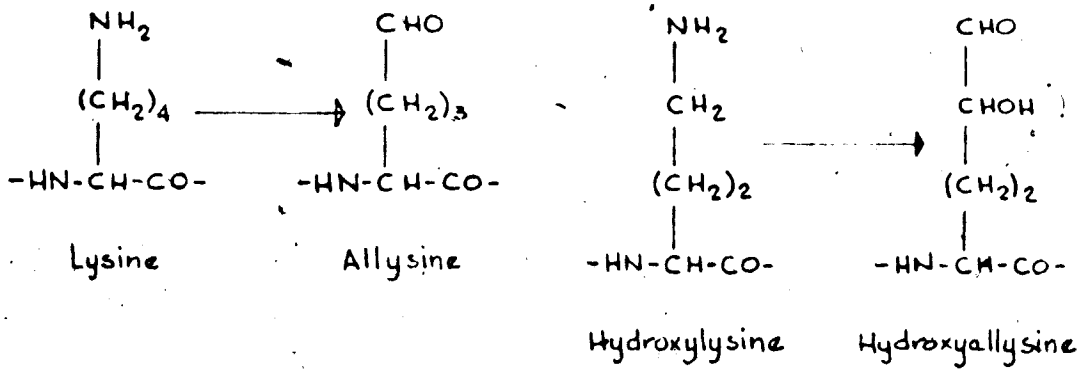
Fig. 4. Two dimensional representation of the packing arrangement of collagen macromolecules in the native-type fibril where overlap zone = .4D and hole zone = .6D

Much interest has been generated by the hole region of the collagen fibrils. It is thought that perhaps it provides a nidus for the deposition of hydroxyapatite in the process of calcification.

III. COLLAGEN CROSS-LINKING

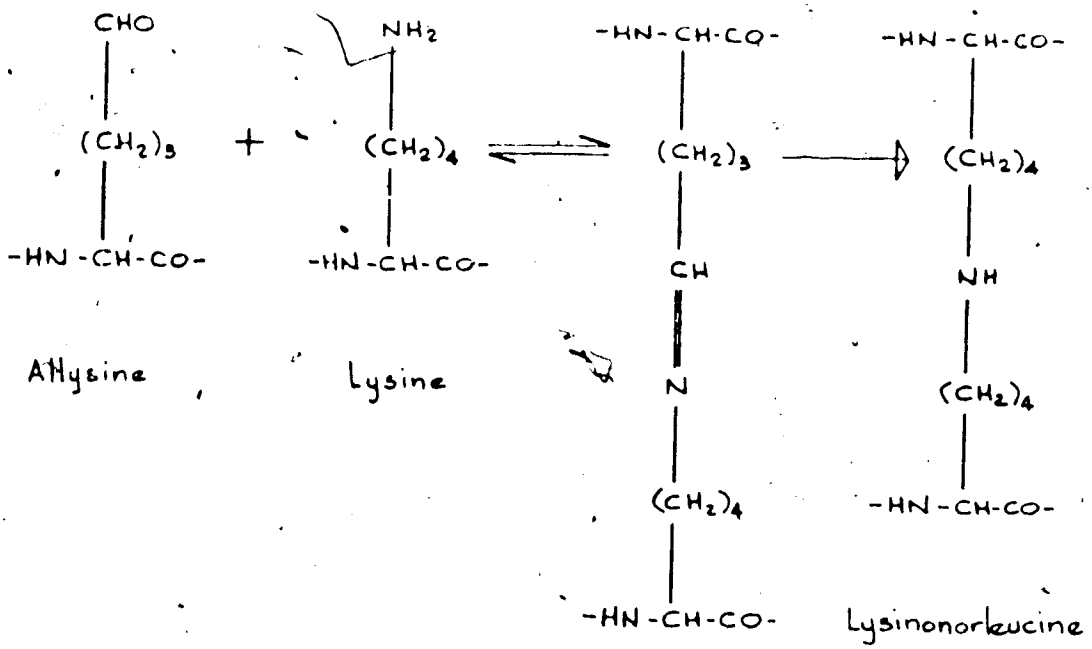
Cross-links form extracellularly once the molecules have aggregated into fibres. Two types of cross-links occur in collagen: intramolecular and intermolecular.

Both types of cross-links arise from lysyl or hydroxylysyl residues which are converted to the α-aminoadipic-γ-semialdehyde, more often called allysine and hydroxyallysine. In other words, the first step in cross-linking is the removal of the ϵ-amino group of a lysyl or hydroxylysyl residue and the formation of an aldehyde[104].



The formation of these precursor aldehydes is catalyzed by lysine oxidase[105] and are located most often in the nonhelical N-terminal region of the peptide although some have been found elsewhere in the chains[106,107].

From these aldehydes, crosslinks are formed via two types of reactions. The first is a Schiff base formation by a condensation of an allysine with the amino group of lysine or hydroxylysine on another α chain. This precursor of desmosine and isodesmosine[108,109] is not a stable cross-link and is considered not to be very important in collagen. However, Bailey[110] believes it is a precursor for the intermolecular cross-link lysinonorleucine.

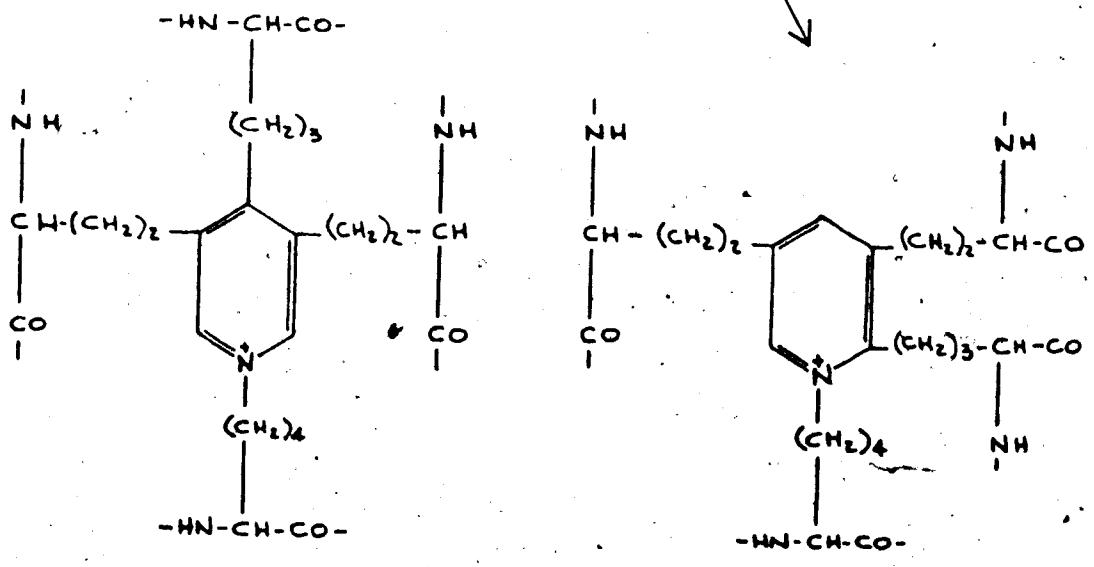


Allysine

Lysine

Lysinonorleucine

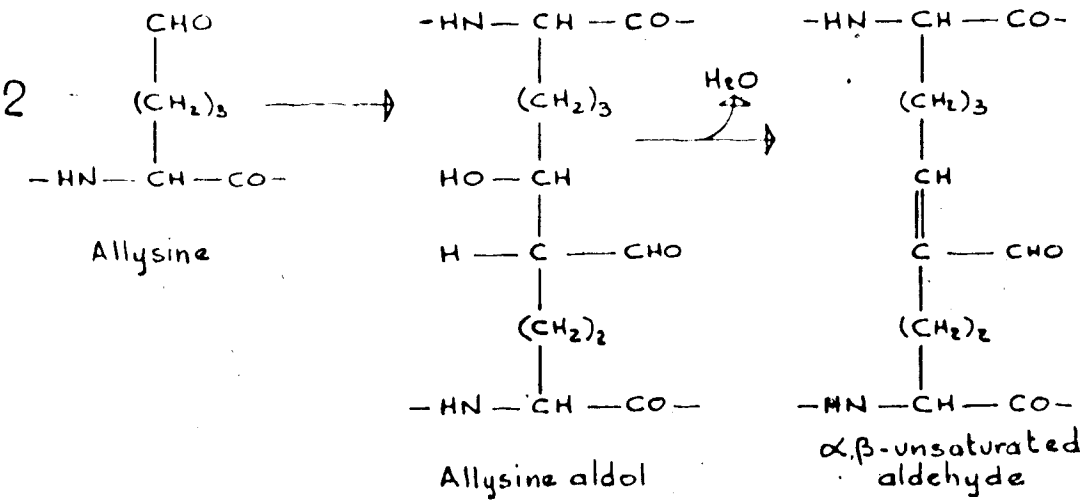
Dehydrolysinonorleucine



Desmosine
in elastin only

Isodesmosine
in elastin only

The second type of reaction involves an aldol condensation between two allysines on two adjacent α chains. The product is the intramolecular cross-link, allysine aldol, present only near the NH_2-terminal of collagen chains, dehydrates readily and is not stable to acid hydrolysis[108, 111]. It transforms during fibril formation and is thought to be a precursor of the intermolecular cross-link, hydroxylysinonorleucine[112] since it disappears as this cross-link appears.

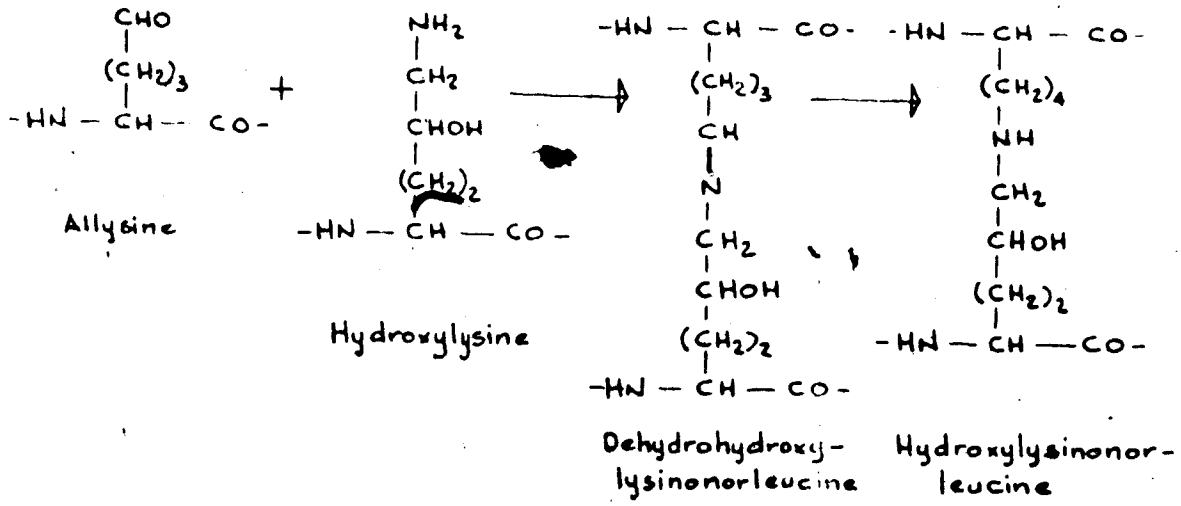


The role of intramolecular cross-links is in doubt since allysine aldol does not contribute significantly to molecular stability nor does it contribute to the stability of fibrils. Some researchers[110,113] believe that intramolecular cross-links are precursors of intermolecular cross-links. The strongest evidence supporting this thesis arises from studies on lathyrism. Lathyrogens inhibit the conversion of lysine to the aldehyde and since both intramolecular and intermolecular cross-linking is inhibited, perhaps the aldehyde is involved in both processes[108].

The role of intermolecular cross-links is better known. Collagen fibers have the unique ability to withstand stress due to the system of covalent cross-links between the individual collagen making up the fiber. Experimental lathyrism clearly demonstrates the need for these

cross-links, as it produces an extremely fragile fibre due to the slippage of adjacent molecules under tension[14]. It is possible to recognize a time-dependent process of maturation of the fibre by the changes in physical properties. An extensive system of these covalent intermolecular bonds between adjacent molecules would increase the tensile strength of the fibre by preventing slippage and would also be a logical explanation of the formation of insoluble collagen.

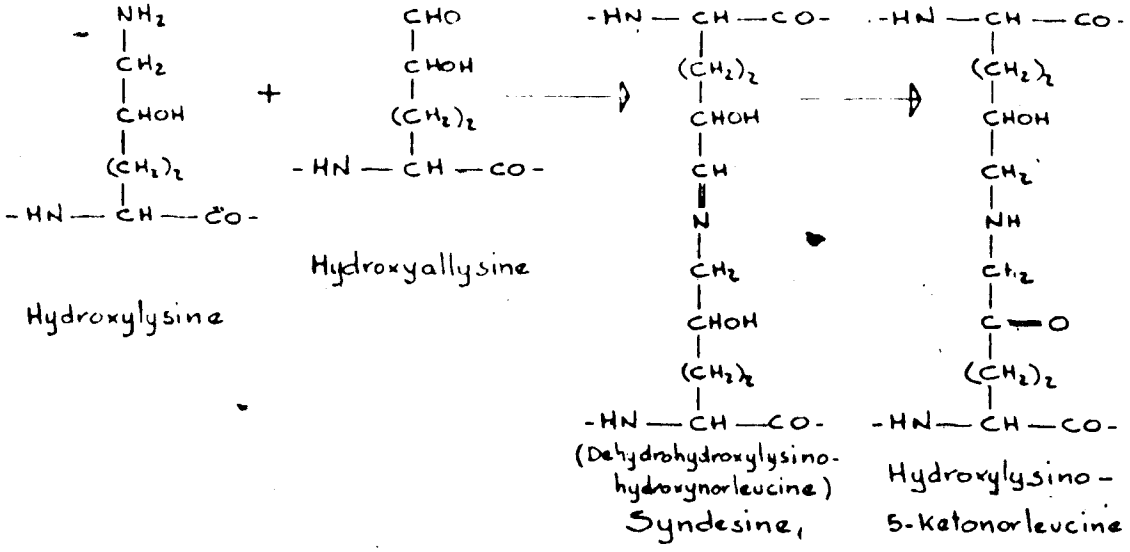
Chemical information on intermolecular cross-linking has been more difficult to obtain. Bailey and Peach[115,116] isolated a reduced product of a Schiff base condensation between a hydroxylysyl residue and an allysyl residue. The labile cross-link had the composition, hydroxylysinonorleucine.



It has been isolated in tendon as well as in reconstituted fibrils. This cross-link decreases with age and is thought to be, in part at least, an intermediate.

Bailey[115,117] isolated in insoluble collagen from chick bone and human and bovine teeth another cross-link which seems to be a major component. It is also present in chick and bovine tendon[118] from old animals and is a minor component of skin. This cross-link, syndesine, was first thought to be the product of an aldol condensation between an

allysyl residue and a hydroxyallysyl residue. It was later shown to be an aldimine derived from the condensation of hydroxyallysine and hydroxylysine[119,120].

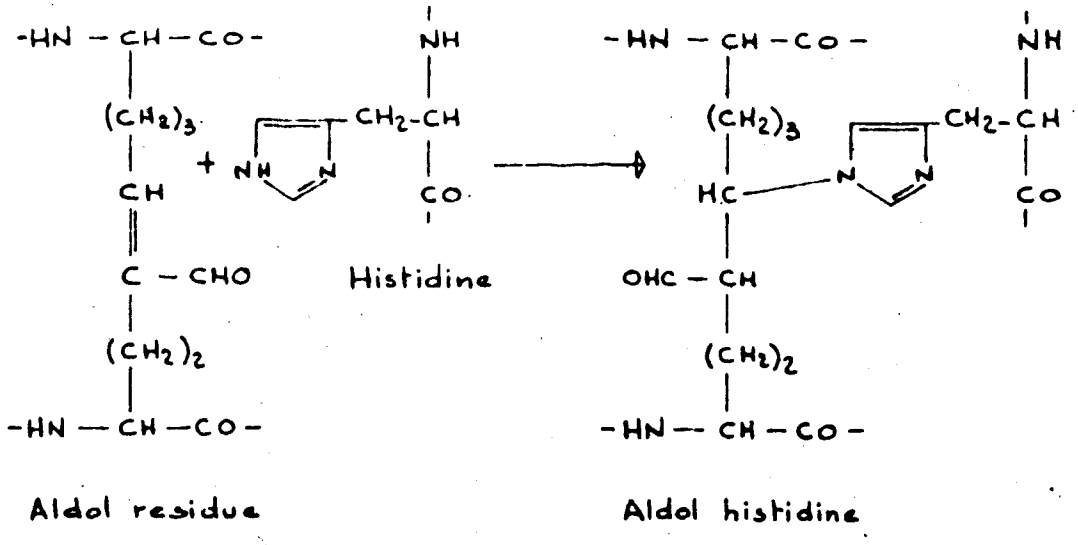


However, this is inconsistent with the unusual stability of dehydrohydroxylysinohydroxynorleucine to heat, dilute acids and D-penicillamine. Subsequently, it was found that there occurs a migration of the double bond to form the stable keto form by a spontaneous Amadori rearrangement[121]. The cross-link must therefore exist *in vivo* as hydroxylysino-5-ketonorleucine.

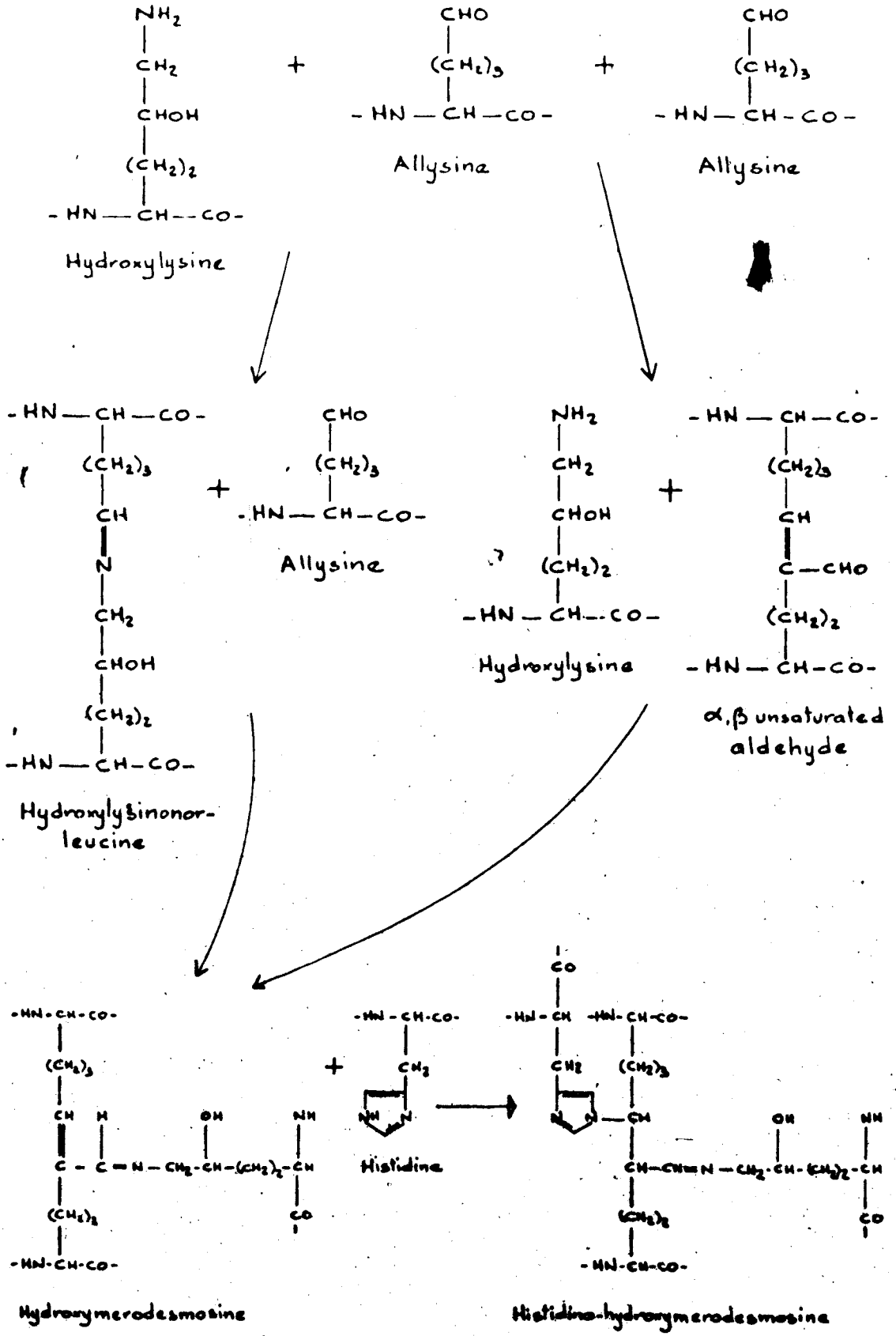
The above accounts for all products resulting from an aldol or Schiff base condensation between lysine, hydroxylysine and their aldol condensation of two hydroxyallysine residues which has not been reported. However, a higher molecular weight compound (or compounds) has been reported and may be a cross-link.

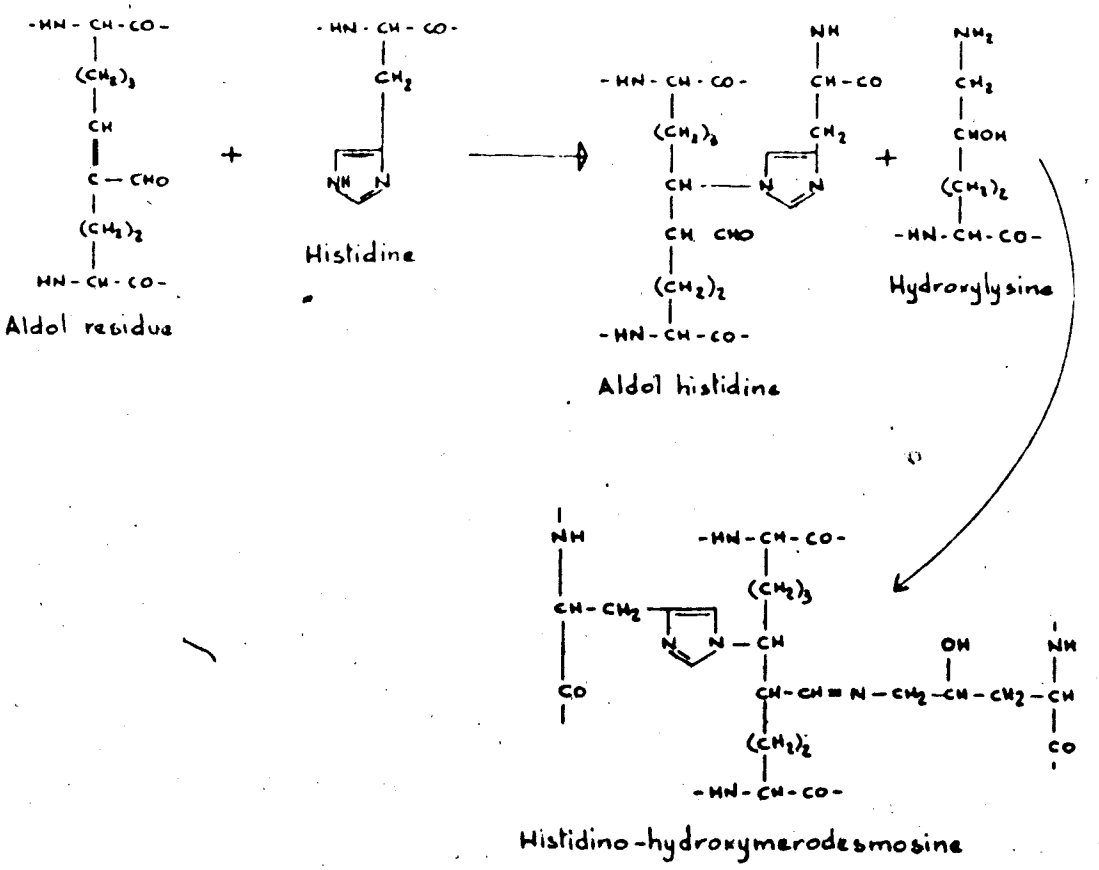
Cross-links arising from amino acids other than lysine residues and the result of reactions other than aldol and Schiff base condensations have been isolated. Disulfide bonds[122,123] are found in Ascaris cuticle collagen. There is also the aldol histidine which is found

predominant only in reconstituted cow skin collagen. It can potentially unite 3 polypeptide chains and is formed by a Michael addition of the imidazole of histidine, to the β-carbon of the α,β-unsaturated bond of an aldol condensation product[124,125]. Robins and Bailey[121] now believe this cross-link to be an artifact. The absence of the reduced form of this component, histidino-hydroxymerodesmosine on reduction under acid conditions and a concomitant increase in the amount of the aldol condensation product support this strongly. They believe that the Michael addition of the histidine residue to the aldol is base catalyzed by the borohydride.



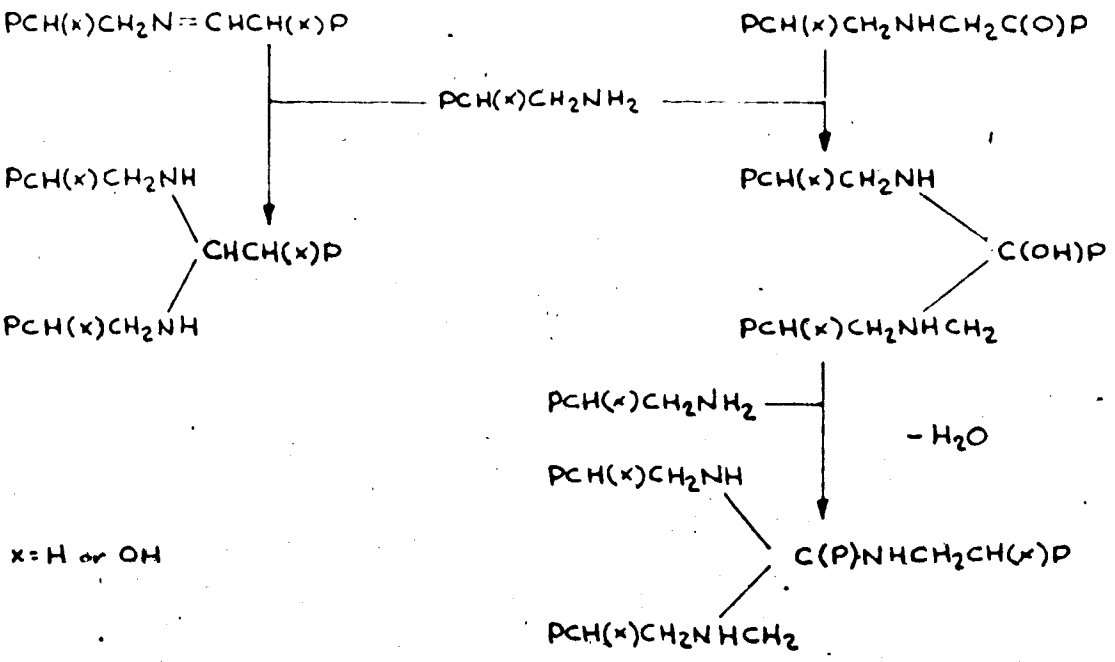
Merodesmosine, a trifunctional cross-link is found in collagen in small amounts in its monohydrated form. It is formed in reconstituted collagen and may arise via three alternate biosynthetic pathways. It may be an intermediate in cross-linking and it was proposed to be a precursor of histidino-hydroxymerodesmosine[125,126]. However, in view of the current findings, it is difficult to assess the role of this cross-link.


Hydroxylysine
Allysine
Allysine
Allysine
Hydroxylysinonor-leucine
Hydroxylysine
α,β unsaturated aldehyde
Histidine
Hydroxymerodesmosine
Histidino-hydroxymerodesmosine



Stable intermolecular cross-link formation is important in the maturation of collagen fibers. Bailey[127] proposed that these reducible electrophilic cross-links, which are progressively lost during aging[128], are transformed into more stable non-reducible cross-links whose structures have not yet been determined. Davis and Risen[129] tested this hypothesis and found that nucleophilic addition of lysine and/or hydroxylysine residues to the electrophilic double bond of the reducible cross-links transforms them into more stable, non-reducible cross-links. He also found that modification of lysine and hydroxylysine residues by destroying their ability to initiate nucleophilic attack on the electrophilic double bonds of the reducible cross-links completely blocks this transformation while the modification of histidine, arginine, glutamic

acid and aspartic acid is without effect. They proposed the following tentative structures.



These cross-links are capable of joining more than two collagen molecules. Davis and Risen hypothesized that the formation of these cross-links would explain the increasing resistance of maturing collagen fibers to dissolution in cold acid or alkali, to mechanical disruption, as well as to thermal disruption.

The location of intermolecular cross-links is difficult to ascertain. However, this information may soon be available as sequencing of the CNBr peptides of the α chains is well underway and cross-links have been isolated on various CNBr peptides[112,130].

IV. SEQUENCING OF CHAINS

The primary structure of collagen must carry information of higher levels of structure. It was with this thought in mind that researchers began concentrating on the sequencing of the α chains.

Sequencing of the α (I) chains is gradually being accomplished

as a result of CNBr cleavage. This method developed by Gross and Witkop[131,132], is based on the fact that CNBr cleaves the α chains at the methionine residues. The resulting peptides, 6 to 9 from the α 1 chain and 6 from the α 2 chain, are then separated by column chromatography and further cleaved if necessary by various enzymes, such as trypsin. Investigations of calf and rat skin collagen have provided the entire sequence of the amino acid residues of the α 1 chain. See Table 2 for the combined result as the differences between the two are few. Attempts to sequence the α 2 has not been quite as fruitful. Three of its 6 CNBr peptides are very long and as a result difficult to sequence. See Table 3 for α 2 CNBr peptides sequenced.

When the sequence of the α 1 chain is examined, one notices some striking features. Prior to sequencing, it was postulated that although glycine had to be in every third position of the helical portion, the other amino acids could occur at random, that is, in either position X or Y of the tripeptide Gly-X-Y. However, this does not appear to be so. There is a preponderance of some amino acids in a particular position of the tripeptide. Leucine, phenylalanine and proline are restricted to position X and glutamic acid occurs frequently in this position. On the other hand, threonine, arginine, methionine, lysine and hydroxylysine occur frequently in position Y and hydroxyproline occurs exclusively in position Y. Another interesting feature is that the hydroxylation of proline and lysine is often incomplete. The significance of these observations has yet to be elucidated.

An interesting paper recently published by Hulmes et al[133] examines the primary structure of collagen for the origin of molecular packing. They looked at the amino acid sequence of the triplet region

of the α 1 chain to see if there would be complementary relationships that would explain the stagger of multiples of 670 Å between the molecules of the fibril. They found that when the chains are staggered 0D, 1D, 2D, 3D and 4D (D = 234 ± 1 residues) the interactions are maximal between amino acids of opposite charge and between large hydrophobic amino acid residues. Unexpectedly they found that the hydrophobic interactions showed a fine structure in their pattern whereas the charged residues were less regular. Positively-charged residues tended to be near negatively-charged residues but did not show a distinct periodicity.

Fietzek et al[138] have recently sequenced the α 2-CB2 from calf, human, rabbit and pig skin collagen and compared them to that of rat and chick skin sequenced by Highberger et al. See Table 4. They observed 6 positions of high interspecies variability. However, the substitutions were conservative and in all cases the functionally important side chains were preserved in definite positions. The substitutions involved uncharged polar or apolar residues except in position 15 where a lysine-arginine substitution is found.

Much work is still required on the α 2 chain. However, once the α 2 chain is completely sequenced, a more detailed diagram of the interactions between the α chains and between collagen molecules will be possible.

V. TYPES OF COLLAGEN - CONTINUATION OF STRUCTURE: Special and Particular Cases

Until recently collagen was considered to have a chain composition $(\alpha 1)_2 \alpha 2$. Tissues of such a chain composition include rat skin[1], dogfish skin[139], human skin[140], chick skin[141] and chick bone[142]. Ex-

ceptions include codfish[143] where the three chains are different, and collagen from lower vertebrates and invertebrates where the three chains are identical[144-146]. However, several researchers have isolated different types of collagen which are genetically distinct. Miller and Matukas[147] observed a lack of stoichiometry when trying to isolate α 1 chains from α 2 chains in chick cartilage. Prior to this collagens examined had had α 1: α 2 ratios of 2:1. They found that although a proportion of cartilage collagen was identical to that of bone and skin of the chick, the cartilage contained another type of α 1 chain which they designated as Type II. They suggested that cartilage consists of 2 distinct collagen molecules, the $(\alpha 1)_2 \alpha 2$ and the $\alpha 1(II)_3$. Trelstad et al[148] supports this view and added that the α1 Type II had a much higher amount of carbohydrate, 5.5%, associated with it as compared to the .5% by weight of carbohydrate associated with Type I. Miller et al[4] have more recently isolated a third type of collagen, Type III, in newborn human skin. They suggest that human skin collagen is a mixture of molecules with the chain composition $\alpha 1(I)_2 \alpha 2$ and $\alpha 1(III)_3$. See Table 5 for a comparison of amino acid compositions of selected CNBr peptides from the 3 types, and Table 15.

Collagen from basement membranes[149] has not been formally numbered but by convention would normally be termed Type IV. It is also composed of 3 identical chains and has a much higher content of hydroxyproline, hydroxylysine and carbohydrate than interstitial collagens. The proline and hydroxyproline make up about 20% and the lysine and hydroxylysine make up about 5%. The 10-12% carbohydrate is virtually all glucosylgalactose linked to hydroxylysine; that is, glucose and galactose exist in almost equimolar amounts and account for all hexose.

Unlike other mammalian collagens it contains half-cystine. Another feature which distinguishes basement membrane is the low alanine content and its high phenylalanine content which is about twice that found in tendon collagen. See Table 6.

Recent developments indicate that vertebrate collagens are a heterogeneous population of molecules which are made up of five distinct genetic types: α 1(I), α 1(II), α 1(III), α 1(IV) and α 2. More types may be discovered. Cited earlier was codfish collagen[143] which had a molecule composed of 3 α chains, each of which appears to be unique.

As a result of these findings, structure and function can be more easily correlated. Several examples come to mind such as the cartilage transition to bone accompanied by a change from the cartilage species of collagen, $\alpha 1(II)_3$ to the bone species, $\alpha 1(I)_2 \alpha 2$, and the change in collagen type occurring during the maturation of fetal skin $\alpha 1(III)_3$ to the adult form $\alpha 1(I)_2 \alpha 2$. It can be seen, from these examples, that as function changes, so do the collagen types. Thus future investigations in particular to the role of collagen must now take into account the different molecular species.

VI. MINERALIZATION

Calcification or tissue mineralization is the deposition of inorganic crystals in or on an organic matrix. The inorganic crystals are composed of calcium and phosphate, bound in a form resembling hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. The organic components are the protein matrix, collagen: the mucopolysaccharide ground substance, thought to be chondroitin sulfate and acidic glycoproteins; and lipids.

The mechanism of calcification is still poorly understood. The fact that only teeth and bone normally calcify whereas other tissues do not even though the extracellular fluids are supersaturated with calcium phosphate remains a perplexing question. Several theories have been put forward to explain the phenomenon.

Role of Glycosaminoglycans

In the body, glycosaminoglycans are found in the form of hyaluronic acid, sialic acid, chondroitin sulfates and various other protein polysaccharides. They form the basis of the ground substance but their function is still unknown. Several workers[130-152] have found by progressive analysis of cartilage toward the ossifying front that chondroitin sulfate decreased abruptly as the calcium content increased. Glimcher[153] suggested that free anionic groups of acid mucopolysaccharides may inhibit calcification by binding calcium ions thus making them unavailable for crystallization. Perhaps in calcifying tissues the mucopolysac[illegible]es are removed destroying their inhibitory action. At the [illegible]ium is released, raising its local concentration and maki[illegible]e for calcification. The opposite view that the glycosam[illegible]romote calcification has also been proposed. Rubin and How[illegible]on the basis of a conformity between metachromatic staining and [illegible]uent mineral deposition, suggested that the property of calcifia[illegible] of epiphyseal cartilage and of tendon was associated with an [illegible]ased base-binding power of the matrix, the base-binding power be[illegible] chondroitin sulfate. On the other hand, Bowness[272] suggests the acid mucopolysaccharides, which he believes exist in 2 tissue compartments, play both roles, that of nucleation and inhibition. These compartments consist of 2 main fractions of chondroitin sulfate; one

fibrous and concerned with nucleation, the other being true ground substance and concerned with inhibition of calcification. However, other experiments have been carried out to further clarify the role of chondroitin sulfates using the uptake of ^{35}S as an indicator of mucopolysaccharide formation and ^{32}P as an indicator of calcification. It was found that the fall in S compounds did not occur in the same zone as the initiation of calcification. When glucose was added to the medium, ^{35}S uptake was increased whereas the enzyme inhibitor phloridzin reduced it. However, calcification was unaffected in both cases suggesting that mineralization is independent of mucopolysaccharide degradation[154]. Recent work on cathepsin D_1, which is thought to be one of the enzymes involved in the degradation, show it is not inhibited by most of the chemical agents which inhibit other proteinases.

Thus, the role of glycosaminoglycans in calcification still remains an open question.

Role of Lipids

In areas of teeth and bone where calcification is occurring, a substance which stains as a lipid has been detected. It exists in a form which is resistant to stain and must be treated with pyridine or alcohol to unmask it, that is, these solvents must remove a substance which protects it from the stain[155]. Wuthier[156] studied the distribution of this lipid component in the growth plate of bone and found its concentration increasing toward the active calcification site and subsequently decreasing dramatically when calcification was complete.

This lipid disappears in vitamin D deficiency and reappears when vitamin D is administered. Many workers have shown that the vitamin encourages the incorporation of phosphorus into phospholipids.

Cotmore et al[157] provided further evidence to the concept that phospholipids are involved in biological mineralization when they studied the calcium binding properties of phospholipids. Submicroscopic spherules were observed to form when Ca^{+2} and PO_4^{-2} ions combined with phosphatidyl serine, an acid phospholipid which Wuthier[156] isolated and characterized from calcifying cartilage.

Cellular Activity in Mineralization

Mitochondria: Isolated mitochondria were found, by Vasington and Murphy[158,159] and independently by De Luca and Engstrom[160] to accumulate large net amounts of Ca^{+2} from the suspending medium during electron transport. They observed that the mitochondria could accumulate the calcium up to several hundred times the initial Ca^{+2} content. Then in 1970, Lehninger[161] proposed that micro packets of amorphous calcium phosphate could be released from the cell and diffuse to specific calcifying sites.

Matthews[162] observed in the growth plate that ^{45}Ca was concentrated in the cells nearest the calcifying front. Subsequently, Martin and Matthews[163] found a gradient in ^{47}Ca concentration in the mitochondria in the chondrocyte. The gradient showed the concentration of ^{47}Ca gradually increasing in the mitochondria from the proliferative zone to that of provisional calcification at which point there was a decrease in mitochondrial calcium granules. In a later experiment, Matthews et al[164] examined and compared the mitochondrial granules in normal as well as rachitic rat epiphysis. They found that in rickets there were few granules in the mitochondria near the calcification front but when vitamin D was administered normal density and distribution of granules was reestablished. They suggested that both an apatite binding matrix and

inorganic phosphate were required by the mitochondria to form such granules. More recently Arsenis[165] found that the mitochondria of the columnar and hypertrophic zones of growth plate cells contained more enzymes which may be associated with calcification than did those of the resting zone.

Vesicles: Recently there has been another interesting development with the identification of vesicles containing crystallites between the hypertrophic chondrocytes in the growth plate as well as in osteoid and predentine.

Anderson et al[166,167] did an extensive study on the vesicles first described by Bonucci[168]. They found such vesicles, which contained or were lipid and had lipid membranes, in the longitudinal septa of the growth plate from the hypertrophic cells downward. These vesicles were closely associated with calcification, neighboring needle-like apatite structures. Their enzyme content was high in alkaline phosphatase, alkaline pyrophosphatase and ATPases; enzymes thought to be connected with calcification. However, they were very low in acid phosphatase and therefore not of lysozomal origin. These enzymes could raise the local content of orthophosphate, destroy phosphate inhibitors and lead to the formation of hydroxyapatite. The vesicles were found to possess a mechanism for ATP dependent transport of calcium and phosphorus into themselves. The crystals were initially closely associated with the inner surface of the vesicular membrane. Peress et al[169] speculate that the membrane serves as a nucleating center for the crystals. Kashiwa and Komorous[170] found mineralized spherules in cytoplasm of chondrocytes, in the matrix adjacent to the hypertrophic chondrocytes and also in the core of the spicules distal to the hypertrophic chon-

droevtes. They hypothesized that perhaps intracellular spherules might be the source of the extracellular ones.

Bernard and Pease[171] in studying intramembranous bone formation, described nucleating sites for apatite as being extrusions from osteoblasts within the osteoid. Crystals were seen to grow epitaxially into the surrounding collagen forming bone nodules which coalesced to form bone seams. Between coalescing nodules, fully formed collagen was observed.

Eisenmann and Glick[172] studied the calcification process in rat incisor teeth. They observed that the first crystalline material appeared in small round membrane-bound bodies interspersed among, but distinct from the collagen fibrils of predentine matrix. These crystal-containing bodies appeared to be cellular in origin and can be seen only in the very early stage of mineralization. Crystals were later observed to radiate beyond the crystal bodies and only then appear to be associated with collagen fibers.

Bernard[173] studied developing molars of mice and observed that the initial crystal formation occurred, in and adjacent to, cellular extensions of the odontoblasts into the predentine. When crystal growth was more extensive, the plasma membranes of these cellular extensions disappeared. Crystals growing from the calcification loci became spheroidal and coalesced to form mantle dentine. Later Bernard and Pease[171] observed that these vesicles budded off from osteoblasts and odontoblasts. Bernard believes that the involvement of structural protein is secondary to initial crystallization and that protein orients and structures the size of crystals rather than organizes their nucleation.

Role of Collagen

Since collagen fibrils and apatite crystallites have a close physical relationship, it is thought that collagen can act as a seed for the initiation of calcification. Termine et al[174] found that there is a direct physical binding between collagen fibrils and apatite crystallites.

Reconstituted collagen fibers act as a seed when placed in a solution supersaturated with respect to calcium and phosphate (epitaxy). Only native type fibrils having 640 Å banding are able to act as a nidus[175]. Due to the exceedingly minute size of the first crystals laid down and the tight packing of the collagen fibrils observed in most species, it is difficult to detect by electron microscopy whether the crystals are located on, between or within the fibrils when seeding begins. However, once the crystals are detectable, they are seen within the collagen fibrils, 640 Å apart mainly in the region of the interband thus showing a relationship to the bands, that is, to some structure having a period similar to the bands of collagen.

Collagen macromolecules are staggered by one quarter of their length giving rise to "holes" at regular intervals which correspond to the interband (see section on Collagen biosynthesis, p. 14). Glimcher and Krane[153] believe these holes permit the packing of collagen fibrils with calcium phosphate crystals. This ability collagen has to act as a seed may be due to the presence of specific sites. In other words, the initial crystallites have a specific spatial relationship to the collagen fibers. However, evidence of the exact physical relationship between the fibres and crystals at the initial crystallization phase is lacking. Hohling et al[176] found 6-7 sites for nucleation in the hole zone and half as

many in the overlapping zone of dentine and bone collagen. Davis and Walker[177] expound that the carboxyl group of glutamic acid and aspartic acid may be the nidus. They found that if glutamic acid and aspartic acid of decalcified bone and dentine were converted to glutamine and asparagine, recalcification was completely blocked whereas the modification of lysine, hydroxylysine, histidine and arginine residues had no effect. Glimcher[153], on the other hand, thought that certain ϵ-amino groups of lysine and hydroxylysine were nucleation centers. However, Davis (unpublished work) disagrees as he found that there was no effect on the rate or extent of calcification when the lysine and hydroxylysine were completely modified with ethoxyformic anhydride or acrylonitrile.

Katz and Li[178] have postulated that the molecular packing of collagen from different tissues may be a factor in calcification. They studied adult bovine dentine, adult rat bone, adult rat tail tendon and purified reconstituted steerskin collagen which they found to be compatible with the theory that collagen is packaged into hexagonal units containing 7 molecules (in cross section)[179]. Most importantly they observed that the intermolecular gap in bone and dentine was 6 Å whereas in tendon it was 3 Å. Phosphate ions, having a diameter of 4 Å, could therefore penetrate the bone and dentine by ionic diffusion but not the tendon.

Collagen Types: Recent studies on the chemistry of collagen have shown differences in its composition when taken from different sources (see section on Types of collagen, p. 25). Miller et al[4] have studied the composition of the different types of collagen monomers. They found that the α 1(I) and α 1(II) cyanogen bromide peptides differed in

amino acid content such as 4-hydroxyproline, aspartic acid and leucine. They also learned that the collagen of mature skin and bone was of the same genetic type, $\alpha\ 1(I)_2\alpha 2$[142,180] and that the collagen of cartilage was of the $\alpha\ 1(II)_3$ type[181]. It was observed that the type which calcifies is the $\alpha\ 1(I)_2\alpha 2$[182]. Toole et al[182] studied the collagen of rachitic osteoid (uncalcified layer between the osteogenetic fibers and bone) and found it to be of the $\alpha\ 1(I)_2\alpha 2$ type, like that of skin and bone but with one important difference. It had 50% more hydroxylysine than normal bone which they suggested could be glycosylated thus blocking calcification by steric interference with apatite crystal formation in the hole region of the collagen molecule.

Recently, Linsenmayer et al[183] studied the temporal and spatial transitions in collagen types during embryonic chick limb development. They perceived that at early stages of leg mesenchyme development, an $(\alpha 1)_2\alpha 2$ type collagen is produced. At a later stage, the limb cores began synthesizing $(\alpha 1)_3$ type collagen, while the outer portion of the limb still produces $(\alpha 1)_2\alpha 2$. At the stage of bone production, the $(\alpha 1)_2\alpha 2$ type collagen reappears. The $(\alpha 1)_2\alpha 2$ thereafter predominates in bone and $(\alpha 1)_3$ in cartilage. Although the reason for the different types of collagen is still unknown, it can be supposed that this may be related to specific functions and therefore perhaps to calcification.

Phosphoprotein: Soft tissue collagens have a similar amino acid and carbohydrate composition as that of calcified tissues. However, one major difference between the two is that calcified tissue collagens possess a phosphoprotein moiety which soft tissue collagens do not possess [184].

In 1964, Veis and Schleuter[185] isolated this phosphoprotein in

dentine and found it to be, at least partially, a phosphate bound to a serine as phosphoserine. They tentatively postulated that perhaps this phosphoprotein plays a role in the nucleation of mineralization as well as stabilizes the structure of collagen. Subsequently they further characterized the phosphoprotein finding it to be rich in aspartic acid, glutamic acid, depleted in hydroxyproline as well as possessing some hexose[186]. They also found it to be electrophoretically composed of a slow (S) and a fast (F) anionic component. They thought it to be localized in a few chain segments of the collagen molecule. Later Veis and Perry[187] characterized these S and F components. The S component, having a molecular weight of about 25,000, was found to be similar to the parent collagen and that the glycine, alanine, proline and hydroxyproline content made up 63% of the molecule. Also, it was rich in aspartic acid, glutamic acid and serine. The F component, found to be homogeneous by electrophoretic and ultracentrifugal techniques, was distinctly different, being rich in aspartic acid and serine but having small amounts of glycine, alanine and proline. It was of collagenous origin as hydroxyproline and hydroxylysine were present. The F component which was the phosphate rich component had a molecular weight of about 38,000 and contained almost all of the hydroxylysine which survived the periodate oxidation. In order for the hydroxylysine to have resisted periodate oxidation, its amino or hydroxyl group must have been involved in a covalent bond, otherwise it would have yielded ammonia and formaldehyde. Since the disaccharide glucosylgalactose is known to be linked to hydroxylysine[188], Veis and Perry proposed the F component to be composed of three parts: the first section comprised of proline, hydroxyproline and hydroxylysine among other amino acids, as

part of the peptide backbone of dentine collagen; the second portion, an oligosaccharide linked glycosidically to hydroxylysine to which the third part, the phosphoprotein moiety is covalently linked. They found the phosphoprotein to be present in an amount of less than 2% of the total protein or in a ratio of one molecule of phosphoprotein to four to six molecules of collagen. Therefore, this phosphoprotein could serve as the site for nucleation in the hole zone of the collagen fiber.

Carmichael et al[189] later isolated glucose and galactose from degradation products corresponding to the phosphoprotein and suggested that perhaps the anionic moiety is attached to the collagen disaccharide through a serine phosphate diester linkage.

Subsequently, Butler et al[190] found that the phosphoprotein could be extracted via a gentle non-hydrolytic extraction procedure. They decalcified rat incisors in acetic acid and followed this by extracting the decalcified tissue in Tris-NaCl. The Tris-NaCl solubilized most of the phosphoprotein thus providing evidence that the phosphoprotein could not be covalently bound. Davis and Walker[177] support Butler's view that phosphoprotein is not necessary for mineralization, as they were able to get dentine to calcify at the same rate and extent once the phosphoprotein was removed by Tris-NaCl extraction.

Carmichael and Dodd[191] proceeded to do a Tris-NaCl extraction followed by periodate oxidation on bovine dentine. They found that half the phosphoprotein was extracted in the Tris-NaCl but that the other half required periodate oxidation to release it. They also found that the carbohydrate content was greater in the Tris-NaCl extract material than in the periodate solubilized fraction. This they suggested may reflect the degree of degradation occurring during periodate oxidation.

Veis et al[192] characterized the Tris-NaCl soluble and the matrix bound phosphoprotein, finding an overall similarity between the two. They suggested that the soluble and the matrix bound phosphoprotein may serve a dual role in locating the deposition of mineral on the collagen matrix and inhibiting calcification of predentine. Recently, its possible role in locating the deposition of mineral has received support from Weinstock and Leblond's[193] elegant radioautographic studies on the deposition of phosphoprotein at the mineralization front of dentine. They showed the phosphoprotein crossed the predentine zone into the mineralizing front thus suggesting it may play a role in mineral deposition.

Up to now, phosphoprotein has been chemically shown to be present in bone and dentine. Although it has been seen radioautographically in a transitory state in predentine, the tissue has never been chemically analyzed for it and nor has cementum. If the theory, that phosphoprotein plays a role in mineralization is valid, then cementum as a calcified tissue should possess it. Predentine, being a tissue which will mineralize, should contain it only at the mineralization front. As a result, predentine and cementum were analyzed to see if phosphoprotein is a necessary component of calcified tissues.

With the current theories and experimental evidence, mineralization can be explained in general terms. Cellular activity is certainly important. However, the involvement of the mitochondria and its vesicles does not explain how the mineral is able to penetrate the collagen fibers. It must be remembered that 60-80% of the mineral is within the collagen and that the intermolecular gap in the collagen fiber of bone and dentine is just large enough to accomodate a phosphate ion. Perhaps

the mitochondria and/or vesicles bring high concentrations of calcium and phosphate to the collagen fiber and deperding which way the collagen fibers are packed, and what type it is, the phosphate is able to penetrate the intermolecular gap along with the calcium. Phosphoprotein and/or functional groups such as the carboxyl groups of glutamic acid and aspartic acid, act as the nidus for the initial crystal formation. The mitochondria and/or vesicles may then bring high concentrations of calcium and phosphate ions (or some compound containing these species) to the calcifying matrix allowing mineralization to continue on the exterior of the fibers.

Not much is known about how calcification is inhibited or controlled, what causes the local build up of calcium and phosphate nor why some tissues calcify more than others. From the above discussion, it is obvious that there is still much to be learned about the finer details of the process of mineralization.

VII. COLLAGEN: A GLYCOPROTEIN

Glycoproteins are proteins which have variable amounts of carbohydrate covalently linked to their peptide portion. They do not have characteristic amino acid compositions, although collagen is an obvious exception. On the other hand, their sugar components are distinctive and include D-galactose, D-mannose, D-glucose, L-fucose, D-xylose, L-arabinose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and the N- and O-acetyl and N-glycolyl derivatives of neuraminic acid.

Collagen's carbohydrate unit is an α-1,2-glucosyl-galactose disaccharide[194-196]. As in almost all glycoproteins collagen contains its carbohydrate units in varying stages of completion or with minor

modifications (microheterogeneity). The type of glycopeptide bond in collagen is a β -glycosidic linkage between galactose and the hydroxyl group on the γ carbon of hydroxylysine[188,195,197]. It is an unusual linkage as it involves a glycosidic substitution onto a hydroxyl group vicinal to an unsubstituted amino group. In other glycoproteins, asparagine, serine and threonine are the residues to which the carbohydrate units are usually linked[198]. Collagen is unique in that only hydroxylysine is involved in the carbohydrate linkage. There is one exception. The carbohydrate units of annelid cuticle collagen are linked to serine and threonine and consist only of galactose mono-, di- and trisaccharides. This collagen contains no hydroxylysine but much larger amounts of serine and threonine than do other collagens[199,200].

Butler[201] has isolated the cyanogen bromide peptide which is rich in carbohydrate; the α 1-CB5. It is the fourth cyanogen bromide peptide from the amino terminal end and its probable sequence in the carbohydrate linkage region in guinea pig skin is -Gly-Met-Hyl(Glc,Gal)-Gly-His-Arg[188]. Morgan et al[202] more recently determined the sequence in carp swim bladder and human skin collagen and found it to be only slightly different from that of the guinea pig skin isolated by Butler.

Skin and dentine collagen contain less than 1% hexose whereas cartilage collagen contains up to 5% hexose. In skin, approximately half of the hexose is present in the disaccharide form[4].

The effect of the carbohydrate in collagen is still speculative. It is thought that the density of carbohydrate units may have an effect on the organization of collagen into fibrils. Tissues such as basement membranes having the largest number of hydroxylysine linked carbohydrate units show the least organization and no fibrillar structure. On

the other hand, tissues such as skin, tendon and sclera have the smallest number of hydroxylysine-linked carbohydrate units and display wide fibrils with distinct 640 Å banding. Tissues with intermediate amounts of hydroxylysine-linked carbohydrate units, such as corneal collagen, have narrow fibrils even though they possess 640 Å banding[194,202,203]. Morgan et al[202] feel that due to the size of the disaccharide prosthetic group, regular hexagonal packing of collagen in a quarter-stagger array could seriously be impaired.

The role of hydroxylysine linked carbohydrate units in collagen is as yet unapparent. Eylar[204] proposed some time ago that proteins are glycosylated so as to provide a passport for excretion from the synthesizing cell. Winterburn and Phelps[205] dispute this hypothesis and hypothesize that the carbohydrate tag determines the fate of the protein once it is extracellular. An example of this comes from Veis and Perry[187] who proposed that the phosphoprotein linked to the carbohydrate units in calcifying tissues may serve as a nucleation site for the initial epitactic nucleation of calcium ions (see section on Mineralization, p.35).

Spiro[207] has speculated that the sugars attached to hydroxylysine could possibly play a regulatory role indirectly, that is, the carbohydrate units may be attached to the hydroxylysine so that the residue could not participate in the types of cross-links occurring in collagens; this effect occurring due to steric hindrance. However, Eyre and Glimcher[208,209] and Robins and Bailey[206] have found evidence for a glycosylated cross-link in collagen. Therefore, steric hindrance of cross-link formation apparently does not occur.

As no work was undertaken on the glycosaminoglycans of predentine and cementum in this project, the topic will not be discussed here.

For a review on the subject see reference 210.

Proteoglycans and glycoproteins - Terminology: To understand the histochemical studies which are discussed below, a brief explanation of the terms glyc[redacted]an, proteoglycan and glycoprotein are given first. Protein-ca[redacted]mplexes in which the components are covalently link[redacted]sues. The properties of the complex, depending on [redacted]portions of the protein and carbohydrate, will be more [redacted]e peptide or sugar component. Such influences can be seen i[redacted]ycans and glycoproteins.

[redacted]glycans are protein-carbohydrate complexes in which polysacchari[redacted]ke up as much as 90% or more of the complex. The protein component[redacted]on-collagenous and the carbohydrate, called glycosaminoglycans ([redacted]mes called mucopolysaccharide), are polysaccharides containing he[redacted]ne. These polysaccharides include hyaluronic acid, chondroitin-[redacted]ulfate, chondroitin-4-sulfate, keratan sulfate, dermatan sulfate, hepar[redacted]sulfate. The polysaccharide chains are unbranched except perha[redacted]arin sulfate and they also contain uronic acid except keratan[redacted]te. If present in the free form, the constituent polysacchari[redacted] chains show little affinity for collagen, apart from the ionic interaction which is fairly easily broken. However, when the polysaccharide chains are linked to the non-collagenous protein, the large molecules associate more firmly with the collagen.

Glycoproteins, of which collagen is a special case, are a covalent compound of non-collagenous protein and sugar chains. The sugar chains are sometimes branched and do not contain more than 20 residues. A large variety of sugars are found in glycoproteins. These include hexosamines and galactose (which is found in only one proteoglycan, that

containing keratan sulfate) as well as mannose, fucose and sialic acid, the latter two often occupying terminal positions on the oligosaccharide chains. Glycoproteins do not contain uronic acid but a few appear sulfated like most of the glycosaminoglycans and proteoglycans.

VIII. DENTINE, PREDENTINE AND CEMENTUM: The Present State of Knowledge

Teeth are composed of three calcified tissues: enamel, dentine and cementum. Dentine forms the bulk of the tooth and encloses the pulp cavity. Enamel envelops the dentine of the crown whereas cementum covers the root dentine. In calcification of dentine, a collagenous matrix must be laid down first. The collagen fibers, arranged in a trellis-like framework, are produced by the odontoblasts which line the pulpal surface of the dentine. Since mineralization lags behind matrix formation, there exists a zone of uncalcified tissue called predentine especially noticeable at the apex of the immature tooth[211].

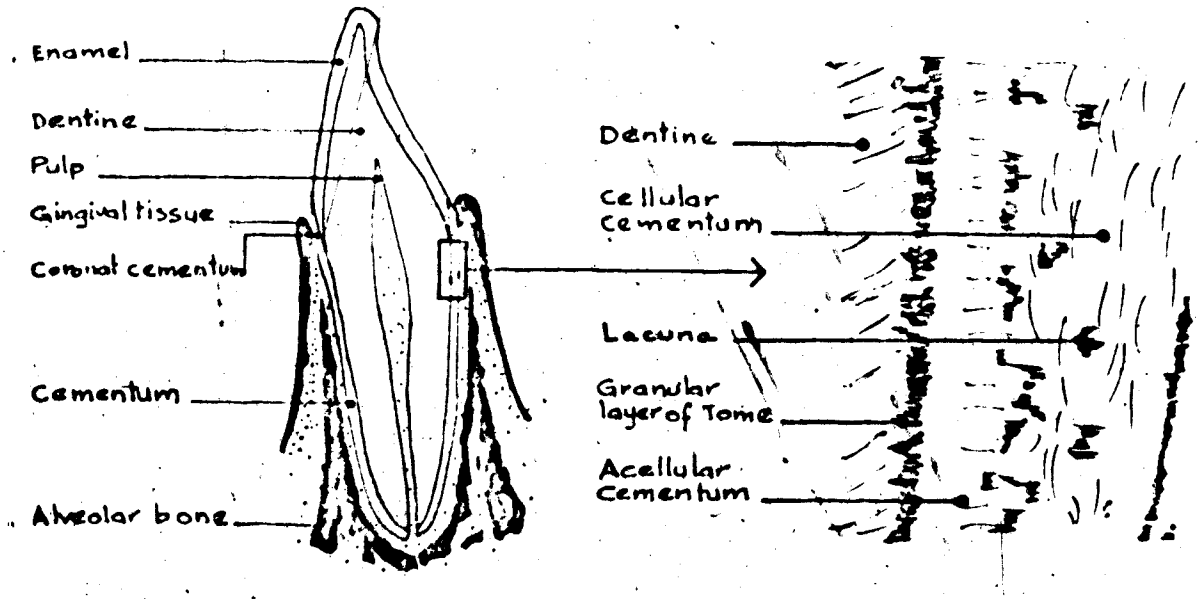


Fig. 5. Relation of the main dental tissues

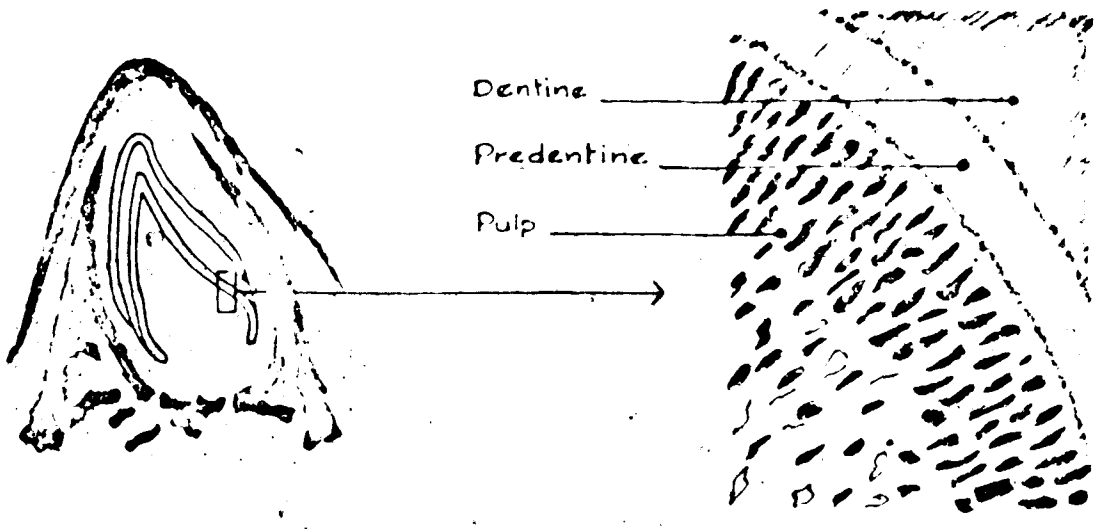


Fig.6. Diagrammatic illustration of an unerupted tooth

Dentine and Predentine: Little is known about predentine due to the small amount of tissue in teeth. As a result most studies on predentine have been histochemical in nature.

Considerable work has been done on dentine. Wislocki et al[212] were the first to observe histologically the presence of mucopolysaccharides in dentine using the periodic acid-Schiff (PAS) technique. Subsequently, using the same technique, Matthiessen[213] expanded these results to include predentine which he found to be PAS positive. Other histochemical investigations have shown significant amounts of acid mucopolysaccharide in predentine. Bevelander and Johnson[214] as well as Weill[215] found predentine intensely metachromatic. However, Greulich and Leblond[216] found that predentine stained weakly compared to dentine,

indicating a relative absence of glycoprotein in predentine. This has been the observation of several other workers[217-219]. Fullmer and Alpher[220] were unable to detect any glycoprotein in predentine.

Recently Weinstock[221], using the acidified phosphotungstic acid technique[222] for glycoprotein, observed that the stained material in dentine occurred between and associated with the collagen fibers. In predentine the interfibrillar material was unreactive although the collagen fibers did stain slightly. Weinstock suggests that glycoprotein may play a role in the initiation of calcification as predentine acquires a carbohydrate rich material, probably glycoprotein, as it is transformed into dentine.

The proteoglycans of dentine have been [redacted] subject of several recent studies. The limiting factor in these studies has been their low concentration in dentine. Clark et al[223] found that the major acid glycosaminoglycan of human dentine-cementum was chondroitin-6-sulfate. They also reported a small amount of hyaluronic acid (2% of the total hexosamine) and were unable to detect keratan sulfate and dermatan sulfate. Engfeldt and Hjerpe[224] have more recently characterized the glycosaminoglycans of both canine predentine and dentine. The major polysaccharides in both these tissues were galactosaminoglycans tentatively identified as chondroitin sulfate. They could not identify a small amount of material which corresponded to the amount of hyaluronic acid Clark et al had isolated from human dentine-cementum. Thus Engfeldt and Hjerpe could not conclude that hyaluronic acid was excluded from predentine and dentine. These authors reported that the glycosaminoglycan hexosamine content of dentine was 0.06% of the organic dry weight and that of predentine was 0.12%. Their results indicate that there is more

chondroitin-6-sulfate in predentine than dentine. Jones and Leaver attempted to clarify the conflicting reports concerning the nature and amounts of the constituent glycosaminoglycans of dentine. They demonstrated that the major glycosaminoglycan of human dentine is chondroitin-4-sulfate but it was suggested that the chondroitin-4-sulfate:chondroitin-6-sulfate ratio may vary with age since chondroitin-6-sulfate may be lost during maturation. Jones and Leaver also found 2-6% of hyaluronic acid, 2-3% of dermatan sulfate and a definite but small amount of keratan sulfate in human dentine.

Linde[226] has recently isolated the glycosaminoglycans of the odontoblast predentine layer of porcine teeth. The total amount of hexosamine was found to be 0.29% of the dry weight. This figure includes hexosamine from the glycoprotein and glycosaminoglycans. He obtained five hexosamine containing fractions when the glycosaminoglycans were separated on CPC cellulose micro columns, each of which was predominantly composed of one of the following components: chondroitin-4-sulfate (30% of the total), chondroitin-6-sulfate (16%), hyaluronic acid (14%), dermatan sulfate (7%), and keratan sulfate and glycoproteins (32%). The keratan sulfate was found to account for about 4% of the total hexosamine.

Veis and coworkers[185,187,192,227] have also done considerable work on dentine. They found that, although dentine collagen resisted acid swelling and had no neutral salt soluble or acid soluble collagen, there were few differences in composition from corium (dermis) collagens except for the presence of phosphoprotein in dentine (see section on Mineralization, p. 35). They reported that the two collagens contained a comparable quantity of hexose (2.2 residues/1000 amino acid residues).

Recently Volpin and Veis[228] have studied the CNBr peptides of skin and dentine collagens. Except for variations in hydroxylation of proline and lysine, they found few differences of significance between the CNBr peptides derived from the α 1(I) and α 2 chains of insoluble bovine corium, dentine collagen and the corresponding CNBr peptides from the soluble bovine collagens. However, in the insoluble bovine corium and dentine collagens, three distinct new classes of CNBr peptides were detected. Both collagens were found to contain an acid stable intermolecular cross-link component, α 1-CB6' [α 1-CB6 + α 1-CB(0, 1)]. Both also contained two additional peptides attributed to the presence of an α 1(III) chain, the α 1(III)-CB3 and α 1(III)-CB (4,5). Evidence was also obtained indicating the presence of a peptide α 2-CB4' consisting of α 2-CB4 and a noncollagenous polypeptide attachment. Both corium collagen and dentine collagens were analyzed for hexose and bound phosphate groups. In dentine collagen, only α (I)-CB5 contained the glucosegalactosyl hydroxylysine, while α 1(I)-CB3 contained only the galactosyl hydroxylysine which was also present in α 1(I)-CB5. The α 2-CB4 and α 2-CB5 contained mixtures of mono- and disaccharide attachments. In addition, Volpin and Veis were able to isolate from the dentine collagen α 1(I)-CB6, α 2-CB4 and α 2-CB5 containing covalently bound phosphate groups which were not found in corium collagen. It seems likely that the hexose is present as a hexose phosphate rather than the phosphate attachment being via a serine hydroxyl. The acid-insoluble CNBr digestion residues were found to contain portions of collagen with uncleaved methionyl residues in close association with a highly acidic polypeptide. This was particularly true in dentine collagen where this polypeptide was rich in phosphoserine groups.

As mentioned above, Veis and Schleuter[185] have identified in dentine an acidic peptide containing a high content of organically bound phosphorus. Weinstock and Leblond[193] found that this phosphoprotein passed through the predentine to reach the mineralization front possibly playing a role in calcification at the dentine-predentine junction.

Cementum: Cementum, the root covering of the tooth, is the calcified dental tissue which most resembles alveolar bone in its composition, structure and behaviour. It provides a medium for the attachment of the collagen fibers of the periodontal ligament that bind the tooth to the surrounding structures as well as playing a role in preserving the width of the periodontal ligament. It assists in maintaining the length of the root available for support of the tooth and effects repair of damage of the root[229].

Cementum is formed in much the same way as dentine. Cementoblasts lay down an uncalcified collagenous tissue called cementoid or precementum which is subsequently mineralized to form cementum. However, since calcification is not as rapid as matrix formation, a thin layer of cementoid tissue is seen on the surface of the cementum.

Two types of cementum can be differentiated morphologically depending on the presence or absence of cells and they are therefore known as acellular (primary) cementum and cellular (secondary) cementum. See Fig. 5. Acellular cementum may cover the root dentine from the cementoenamel junction of the apex, but is often missing on the apical third of the root. The more predominant cellular cementum usually covers the apical root dentine and acellular cementum and includes cementocytes representing cementoblasts which have been left behind and surrounded during cementum formation.

Collagen fibrils of cementum are orientated two ways[230,231]. Sharpey's fibers, the group of fibers which run at right angles to the cementum surface, originate from the periodontal ligament and are incorporated in the cementum as it mineralizes. The second group of fibers are thought to be produced by the cementoblasts. These fibers are not directly attached to the bone but are fundamental to the inherent structure of cementum. The two groups of fibers tend to run at right angles to one another.

Cementum is less calcified than dentine. Selvig and Selvig[232] found that the combined calcium plus magnesium content of human cementum was in the range of 25.7 to 26.6% whereas that of dentine was 26.8 to 27.6%. The phosphorus content of cementum ranged from 11.8 to 12.5% compared to 12.2 to 13.2% in dentine. Neider et al[233] report similar findings. Selvig also observed differences in the distribution of mineral in the matrix fibers of cellular cementum as well as in those of acellular cementum. The acellular cementum, he noted to be more calcified than the cellular form, the latter also containing uncalcified cores of Sharpey's fibers.

Glimcher et al[234] have determined the amino acid composition of coronal cementum (see Fig. 5) which was found to be characteristic of collagen, that is, the organic matrix contains at least 90% collagen. Cementum matrix collagen resembles bone and dentine collagens in that it is virtually insoluble in neutral and acidic buffers.

The non-collagen constituents of cementum have not been characterized nor has its collagen been studied in great detail. For example, it has yet to be determined whether the phosphoprotein moiety found in

bone and dentine, which is thought to serve as the nucleation site for mineralization, occurs in cementum[185,190,191,184].

From the above it is apparent that further chemical analyses are required to gain additional insights on predentine and cementum.

I. MATERIALS

Fresh bovine jaws were obtained from animals between 1 and 2 years old at slaughter. Unerupted and erupted incisors and canines were extracted as well as unerupted molars. The unerupted teeth were freed of their dental sacs, and the pulps were removed. These as well as the erupted teeth were then washed by soaking in distilled water at 4° overnight.

Predentine was obtained from the unerupted teeth by cutting away the translucent zone at the apex of the root. The fragments were then rewashed in distilled water and lyophilized. The predentine was subsequently dissected under a dissecting microscope to remove the adhering odontoblasts and the opaque dentine.

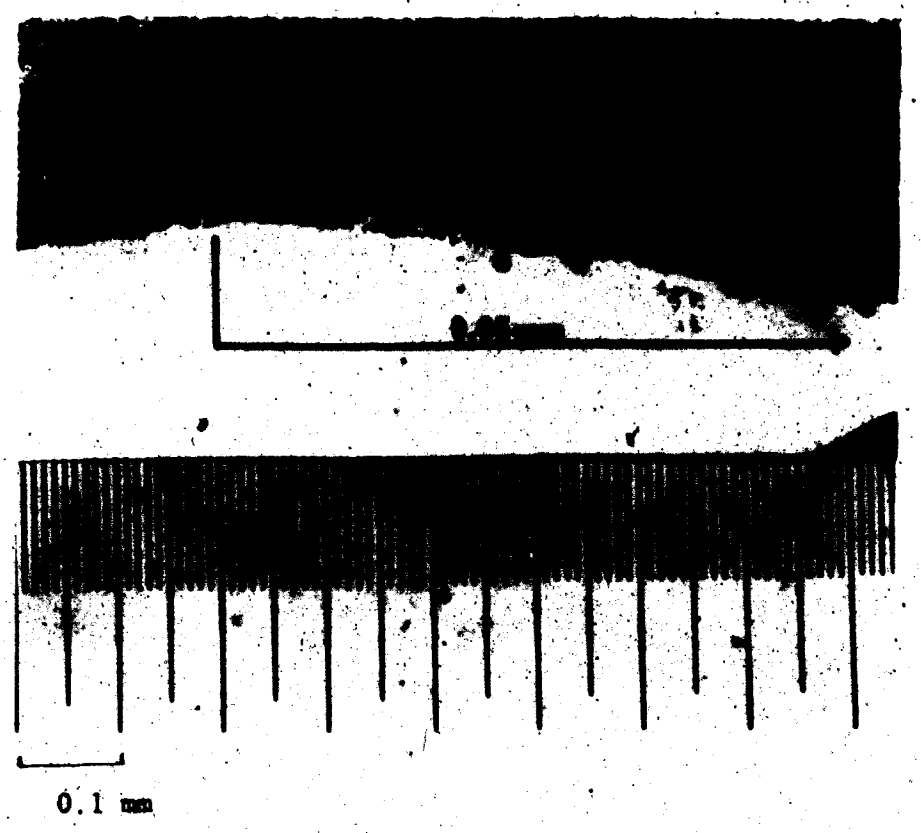


Fig. 7. Hemotoxylin-eosin stained predentine showing mineralization front. The length of the predentine region is indicated. (By courtesy of Dr. K.A. McMurchy)

The erupted teeth were scraped clean of any adhering soft tissue. The crowns were sectioned below the cervical line with a Gillings Hamco Fine Sectioning Machine. The root was then sliced longitudinally into thin lamellae of about 250 microns. Under the dissecting microscope, the somewhat more translucent cementum, which appears as a thin layer next to the dentine, was carefully separated from the dentine and then lyophilized.

II. METHODS

Density Gradient Isolation of Cementum

To ensure that the cementum was not significantly contaminated with dentine, the Manly and Hodge[235] flotation method was performed on a sample of cementum. The sample was ground dry in a Heidelberg Colloidal Mill, and the resulting powder passed through a 60 μ sieve. The powder was then centrifuged in a mixture of bromoform and acetone having a specific gravity of 2.11. The sample separated into a light (cementum) and heavy (dentine) fraction. As an additional check for purity, the heavy fraction was examined for dentinal tubules under the light microscope.

Calcium

In order that any organically bound calcium might be freed, the calcium content of predentine and cementum was determined following acid hydrolysis in 10N H_2SO_4. The calcium was determined by EDTA titration according to the method of Ericsson[236]. This method involves the firm binding of calcium in an un-ionized complex with EDTA. A small amount of $MgCl_2$ (.4 mls of 1mM solution) was added so that the indicator, Eriochrome Black T, would shift from red to blue when all Mg ions had

been bound by the EDTA titration solution as with calcium alone the color shift is too gradual and indistinct.

Phosphorus

The phosphorus content was determined using the modifications by Eastoe[237] of the Fiske and Subbarow[238] method with the exception that the amino-napthol sulfonic reagent was prepared according to the procedure of Bartlett[239]. This method is based on the reduction of phosphomolybdic acid by 1-amino-2-napthol-4-sulfonic acid which is blue in the presence of sulfite.

Decalcification

The predentine and cementum fragments were demineralized by repeated extraction at 4° in 0.5M EDTA adjusted to pH 7.4 with NaOH. Extraction of cementum continued for 17 days and predentine, which contained little calcium, for 4 days, at which time calcium could no longer be detected in the residual matrix by atomic absorption spectrometry. The method employed here was a modification of the procedure of Willis[240]. Lanthanum was used in the analysis to prevent interference by phosphorus. With this procedure, calcium content cannot be detected below 0.15 parts per million. The residual cementum and predentine were washed with distilled water until EDTA could no longer be detected. The EDTA test involves the Ericsson[236] method where adding Mg to a 5 mls portion of the water wash indicates the presence of EDTA using Eriochrome Black T as the indicator.

Tris-NaCl Extraction

Butler et al[190] showed that phosphoprotein could be removed by a gentle non-hydrolytic extraction with Tris-NaCl. Thus, the decalci-

fied predentine and cementum residues were extracted for 3 periods, each of 24 hours, in 500 volumes of 0.5M Tris-NaCl (pH 7.4) containing 1.0M NaCl. The remaining residues were washed in distilled water and lyophilized. The extracts were dialyzed against distilled water and lyophilized.

Phosphoprotein Determination

Carmichael and Dodd[191] found that not all the phosphoprotein was Tris-NaCl soluble and that some required periodate oxidation to remove it. As a result, the EDTA and Tris-NaCl extracted predentine and cementum matrices were subjected to oxidative degradation for 44 hours with sodium metaperiodate following the procedure of Carmichael et al[189]. The 54 mg of predentine and 71 mg of cementum which were solubilized in 10 mls of sodium metaperiodate (0.025M $NaIO_4$ + 0.025M $NaHCO_3$, pH 7.75) were dialyzed at 4°C against 4 liters of 0.01M sodium bicarbonate for 3 days, changing the sodium bicarbonate twice daily. The retentate was subsequently dialyzed exhaustively against distilled water and then lyophilized. The dried residue of each weighed about 45 mg, and each was analyzed for calcium and phosphorus. Using the procedure of Clark and Veis[241], electrophoresis of 50 µl and 100 µl of predentine (270 µg lyophilized periodate soluble material/ml Tris-Glycine buffer) and cementum (310 µg lyophilized periodate soluble material/ml Tris-Glycine buffer) in a polyacrylamide gel at pH 8.4 and run at 4mA/tube, separated the non-diffusible components. Following electrophoretic separation, the gels were sliced transversely and analyzed for phosphorus.

Determination of Lysine-derived Cross-links

Suspensions of 12 mg each of predentine preparation and cementum

preparation in 1.8 mls of 0.15M saline (pH 7.4) were reduced with 0.2 mls of tritiated sodium borohydride (18.4 mCi/mmole) dissolved in 0.01M NaOH (30:1 w/w ratio of predentine and cementum preparations to $NaBH_4$). The tritiated sodium borohydride was standardized, according to Paz et al[242], by reduction of 4-(p-nitrobenzamido)-butyraldehyde to 4-(p-nitrobenzamido)-butanol which had a specific activity of 5.45 mCi/mmole. The pH was adjusted to 9.0 and the reaction mixture stirred at room temperature for 1 hour. After acidification to pH 4.0 with 50% acetic acid, the reaction mixture was exhaustively dialyzed against distilled water at 4° and then lyophilized.

Acid hydrolysis in 6N HCl under N_2 in sealed tubes at 108° for 22 hours or alkaline hydrolysis in 2M KOH under N_2 in teflon bottles at 108° for 20 hours was carried out on portions of the reduced cementum and predentine preparations. Reduced aldimine cross-links were determined in the acid hydrolysates using the Jeol 5AH amino acid analyzer (0.8 x 45 cm column, LCR-2 resin, 60°) eluting with 0.35M (Na^+) sodium citrate buffer, pH 5.28 at a flow rate of 0.62 ml/min. Reduced cross-link precursors ϵ-hydroxynorleucine and δ, ϵ-dihydroxynorleucine were determined in alkaline hydrolysates using the amino acid analyzer (0.8 x 45 cm column, LCR-2 resin, 54°) eluting with 0.2M (Na^+) citrate buffer, pH 2.93 containing 3% propanol, 0.2M (Na^+) sodium citrate buffer, pH 3.35, and 0.2M (Na^+) sodium citrate, pH 4.10, at a flow rate of 0.80 ml/min. For both the reduced cross-links and the cross-link precursors, fractions were collected using a split stream device, and each fraction was mixed with 10 ml Aquasol (New England Nuclear) and counted in a Nuclear Chicago Mark I Liquid Scintillation System.

Amino Acid Analysis

Samples of predentine and cementum matrices were hydrolyzed in 6N HCl at 110° in sealed tubes under N_2 for 22 hours. Amino acid analyses were carried out using the two-column system on a Jeol 5AH analyzer. No corrections were made for hydrolysis losses.

Separation of Collagen and Non-collagen Glycopeptides[243]

EDTA and Tris-NaCl extracted predentine and cementum matrices were each digested 16 hours at 65° with 0.03 ml of previously activated papain (Sigma, 2x recrystallized) in 4 mls of 0.1M acetic acid sodium acetate buffer (pH 6.0) containing 88 mg of cysteine hydrochloride and 186 mg of EDTA/100 mls. 3.0 mls of the digest were added to a Sephadex G-25 column (fine grade, 33 x 1.5 cm) and eluted with water at room temperature. The flow rate was 45 mls/hr and 2.5 - 3.0 mls fractions were collected. A monitor for non-amino sugars was performed on 0.1 ml of each fraction. This was done by modifying the orcinol-H_2SO_4 method for increased sensitivity; that is, 1.5 mls of 80% H_2SO_4-1% orcinol solution was added to the 0.1 mls portion of each fraction previously diluted to 0.5 mls. Two orcinol positive peuks were detected; peak 1 at the void volume containing the higher molecular weight, non-collagen glycopeptides and the retarded peak 2, containing the collagen glycopeptides[243].

Neutral Sugar Analysis

The fractions containing peak 1 were pooled as well as those for peak 2. The pooled fractions were then lyophilized and hydrolyzed in 2N HCl for 4 hours at 105°. However, before hydrolysis, 25 µg of L-Rhamnose was added to each to act as an internal standard. The hydro-

lysates were neutralized with the bicarbonate form of Dowex 1 x 8 (20-50 mesh beads). The slurries were then poured into small chromatography columns collecting the effluent and several water washes. These were lyophilized and analyzed for neutral sugars on a Technicon Carbohydrate Autoanalyzer. The dry residue was dissolved in 1.0 ml of borate buffer 1 and added to the carbohydrate column (type S chromobeads). A similar buffer gradient as the standard method was used except that the volume per chamber was reduced from 50 ml to 30 mls. The temperature was as in the standard method.

In the standard method, the column was 68 cm x 0.6 cm long which corresponds to an 8 hour run. As a more rapid separation was desired and this column length separates a rather large number of sugars (including disaccharides) some of which were not expected in our analyses, the column length was shortened to 33.5 cm. The sugars of interest, mannose, fucose, galactose, xylose and glucose, were well separated.

Elution was achieved at 45 mls/hr at 53.5° C with the following gradient in a nine chambered autograd: chambers 1 and 2, 30 mls of buffer 1 (0.1M H_3BO_3, pH 8.00); chamber 3, 15 mls of buffer 1 and 15 mls of buffer 2 (0.1M H_3BO_3 + 0.05M NaCl, pH 8.00); chamber 4, 12 mls of buffer 3 (0.1M H_3BO_3 + 0.1M NaCl, pH 8.00) and 18 mls of buffer 4 (0.2M H_3BO_3, pH 8.00); chambers 5, 6 and 7, 15 mls of buffer 4 and 15 mls of buffer 5 (0.2M H_3BO_3 + 0.2M NaCl, pH 9.50); chambers 8 and 9, 30 mls of buffer 5. The effluent was analyzed for neutral sugars with orcinol-H_2SO_4 (70% H_2SO_4-0.1% orcinol, Sigma). This colorimetric system was as in the standard Technicon system for carbohydrates (Technicon Development Bulletin 124). The individual monosaccharides were quantitated by comparing their areas with that of Rhamnose. See Table 8 for a diagrammatic illustration

of typical chromatograms of G-25 Peak 1 and Peak 2. It can be seen that the sugars of interest were very well separated but one or two unknown orcinol positive compounds were detected especially from the G-25 Peak 1 hydrolysate. The final unknown peak immediately following glucose appeared in all chromatograms including those from unhydrolyzed standard mixtures of pure sugars and therefore did not appear to be a hydrolysis artifact. This unknown could possibly be due to degradation products of more than one sugar, formed on the column under the alkaline conditions and elevated temperature.

The color yields in the orcinol method are different for each sugar. The acid hydrolyses losses were different for each sugar. There was also a possibility of mechanical losses particularly during the lyophilization of the resin neutralized hydrolysate. The addition of a known quantity of L-Rhamnose before acid hydrolyses eliminates some of these problems. However, it was necessary to carry out analysis of hydrolyzed standard mixtures of sugars, including L-Rhamnose, in order to obtain reliable colour yields relative to L-Rhamnose.[3] The colour yields relative to Rhamnose also varied to some extent with the total quantity of sugar in a given peak; in other words, the standard curve was not linear. This had to be taken into account when applying the colour factors.

Hydroxyproline

Hydroxyproline content was determined by the method of Stegemann and Stalder[244] on a 0.1 ml portion of the papain digest which was hydrolyzed in 6N HCl at 103° for 23 hours. The hydrolysates were evaporated to dryness in vacuo over P_2O_5 and NaOH. The small amount of acid remaining in the residues were neutralized before the analyses were performed.

The range of this method is from 1 µg to 8 µg hydroxyproline per 2 mls of sample.

1. PREDENTINE

Prior to EDTA extraction, the calcium content of predentine was determined to be 0.4% (Table 7). This value corresponds to a hydroxyapatite content of 1% which in turn corresponds to a phosphorus content of 0.18%. However, the phosphorus content was found to be 0.37%. The predentine fraction was 99% organic material.

The EDTA extract was dialyzed against distilled water and then lyophilized. The dry residue was found to have a phosphorus content of 0.07%. The phosphorus content of the pooled Tris-NaCl extracts was 1.7%, this being considerably lower than the comparable Tris-NaCl extract of dentine.

In the EDTA-Tris-NaCl extracted predentine residue neither phosphorus nor calcium (by the method of Ericsson[236]) could be detected.

When subjected to oxidative degradation with sodium metaperiodate, the EDTA-Tris-NaCl extracted predentine was completely solubilized. The resulting extract, which had been dialyzed first against sodium bicarbonate and then against distilled water, was lyophilized and analyzed for phosphorus. The phosphorus content was found to be 0.02%. Several anionic components were revealed by electrophoresis of this fraction, including a lightly staining band with a mobility corresponding to the phosphoprotein component previously demonstrated in dentine[185]. See Table 9 for gel results. However, no phosphorus could be detected in this band. Total absence of phosphorus in this fraction cannot be regarded as established in view of the small sample size, low phosphorus content of the sample, and low staining intensity of this band. However, the use of a comparable sample size of dentine produced a signifi-

cant amount of phosphoprotein at this position.

The amino acid composition of the EDTA-Tris-NaCl extracted predentine (Table 10) generally resembles that of dentine matrix collagen. The glycine content was reduced from a value of about 320 residues/1000 amino acids to 281 residues/1000. The hydroxyproline content of 90 residues/1000 was also low compared to the normal value of about 100 residues/1000. The proline (174 residues/1000) and the hydroxylysine (12.4 residues/1000) were high as compared to the normal values for proline (about 115 residues/1000) and hydroxylysine (about 4-8 residues/1000) as in skin or dentine collagen.

Chemically reduced aldimine cross-link content as well as cross-link precursor content are recorded in Table 11 accompanied with similar data for dentine matrix collagen. The hydroxylysinohydroxynorleucine content of predentine was 4.8×10^{-1} lysine derived residues/1000, while the hydroxylysinonorleucine content was 3.5×10^{-2}. Thus, the major cross-link of predentine is hydroxylysinohydroxynorleucine. The cross-link precursors, dihydroxynorleucine and hydroxynorleucine, were present in concentrations of 1.2×10^{-2} lysine derived residues/1000.

The neutral sugar results are given in Table 12. The total neutral sugar content of the Sephadex G-25 peaks was considered more accurate when obtained from the sum of the results derived from the autoanalyzer than from the modified orcinol results. The total in each case was expressed as g neutral sugar/14g hydroxyproline. (The hydroxyproline content of the papain digest was used in this calculation as this avoids errors due to the possibility of incomplete digestion of the collagen by papain.) This value gives quantities which are close to a percentage on the dry weight of a pure collagen. The G-25 peak 2 re-

sults (collagen hexose) for predentine collagen corresponded to 4.9g hexose/14g hydroxyproline, of which 52% was galactose and 41% glucose. The hexose of the non-collagen glycoprotein associated with the predentine collagen (G-25 peak 1) corresponded to 2.2g hexose/14g hydroxyproline. This hexose was made up of 20% glucose, 33% galactose, 23% mannose, and 10% fucose.

II. CEMENTUM

Undecalcified cementum, examined by the flotation procedure, contained less than 1% of the heavier "dentine" fraction. However, when scrutinized under the microscope, this heavier fraction was also found to be mainly cementum.

Cementum was shown to have a calcium content of 26.3% by weight (Table 7). This percentage of calcium corresponds to a hydroxyapatite content of 66% and an organic content of 34%. Phosphorus content was determined to be 11.4%, and hence the Ca/P ratio is 2.3 (w/w). The Ca/P ratio of hydroxyapatite is 2.2 (w/w). Thus, the mineral of cementum is hydroxyapatite, like that of other calcified tissues.

The phosphorus content of the EDTA extract was 0.14%, whereas that of the pooled Tris-NaCl extracts was 1.76%. No phosphorus or calcium could be detected in the decalcified Tris-NaCl extracted cementum.

Decalcified Tris-NaCl extracted cementum was completely solubilized when subjected to oxidative degradation with sodium metaperiodate. The resulting extract was dialyzed against 4 liters of 0.01M sodium bicarbonate, and then exhaustively dialyzed against distilled water. The retentate was lyophilized and the calcium content was determined to be 0.3% and the phosphorus content to be 0.03%. Upon electrophoretic examination, a large diffuse anionic component and a discrete band with a

mobility approximately that of the phosphoprotein found in dentine were revealed. See Table 9. The gels were analyzed for phosphorus and found to be free of this element. Since the sample size was small and the consequent component concentrations were low, a control experiment was set up with a comparable weight of decalcified Tris-NaCl extracted dentine. The periodate extract was analyzed just as that of the cementum, and then lyophilized. The dried residue was then subjected to polyacrylamide gel electrophoresis, and the phosphoprotein fraction was analyzed for phosphorus. The presence of phosphorus was clearly established in this fraction.

Amino acid analysis of the decalcified Tris-NaCl extracted cementum matrix revealed its composition to be characteristic of collagen (Table 13). The arginine (57.6 residues/1000 amino acids) and the proline (156 residues/1000) are higher than in bone and dentine.

Table 11 shows the contents of reduced aldimine cross-links and cross-link precursors, jointly with similar results for dentine matrix collagen. The cross-link, hydroxylysinohydroxynorleucine, was present in the concentration of 2.6×10^{-1} lysine derived residues/1000 whereas hydroxylysinonorleucine was detected to be 5.2×10^{-2}. Thus, the major cross-link of cementum is hydroxylysinohydroxynorleucine. The cross-link precursor, dihydroxynorleucine, was found to occur in concentrations of 3.2×10^{-2} lysine derived residues/1000 and hydroxynorleucine content was 1.1×10^{-2}.

The neutral sugar results are given in Table 12. The G-25 peak 2 result for cementum collagen was 1.5g hexose/14g hydroxyproline of which 62% was galactose and 38% glucose. The hexose of the non-collagen glycoprotein associated with the cementum collagen (G-25 Peak 1)

corresponded to 0.9μ hexose/14μ hydroxyproline. This hexose was composed of 40% galactose, 26% mannose, 7% fucose, 16% glucose, and 11% of an unknown.

DISCUSSION

Few chemical analysis have been carried out on predentine. Most studies have been histological in nature apart from the recent studies on glycosaminoglycans. Cementum has not been studied chemically in great detail either. Selvig[245] has studied its genesis and fine structure histologically as well as with the electron microscope and determined its mineral content. Glimcher[234] has reported the amino acid composition of the less typical coronal cementum whereas Birkedal-Hansen et al[246] have reported the amino acid composition of bovine cementum. As a result further chemical analysis were carried out on these tissues in order to acquire more information about their nature. These included four major analyses: reducible cross-link, amino acid and neutral sugar determinations and an investigation of the occurrence of phosphoprotein in predentine and cementum.

I. PHOSPHOPROTEIN

Predentine: The inorganic crystals of teeth closely resemble hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. From its molecular weight of 1000, the calcium content of hydroxyapatite is calculated to be 40%. The calcium content of predentine (0.4%) therefore corresponds to 1% hydroxyapatite. Based on this figure of 1% hydroxyapatite one can now estimate the phosphorus content of predentine to be 0.18%. However, upon analysis, predentine was found to contain 0.37% phosphorus. This additional phosphorus may be organically bound and/or inorganic.

Veis and coworkers[185,187,189] have isolated, from decalcified bone and dentine matrix, a highly anionic phosphoprotein which is covalently linked to the collagen. They suggested it may provide the

sites for the epitactic nucleation of mineralization for the matrix. This phosphoprotein was also found in Tris-NaCl extracted decalcified dentine matrix[191]. Thus it was logical first to extract this soluble phosphoprotein from predentine. However, once the predentine had been extracted with EDTA and Tris-NaCl, phosphorus could no longer be detected in the residue. This would tend to indicate that the collagen bound phosphoprotein was absent in predentine but the possibility that phosphorus was present below the limits of detection (0.3 μg phosphorus/aliquot analyzed) could not be excluded. Thus the insoluble residue was examined for the presence of small quantities of phosphoprotein. Oxidative degradation, carried out on a sample of predentine, led to the solubilization of a fraction containing a low concentration of phosphorus (0.02%). This fraction, upon electrophoretic examination, was shown to be composed of several anionic components, one of which displayed an electrophoretic mobility closely similar to that of dentine phosphoprotein. No phosphorus could be detected in this band. However, calculation showed that even if all the phosphorus known to be present in the fraction obtained after periodate treatment was in one electrophoretic band, the concentration in the final analytical solution was below the limit of detection (0.3 μg phosphorus/aliquot analyzed). Therefore, there was no proof that the phosphoprotein was absent. However, the predentine component isolated by electrophoresis could not be the same as the dentine phosphoprotein as the absence or virtual absence of phosphate from the protein would result in it having a significantly different mobility. Hence, it is probable the two components differ in primary composition, but this could only be determined by an amino acid determination and there was insufficient sample for this.

The above results suggest the absence of a bound phosphoprotein in predentine and thus support Weinstock and Leblond's[193] radioautographic study on the deposition of a phosphoprotein in dentine. These researchers, using ^{33}P and ^{3}H proline labels, were able to demonstrate that a phosphoprotein, secreted by the odontoblasts, traverses the predentine to the mineralization front where it is deposited in the dentine.

EDTA extracts[192] as well as Tris-NaCl extracts[191] have been found to contain soluble phosphoprotein. Both the EDTA extract and the Tris-NaCl extract of predentine contained non-diffusible fractions containing phosphorus. However, the EDTA extract and the Tris-NaCl extract of dentine contain much more phosphorus than those of predentine. The Tris-NaCl soluble non-dialysable fractions of both dentine and predentine matrices form about 5% of the organic matrix[191]. Hence, if a soluble phosphoprotein is present in predentine, it is present in a much lower concentration than in dentine. One should expect to find a very small transitory fraction.

Cementum: The values obtained in this study for the calcium (26.3%) and phosphorus (11.4%) contents of bovine cementum are in agreement with the results given below for human tissue. The calcium and phosphorus contents of human cementum range from 25.7 to 26.6% and 11.8 to 12.5% dry weight respectively[232]. The organic content of bovine cementum was found to be 34%.

Phosphoprotein is thought to be the site of nucleation for mineralization[185]. The content of this component found in cementum closely resembles that of predentine. The Tris-NaCl extraction solubilized about 5% of the decalcified cementum matrix. The non-diffusible components of this extract contained 1.76% phosphorus.

When the decalcified Tris-NaCl extracted cementum matrix was analysed for phosphorus and calcium, neither of these two elements were detected. Oxidative degradation solubilized a non-diffusible material which contained 0.03% phosphorus and 0.3% calcium. Electrophoretic examination of this material revealed a large diffuse anionic component and a discrete band which had a mobility similar to that of dentine phosphoprotein. However, no phosphorus could be detected in the gel although phosphorus was clearly discernible in a control experiment utilizing an equal weight of dentine. As in the case of the predentine, the level of phosphorus in the fraction obtained after periodate treatment was so low that when the resulting electrophoretic band was analyzed, the concentration of phosphorus would have been below the limits of detection. Therefore, the complete absence of the phosphoprotein was not proved.

The lack of phosphoprotein in cementum is very surprising. Veis and coworkers[140,185,187,189] isolated the phosphoprotein in the mineralized tissues, bone and dentine[184], but were unable to detect it in skin. As a result they hypothesized that it may play a role in mineralization. As this fraction is imperceptible in cementum, the function of phosphoprotein in calcified tissue matrices must be reviewed.

II. REDUCIBLE CROSS-LINKS:

Because of the fundamental role of cross-links in preserving tissue integrity, it was important to establish the cross-link pattern in these tissues. It is important to remember that with the techniques presently available, only reducible cross-links can be investigated. Perhaps other cross-links, which are not reducible, are present in collagen in addition to the reducible ones.

Predentine: The most abundant reducible intermolecular cross-link in mineralized[117,120] and embryonic tissues[247] has been reported to be dehydrodihydroxylysinonorleucine. During aging this cross-link is replaced by other polyfunctional cross-links[247,128]. It is therefore not surprising that the major reducible cross-link of predentine (4.8×10^{-1} lysine derived residues/1000 amino acid residues) and dentine (5.8×10^{-1} residues/1000) from unerupted teeth is dehydrodihydroxylysinonorleucine, detected as the reduced aldimine. There is a slight increase of the cross-link in dentine which may be accounted for by taking into consideration the age of the tissue. The source of the dentine was unerupted teeth, that is, a young organ in which growth and cross-link formation is at a peak. Davis[248] found dehydrodihydroxylysinonorleucine to be reduced to 1.6×10^{-1} residues/1000 in erupted bovine molar dentine collagen. As predentine matures, the cross-links peak in unerupted dentine and subsequently drop significantly in the erupted dentine.

Dehydrohydroxylysinonorleucine also decreases in concentration with age after having peaked during the period of maximum rate of growth[128]. Comparing the results obtained for predentine and dentine, one observes a considerable drop in the concentration of this reducible cross-link in dentine. Hence, the decrease of dehydrohydroxylysinonorleucine occurs much more rapidly than is the case for dehydrodihydroxylysinonorleucine.

Bailey[127] proposed that the reducible cross-links were intermediates which are transformed into more stable non-reducible cross-links. Davis and coworkers[129,249] have recently shown that lysine and/or hydroxylysine residues can be added to the electrophilic double bond of the reducible cross-links, transforming these into stable non-reducible

cross-links which in turn can join more than two collagen molecules.

Thus the above observations that dehydrodihydroxylysinonorleucine and dehydrohydroxylysinonorleucine decrease, that is, are transformed with age in predentine, are in accord with the current theories on the fate of collagen cross-links in maturing tissues.

Although less marked, the cross-link precursors show a similar relationship to development as the cross-links. Dihydroxynorleucine is slightly increased in the dentine matrix collagen while hydroxynorleucine is decreased, because there is more hydroxylysine in regions susceptible to lysine oxidase.

The equilibrium between reducible cross-links and their precursors is much more favourable in the case of hydroxyallysine derived reducible cross-links than allysine derived reducible cross-links. This is a result of isomerization of the hydroxyallysine derived cross-links to the more stable α-keto-amine cross-links. Therefore, one equivalent of hydroxyallysine will be in equilibrium with more of its derived cross-links than will one equivalent of allysine.

Cementum: As in other calcified tissue collagens, as well as predentine, the major reducible cross-link of cementum is dehydrodihydroxylysinonorleucine. This reducible cross-link decreases in concentration with maturation of the tissue probably brought about by its modification to a more stable non-reducible cross-link. Therefore, it is to be expected that this cross-link is considerably lower in concentration in cementum matrix than in dentine matrix as the cementum was collected from erupted teeth and the dentine from younger unerupted teeth. On the other hand, the minor cross-link of cementum, dehydrohydroxylysinonorleucine, occurs in greater concentration than in dentine.

Cementum is the calcified tissue which most resembles alveolar bone in its composition, structure and behavior. Comparison of the cementum cross-link results with those obtained by Davis[248] on bone (Table 14) shows the cross-link content of the two matrices to be very similar. In this comparison, it will also be noted that less calcified tissues, cementum included, seem to contain more dehydrohydroxylysinonorleucine than the more calcified tissues.

The cross-link precursor results are similar to those of dentine, that is, the dihydroxynorleucine in cementum is more plentiful than hydroxynorleucine. Mechanic[120] found this to be also the case in bone. As discussed in section on predentine cross-links, this indicates that there is more hydroxylysine in regions susceptible to lysine oxidase.

The implications of the cementum cross-link and precursor results are the same as that for the predentine results, that is, the equilibrium between reducible cross-links and their precursors is more favorable in the case of hydroxyallysine derived reducible cross-links than allysine derived reducible cross-links.

III. AMINO ACID COMPOSITION

Predentine: The EDTA-Tris-NaCl extracted predentine matrix proved to have an amino acid composition generally similar to that of dentine matrix collagen. Comparison of these results reveal that the lysine (23 residues/1000) and the hydroxylysine (12.4 residues/1000) of predentine are higher than the lysine (18.9 residues/1000) and hydroxylysine (9.2 residues/1000) of dentine. The sum of hydroxylysine and lysine (35.4 residues/1000) is slightly higher than the normal range for tissue collagens (29-33 residues/1000) and significantly higher than the sum in den-

tine (28.1 residues/1000). The hydroxylysine content of collagen is known to vary widely from tissue to tissue and with the age of the animal in the same tissue[250,142] even though the total of lysine and hydroxylysine remains constant. A progressive loss of hydroxylysine and an equivalent increase in lysine content has been observed in maturing rat and chick skin[250] as well as in mineralizing turkey tendon collagen[251], that is, the sum of these two amino acids remained constant. This constancy was not observed in the transformation of predentine to dentine. It must be remembered that only one amino acid analysis was performed on the predentine matrix and that the above observation may be due to experimental error. However, if the experimental errors are not significant, this finding may be significant and may indicate that maturation is not the only process occurring in the transformation of predentine to dentine.

Cartilage collagens[253], are known to contain higher levels of hydroxylysine than the Type I collagen of skin, tendon and bone. This is due to the genetically different types of collagen known to occur in this type of tissue. The amino acid composition of these different types of collagen are being determined. Type III collagen which was first isolated from fetal skin[4] has been recently isolated from dentine[228] and gingival tissues[254]. Its amino acid composition does not exhibit higher levels of hydroxylysine than the Type I (skin, bone, tendon) and as a result it is difficult to compare predentine with Type III collagen[253,255,256]. See Table 15. However, Type II (cartilage) does have an increased hydroxylysine content[257-260]. Recently Linsenmayer[260] compared the α1(II) and α1(I) chains of embryonic chick cartilage. The sum of hydroxylysine and lysine of the α1(II) chain (36 residues/1000) was

higher than that of the $\alpha 1(I)$ (32.2 residues/1000). There was also considerably more hydroxylysine in the Type II collagen (19 residues/1000) than in the Type I collagen (7.2 residues/1000). In both predentine and Type II collagen (see Table 15), the alanine content is low while the tyrosine is high. Thus from the point of view of amino acid composition, predentine approaches the Type II values more closely than the Type I. Hence, the transformation of predentine to dentine may be analogous to bone formation, that is, Type II collagen is replaced by Type I collagen.

The proline and hydroxyproline results are more difficult to account for. The sum of proline (174 residues/1000) and hydroxyproline (90 residues/1000) in predentine is 264 residues/1000, while in dentine it is 216.5 residues/1000. The normal range is from 213 to 218 residues/1000. Bornstein[261,262] had observed incomplete hydroxylation of individual prolyl residues in early sequence studies of rat skin and tail tendon collagen. He hypothesized that age as well as dietary factors may have a possible effect on hydroxylation of proline. Juva and Prockop[69] have shown that the reduced affinity of prolyl hydroxylase for partially hydroxylated chains accounts for the phenomenon of incomplete hydroxylation of individual prolyl residues observed by Bornstein. Berg and Prockop[86] as well as Murphy and Rosenbloom[85] have reached the conclusion that the triple helical conformation prevents its further hydroxylation. Several laboratories[142,263] have reported that there is no change in the overall extent of proline hydroxylation with age. A comparison of the sum of hydroxyproline and proline contents of the $\alpha 1(I)$, $\alpha 1(II)$ and $\alpha 1(III)$ shows no significant differences. See Table 15. The glycine and hydroxyproline contents of predentine proved to be low

whereas the proline content was high. As animal non-collagenous proteins contain no hydroxyproline and are relatively low in glycine but fairly rich in proline[89,264,265], the above discrepancies in the analysis of predentine collagen are understandable. The reduced glycine and hydroxyproline content and the elevated proline value are probably due to the predentine collagen being contaminated with non-collagenous protein. The neutral sugar results suggest that the contaminant is glycoprotein.

Cementum: The amino acid composition of the EDTA - Tris-NaCl extracted cementum matrix is in general agreement with that of other calcified tissues. The hydroxylysine content of cementum (10.6 residues/1000) was similar to that of dentine (9.2 residues/1000) but much higher than that of bone (6.4 residues/1000). See Table 13. This trend is also observed in the less typical coronal cementum[234] as well as in the bovine cementum Birkedal-Hansen et al[246] analyzed. See Table 16. The per cent hydroxylation of lysine in cementum collagen (35%), dentine (32%) and bone (19.5%) also showed the same pattern. This difference between the dental tissues and bone may prove to be significant. The hydroxylysine content of cementum collagen is very similar to that obtained from periodonal ligament collagen from which the Sharpey's fibres originate[269]. The sum of the lysine and hydroxylysine in cementum (30.6 residues/1000) was intmediate between that of bone (32.8 residues/1000) and dentine (28.1 residues/1000); the normal range for mammalian tissues being between 29 and 33 residues/1000.

From the above experimental data, cementum collagen appears to resemble dentine collagen rather than that of bone. This is surprising as cementum has always been thought to resemble bone more than any other tissue. These results are difficult to explain in terms of the genetic

types of collagen as bone and dentine have the same genetic type, the $\alpha 1(I)_2 \alpha 2$. Very recently Butler et al[252] have shown that bovine cementum collagen is predominantly Type I, with less than 5% of Type III present. The effect of the latter on the average amino acid composition as obtained in the present work would be negligible. However, the difference observed between cementum and bone may be due to the presence of some Type II collagen in cementum as this type of collagen has elevated levels of hydroxylysine[257-260]. There are no reports of Type II collagen occurring in bone.

Cementum proved to have a low hydroxyproline content and a high proline content as compared to bone and dentine. The sum of proline (156 residues/1000) and hydroxyproline (81.2 residues/1000) was 237.2 residues/1000 which is higher than the normal range of 211 to 218 residues/1000. The glycine content was somewhat low indicating the cementum collagen was not pure. This is also reflected in the low hydroxyproline and high proline content. One contaminant was an insoluble non-collagenous glycoprotein associated with the collagen.

The differences in amino acid composition observed between cementum, bone and dentine may partly be due to the different purification methods utilized, such as different periods of extraction in EDTA and whether or not the tissue was extracted with Tris-NaCl. It may also be that the same purification methods were more effective on one tissue than another, but if so, this may indicate some fundamental differences such as non-collagenous components more closely associated with one collagen than with another.

Some reservations should be made about comparing average amino acid values obtained here for predentine, dentine and cementum with values

for individual purified α 1 chains of Types I, II and III. For example when looking at the individual α 1 chains of the Type I collagen, the α 2 is not taken into consideration. Also the fact that these chains are highly purified does not show the repercussions of even a very small amount of contaminant on the average amino acid composition.

IV. NEUTRAL SUGARS

Predentine: The separation of collagen and non-collagen glycopeptides released from insoluble tissue fractions by papain is similar to that obtained when collagenase is employed followed by trypsin or pronase[207,266]

Only one analysis for neutral sugars was carried out on the separated glycopeptides and hexosamines and other sugars were not determined due to the small amount of predentine available. Accordingly, the quantitation of collagen hexoses (peak 2) requires confirmation. Although the modified manual orcinol method yielded a lower result than the Technicon carbohydrate method, both values were still much higher than has been found for skin[148,243] and dentine[185] collagens, which contain less than 1% hexose. Predentine collagen seems to resemble cartilagenous tissue collagens[148,243] in its apparent high hexose content. It has yet to be proven that the sugar is linked to the hydroxylysine because there was not enough of the G-25 peak 2, the collagen glycopeptide containing peak, to determine the glycosylated amino acids by the methods of Spiro[194,270].

The galactose content corresponds to 13.5 residues of galactose/1000 amino acids. This indicates, with an 8% discrepancy, complete glycosylation of the hydroxylysine.

The calculation for predentine is as follows:

let y = number of grams of galactose/14 grams of hydroxyproline (4.9g neutral sugar/14g hydroxyproline of which 59% is galactose = 2.9g galactose/14g hydroxyproline)

131 = molecular weight of hydroxyproline (M.W. hypro)

180 = molecular weight of galactose (M.W. gal.)

90 = number of residues hydroxyproline/1000 amino acid residues determined by amino acid analysis (res. hypro/1000 res.)

residues of galactose/1000 amino acids residues

$$= \frac{y}{14} \times \text{M.W. hypro} \times \text{res hypro/1000 res.} \times \frac{1}{\text{M.W. gal.}}$$

$$= \frac{y \times \text{M.W. hypro} \times \text{res hypro/1000 res.}}{14 \times \text{M.W. gal.}}$$

$$= \frac{2.9 \times 131 \times 90}{14 \times 180}$$

= 13.5 moles galactose/1000 amino acid residues

The excess of galactose over glucose indicates that only a part of the hexose is in the disaccharide form. Presumably galactosylhydroxylysine is also present as in other collagens[194-196] such as dentine[228] and skin collagen[4,228].

Peak 1 was found to contain significant amounts of mannose, fucose and galactose. These hexoses are the major sugar components of non-collagenous glycoproteins[89,243,264,265,267]. This strongly suggests that, as in other connective tissues, glycoproteins were important components of the non-collagenous material associated with the insoluble collagen. Glucose, which is frequently found in tissue glycoproteins[243,265,266,267] but not in serum glycoproteins occurred in relatively high concentration. The significance of insoluble glycoproteins has yet to be elucidated. Their wide distribution in connective tissues, some-

times in quite large amounts[243], has become increasingly evident in recent years.

Perhaps the limited PAS staining of predentine, mentioned earlier, may be due to the insoluble glycoprotein. Further work is required on the proteoglycans and soluble glycoproteins of predentine.

Cementum: The soluble glycoproteins and proteoglycans of cementum were not studied in the present work as the sample was small and the EDTA and Tris-NaCl extracts were utilized for the calcium and phosphorus determinations. As a result we concentrated on the insoluble glycoprotein fraction. This non-collagenous fraction appeared in peak 1 of the Sephadex gel chromatography of the cementum papain digest. It was found to contain glucose and generally resemble glycoproteins from other connective tissues in containing significant amounts of galactose, mannose and fucose[243,264,265,268]. This fraction is probably heterogenous like the similar fraction of other connective tissues[243,264,265,268]. Its composition may vary with age and from tissue to tissue[243].

As opposed to skin collagen which contains about 0.5g hexose/14g hydroxyproline[148,243], (0.46g hexose/14g hydroxyproline in our lab - unpublished results) cementum collagen was found by a similar technique to contain 1.5g hexose/14g hydroxyproline, of which 62% was galactose. This corresponds to 3.9 residues of galactose/1000 amino residues. See calculation for predentine on p. 76. Comparing this value with the number of hydroxylysine residues (10.6/1000) indicates 36.8% glycosylation, of which only a part is the disaccharide. However, as with predentine, there was insufficient tissue for proof to be obtained that the hexoses were linked to hydroxylysine.

From the above hexose results on predentine and cementum a

tentative prediction can now be made concerning collagen types. Trelstad et al[148] have found that there is a relatively high amount of carbohydrate, 5.5% by weight, covalently bound to the cartilage Type II collagen as compared to the 0.5% hexose in skin Type I collagen. We, therefore, have indirect evidence that predentine, by its carbohydrate content, may be of the cartilage Type II collagen. The rather high per cent of hydroxylated lysine residues is consistent with the increased sugar content. However, the increase in sugar content (4.9g hexose/14g hydroxyproline in predentine as compared to 0.46g/14g hydroxyproline in skin) was much larger than the increase in hydroxylated lysine (12.4 residues/1000 amino acid residues in predentine as compared to an average value of 6.8 residues/1000 for Type I collagens).

Cementum has a sugar content of 1.5% which is intermediate between dentine and skin, and cartilage. This may be an indication that cementum collagen, which contains two types of fibers, the Sharpey's fibers and the intrinsic, is a mixture of two types of collagen, that of skin and dentine: the $\alpha 1(I)_2\alpha 2$ and that of cartilage: the $\alpha 1(II)_3$, although the hydroxylysine results show no indication of this. The hydroxylysine content is not significantly higher than that of the periodontal ligament[269]. See Table 16.

The hexose content of the collagen of dental tissues is of interest as it is thought to have a relationship with the morphology of the collagen fibers[203,207,243] (see section on Collagen: a glycoprotein, p. 40) as well as with calcification.

It is thought that the seeding site for apatite crystals occurs in the hole region of the collagen fibrils (see sections on Collagen biosynthesis, p. 14 and Mineralization, p. 33), and that modification of the

ϵ-amino groups of lysine and hydroxylysine in this vicinity inhibits nucleation[154]. Since the glucose-galactose moiety attached to the hydroxylysine is thought to project in the hole region[201], calcification may be hindered by steric interference. Transformation of a non-calcified tissue to a calcified one, such as predentine to dentine, might then involve an enzymic modification of these strategically located residues, although there is no evidence for such a change in fully formed collagen fibers. There are other possibilities. The transformation of predentine may involve the resorption of collagen which is Type I, but nevertheless has a high hexose content, with a concurrent replacement with Type I collagen having a low hexose content. On the other hand, the transformation may involve a Type II collagen replacement with Type I. There is no evidence for the above statements, however. Collagen is not thought to be resorbed in the predentine to dentine transformation.

In the section on the role of glycosaminoglycans in mineralization (p. 28), it was suggested that acid mucopolysaccharides can either initiate or inhibit calcification[153,271]. Bowness[272], on the other hand, suggested that they could do both if a two compartment system was considered; one compartment being fibrous and concerned with nucleation and the other being the true ground substance and concerned with the inhibition of calcification. The two compartment theory may also be applicable to glycoproteins. However, as opposed to Bowness's theory, the insoluble glycoprotein, which may be presumed to occur in the fibrous compartment, may act as an inhibitor of calcification. These glycoproteins have high contents of glutamic and aspartic acid which are capable of binding calcium. One would therefore expect that it would be necessary for the insoluble glycoprotein to be removed before

calcification can occur. It is interesting to note that, using similar methods, the non-collagenous neutral sugar content of dentine (Pearson unpublished results) was determined to be one-tenth that of predentine.

V. SUMMARY:

Predentine was found to be virtually phosphoprotein free. As the basic premise was that phosphoprotein provided a nidus for calcification and therefore should not occur in uncalcified tissues and if so, only in very small quantities as in a transitory state, this result was expected. The lack of this component in cementum, however, did come as a surprise and now the role of phosphoprotein in calcified tissues must be reconsidered.

The major cross-links of the calcified tissues bone and dentine are hydroxylysinohydroxynorleucine and hydroxylysinonorleucine. These were found to be the major cross-links in predentine and cementum also. Of the two cross-links, hydroxylysinohydroxynorleucine was the predominant one in both tissues. The cross-link precursors dihydroxynorleucine and hydroxynorleucine showed the same trend as that of the cross-links.

The amino acid compositions of predentine and cementum generally resembled that of other collagens. There is some indication that predentine may possess Type II collagen, this being also reflected in its apparently high collagen hexose content. Cementum is probably mostly Type I collagen judging by its amino acid composition. However, its collagen hexose content indicates a possible mixture of Type I and II collagen. The non-collagenous neutral sugar results indicate that the predentine and cementum collagens are associated with an insoluble glycoprotein as in other connective tissues.

VI. SUGGESTIONS FOR FURTHER WORK

From the discussion above it is obvious that there were many quantitation problems as the amount of sample for each analysis was very limited especially in the case of predentine.

Engfeldt and Hjerpe[224] chose to use dentine and predentine from rachitic beagle puppies for studying the glycosaminoglycans of these tissues, as this provides a large predentine zone and therefore high yield of predentine. This method of obtaining predentine was not satisfactory in this project as it is not known what effects richets may have on the collagen or on the predentine tissue as a whole. Linde[226] chose, on the other hand, to obtain the odontoblast-predentine layer by scraping the pulp cavity of unerupted porcine teeth. This method was not chosen either as it invites contamination of the predentine sample with odontoblasts as well as with dentine. Perhaps in future, whole teeth could be decalcified, although the decalcification process would take much longer than the usual way where the teeth are broken into splinters. The decalcified tooth could then be sectioned longitudinally and the predentine dissected from both the apex of the tooth and the pulp cavity. This method was recently used very efficaciously on erupted bovine teeth by Birkedal-Hansen et al[246,252] to obtain cementum and thus could also be used in future to collect predentine as well.

Having thus obtained sufficient sample of both predentine and cementum, the phosphoprotein determinations should be repeated with enough sample such that the EDTA and Tris-NaCl soluble phosphoprotein (if any) as well as the periodate soluble phosphoprotein (if any) can be isolated and their amino acid and hexose composition characterized. The amino acid composition, cross-link and cross-link precursor content of

both the predentine and cementum matrix should be verified as well as their neutral sugar composition. The glycosylated amino acids could then be identified and quantitated by ion exchange column chromatography [194,270]. The non-collagenous contaminants referred to earlier in the neutral sugar section (p. 76) could be isolated by first digesting the insoluble residue with collagenase so as to rid the sample of collagen. The remaining residue could be treated with reducing agents so as to solubilize the insoluble proteoglycans and glycoproteins thus enabling their separation characterization by usual methods[223-226,243,264,265]. The soluble proteoglycans and glycoproteins of both tissues could also be investigated. It would also be very interesting to see a collagen type determination on predentine.

PUBLICATIONS

The following publications will appear based on the present work:

1. Carmichael, D.J., Chovelon, A. and Pearson, C.H.: The composition of the insoluble collagenous matrix of bovine predentine. Calc. Tiss. Res.: in press, 1975.

2. Chovelon, A., Carmichael, D.J. and Pearson, C.H.: The composition of the organic matrix of bovine cementum. Arch. Oral Biol.: in press, 1975.

TABLE 1

AMINO ACID COMPOSITION OF SEVERAL MAMMALIAN TISSUE COLLAGENS[1]

	Tendon	Skin	Uterus	Renal Reticulum	Bone
Alanine	110.7	114.5	95.9	96.5	113.5
Glycine	324	324.4	337.1	309	319
Valine	25.4	24.5	21.6	26.8	23.6
Leucine	26.0	24.8	24.5	35.8	25.5
Isoleucine	11.1	10.4	10.8	18.0	13.3
Proline	126.4	125.1	108.2	97.2	123.4
Phenylalanine	14.2	12.6	12.3	18.1	13.9
Tyrosine	3.6	3.5	2.2	3.0	4.5
Serine	36.9	36.9	41.9	42.8	35.9
Threonine	18.5	18.3	20.6	21.9	18.4
Cystine	--	trace	trace	--	--
Methionine	5.7	7.0	5.8	8.6	5.3
Arginine	49.0	49.0	49.0	45.3	47.1
Histidine	5.4	5.4	5.4	5.3	5.8
Lysine	21.6	26.6	24.9	21.6	28.0
Aspartic acid	48.4	47.2	52.5	52.9	47.0
Glutamic acid	72.3	77.7	73.6	76.7	72.2
Hydroxyproline	92.1	90.9	108.6	107.7	100.2
Hydroxylysine	8.9	5.9	5.1	12.2	3.5

Values expressed in amino acid residues per 1000 total amino acid residues.

1. Values obtained from Eastoe, J.E.[6]

TABLE 2

THE AMINO ACID SEQUENCE OF THE α1 CHAIN[1]

pGlu-Met-Ser-Tyr-Gly-Tyr-Asp-Glu-Lys-Ser-Ala-Gly-Val-Ser-Val-Pro-

1	Gly-Pro-Met-Gly-Pro-Ser-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Pro-Gln-Gly-Phe-Gln-Gly-Pro-Hyp-
28	Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser-Gly-Pro-Met-Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Lys-Asn-Gly-Asp-Asp-
55	Gly-Glu-Ala-Gly-Lys-Pro-Gly-Arg-Hyp-Gly-Gln-Arg-Gly-Pro-Hyp-Gly-Pro-Gln-Gly-Ala-Arg-Gly-Leu-Hyp-Gly-Thr-Ala-
82	Gly-Leu-Hyp-Gly-Met-Hyl-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu-Asp-Gly-Ala-Lys-Gly-Asn-Thr-Gly-Pro-Ala-Gly-Pro-Lys-
109	Gly-Glu-Hyp-Gly-Ser-Hyp-Gly-Glx-Asx-Gly-Ala-Hyp-Gly-Gln-Met-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Glu-Arg-Gly-Arg-Hyp-
136	Gly-Pro-Hyp-Gly-Ser-Ala-Gly-Ala-Arg-Gly-Asp-Asp-Gly-Ala-Val-Gly-Ala-Ala-Gly-Pro-Hyp-Gly-Pro-Thr-Gly-Pro-Thr-
163	Gly-Pro-Hyp-Gly-Phe-Hyp-Gly-Ala-Ala-Gly-Ala-Lys-Gly-Glu-Ala-Gly-Pro-Gln-Gly-Ala-Arg-Gly-Ser-Glu-Gly-Pro-Gln-
190	Gly-Val-Arg-Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Ala-Ala-Gly-Pro-Ala-Gly-Asn-Hyp-Gly-Ala-Asp-Gly-Gln-Hyp-
217	Gly-Ala-Lys-Gly-Ala-Asn-Gly-Ala-Hyp-Gly-Ile-Ala-Gly-Ala-Hyp-Gly-Phe-Hyp-Gly-Ala-Arg-Gly-Pro-Ser-Gly-Pro-Gln-
244	Gly-Pro-Ser-Gly-Ala-Hyp-Gly-Pro-Lys-Gly-Asn-Ser-Gly-Glu-Hyp-Gly-Ala-Hyp-Gly-Asn-Lys-Gly-Asp-Thr-Gly-Ala-Lys-
271	Gly-Glu-Hyp-Gly-Pro-Ala-Gly-Val-Gln-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Glu-Glu-Gly-Lys-Arg-Gly-Ala-Arg-Gly-Glu-Hyp-
298	Gly-Pro-Ser-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Glu-Arg-Gly-Gly-Hyp-Gly-Ser-Arg-Gly-Phe-Hyp-Gly-Ala-Asp-Gly-Val-Ala-
325	Gly-Pro-Lys-Gly-Pro-Ala-Gly-Glu-Arg-Gly-Ser-Hyp-Gly-Pro-Ala-Gly-Pro-Lys-Gly-Ser-Hyp-Gly-Glu-Ala-Gly-Arg-Hyp-
352	Gly-Glu-Ala-Gly-Leu-Hyp-Gly-Ala-Lys-Gly-Leu-Thr-Gly-Ser-Hyp-Gly-Ser-Hyp-Gly-Pro-Asp-Gly-Lys-Thr-Gly-Pro-Hyp-
379	Gly-Pro-Ala-Gly-Gln-Asp-Gly-Arg-Hyp-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Ala-Arg-Gly-Gln-Ala-Gly-Val-Met-Gly-Phe-Hyp-
406	Gly-Pro-Lys-Gly-Ala-Ala-Gly-Glu-Hyp-Gly-Lys-Ala-Gly-Glu-Arg-Gly-Val-Hyp-Gly-Pro-Hyp-Gly-Ala-Val-Gly-Pro-Ala-
433	Gly-Lys-Asp-Gly-Glu-Ala-Gly-Ala-Gln-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Pro-Ala-Gly-Glu-Arg-Gly-Glu-Gln-Gly-Pro-Ala-
460	Gly-Ser-Hyp-Gly-Phe-Gln-Gly-Leu-Hyp-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Glu-Ala-Gly-Lys-Hyp-Gly-Glu-Gln-Gly-Val-Hyp-
487	Gly-Asp-Leu-Gly-Ala-Hyp-Gly-Pro-Ser-Gly-Ala-Arg-Gly-Glu-Arg-Gly-Phe-Hyp-Gly-Glu-Arg-Gly-Val-Glu-Gly-Pro-Hyp-
514	Gly-Pro-Ala-Gly-Pro-Arg-Gly-Ala-Asn-Gly-Ala-Hyp-Gly-Asn-Asp-Gly-Ala-Lys-Gly-Asp-Ala-Gly-Ala-Hyp-Gly-Ala-Hyp-
541	Gly-Ser-Gln-Gly-Ala-Hyp-Gly-Leu-Gln-Gly-Met-Hyp-Gly-Glu-Arg-Gly-Ala-Ala-Gly-Leu-Hyp-Gly-Pro-Lys-Gly-Asp-Arg-
568	Gly-Asp-Ala-Gly-Pro-Lys-Gly-Ala-Asp-Gly-Ala-Pro-Gly-Lys-Asp-Gly-Val-Arg-Gly-Leu-Thr-Gly-Pro-Ile-Gly-Pro-Hyp-
595	Gly-Pro-Ala-Gly-Ala-Hyp-Gly-Asp-Lys-Gly-Glu-Ala-Gly-Pro-Ser-Gly-Pro-Ala-Gly-Thr-Arg-Gly-Ala-Hyp-Gly-Asp-Arg-
622	Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Phe-Ala-Gly-Pro-Hyp-Gly-Ala-Asp-Gly-Gln-Hyp-Gly-Ala-Lys-Gly-Glu-Hyp-
649	Gly-Asp-Ala-Gly-Ala-Lys-Gly-Asp-Ala-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Pro-Ile-Gly-Asn-Val-
676	Gly-Ala-Hyp-Gly-Pro-Hyl-Gly-Ala-Arg-Gly-Ser-Ala-Gly-Pro-Hyp-Gly-Ala-Thr-Gly-Phe-Hyp-Gly-Ala-Ala-Gly-Arg-Val-
703	Gly-Pro-Hyp-Gly-Pro-Ser-Gly-Asn-Ala-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Lys-Glu-Gly-Ser-Lys-Gly-Pro-Arg-
730	Gly-Glu-Thr-Gly-Pro-Ala-Gly-Arg-Hyp-Gly-Glu-Val-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Glu-Lys-Gly-Ala-Hyp-
757	Gly-Ala-Asp-Gly-Pro-Ala-Gly-Ala-Hyp-Gly-Thr-Pro-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Hyp-
784	Gly-Gln-Arg-Gly-Glu-Arg-Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-Pro-Ser-Gly-Glu-Hyp-Gly-Lys-Gln-Gly-Pro-Ser-Gly-Ala-Ser-
811	Gly-Glu-Arg-Gly-Pro-Hyp-Gly-Pro-Met-Gly-Pro-Hyp-Gly-Leu-Ala-Gly-Pro-Hyp-Gly-Glu-Ser-Gly-Arg-Glu-Gly-Ala-Hyp-
838	Gly-Ala-Glu-Gly-Ser-Hyp-Gly-Arg-Asp-Gly-Ser-Hyp-Gly-Ala-Lys-Gly-Asp-Arg-Gly-Glu-Thr-Gly-Pro-Ala-Gly-Ala-Hyp-
865	Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Ala-Hyp-Gly-Pro-Val-Gly-Pro-Ala-Gly-Lys-Ser-Gly-Asp-Arg-Gly-Glu-Thr-Gly-Pro-Ala-
892	Gly-Pro-Ile-Gly-Pro-Val-Gly-Pro-Ala-Gly-Ala-Arg-Gly-Pro-Ala-Gly-Pro-Gln-Gly-Pro-Arg-Gly-Asx-Hyl-Gly-Glx-Thr-
919	Gly-Glx-Glx-Gly-Asx-Arg-Gly-Ile-Hyl-Gly-His-Arg-Gly-Phe-Ser-Gly-Leu-Gln-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Ser-Hyp-
946	Gly-Glu-Gln-Gly-Pro-Ser-Gly-Ala-Ser-Gly-Pro-Ala-Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Ser-Ala-Gly-Ser-Hyp-Gly-Lys-Asp-
973	Gly-Leu-Asn-Gly-Leu-Hyp-Gly-Pro-Ile-Gly-Hyp-Hyp-Gly-Pro-Arg-Gly-Arg-Thr-Gly-Asp-Ala-Gly-Pro-Ala-Gly-Pro-Hyp-
1000	Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Pro-

Ser-Gly-Gly-Tyr-Asp-Leu-Ser-Phe-Leu-Pro-Gln-Pro-Pro-Gln-Gln-Glx-Lys-Ala-His-Asp-Gly-Gly-Arg-Tyr-Tyr

The amino acid sequence of the α1 chain from rat (residues 1-402) and calf (residues 403-1011) skin collagen. The N terminal (from rat) and the C-terminal (from calf) non helical regions are separated from the triplet region and are not numbered.

1. Table is taken from Hulmes et al[133]

TABLE 3

AMINO ACID SEQUENCE OF α2CNBr PEPTIDES SEQUENCED TO DATE FROM CHICK, RAT AND CALF SKIN COLLAGEN

α2CNBr peptides

α2-CB1[1]

Chick Pca-Tyr-Asp-Pro-Ser-Lys-Ala-Ala-Asp-Phe-Gly-Pro-Gly-Pro-Met

Rat Pca-Tyr-Ser-Asp-Lys-Gly-Val-Ser-Ala-Gly-Pro-Gly-Pro-Met

α2-CB0[2]

Rat Gly-Leu-Met

α2-CB4[2] 42 N-terminal amino acids of 330 residues

Rat Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Ala-Val-Gly-Ala-Hyp-Gly-Pro-Gln

Calf Gly-Pro-Arg-Gly-Pro-Hyp-Gly-Ala-Ser-Gly-Ala-Hyp-Gly-Pro-Gln

Rat Gly-Phe-Gln-Gly-Pro-Ala-Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Gln-Hyp

Calf Gly-Phe-Gln-Gly-Pro-Hyp-Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Gln-Hyp

Rat Gly-Pro-Ala-Gly-Pro-Arg-Gly-Pro-Ala-Gly-Pro-Hyp

Calf Gly-Pro-Ala-Gly-Ala-Arg-Gly-Pro-Hyp-Gly-Pro-Hyp

α2-CB2[3]

Chick Gly-Pro-Ala-Gly-Asn-Arg-Gly-Ala-Ser-Gly-Pro-Ala-Gly-Val-Lys

Rat Gly-Pro-Hyp-Gly-Asn-Arg-Gly-Thr-Ser-Gly-Pro-Ala-Gly-Val-Arg

Chick Gly-Pro-Asn-Gly-Asp-Ala-Gly-Arg-Hyp-Gly-Glu-Hyp-Gly-Leu-Hse

Rat Gly,Pro,Asp,Gly,Asp,Ala,Gly,Arg,Hyp,Gly,Gln,Hyp,Gly,Leu,Hse

α2-CB3

α2-CB5[4] 45 N-terminal residues of 320 residues

Rat Thr-Gly-Phe-Hyp-Gly-Ala-Ala-Gly-Arg-Thr-Gly-[illegible]-Gly-Pro-
Ser-Gly-Ile-Thr-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-[illegible]-Ala-Gly-Lys-
Glu-Gly-Ile-Lys-Gly-Pro-Arg-Gly-Asp-Gln-Gly-Pro-Val-Gly-Arg

Table 3 continued:

1. Sequence for α2-CB1 is from Kang and Gross[134].
2. Sequence for α2-CB4 is from Fietzek et al[135].
3. Sequence for α2-CB2 is from Highberger et al[136].
4. Sequence for α2-CB5 is from Fietzek and Kuhn[137].

TABLE 4

AMINO ACID SEQUENCE OF α2-CB1 FROM CALF, HUMAN, RABBIT, PIG, RAT AND CHICK COLLAGEN[1]

	1										15
Calf	Gly-Pro-Ala-Gly-Ser-Arg-Gly-Thr-Ala-Gly-Pro-Ala-Gly-Val-Arg-										
Human	Gly-Pro-Hyp-Gly-Ser-Arg-Gly-Ala-Ser-Gly-Pro-Ala-Gly-Val-Arg-										
Rabbit	Gly-Pro-Hyp-Gly-Ser-Arg-Gly-Thr-Ser-Gly-Pro-Ala-Gly-Val-Arg-										
Pig	Gly-Pro-Hyp-Gly-Ser-Arg-Gly-Thr-Pro-Gly-Pro-Ala-Gly-Val-Arg-										
Rat	Gly-Pro-Hyp-Gly-Asn-Arg-Gly-Thr-Ser-Gly-Pro-Ala-Gly-Val-Arg-										
Chick	Gly-Pro-Ala-Gly-Asn-Arg-Gly-Ala-Ser-Gly-Pro-Ala-Gly-Val-Lys-										

	20										30
Calf	Gly-Pro-Asn-Gly-Asp-Ser-Gly-Arg-Hyp-Gly-Glu-Hyp-Gly-Leu-Hse										
Human	Gly-Pro-Asx-Gly-Asx-Ala-Gly-(Arg,Hyp,Gly,Glu,Hyp,Gly,Leu,Hse)										
Rabbit	Gly-Pro-Asx-Gly-Asx-Ser-Gly-(Arg,Hyp,Gly,Glu,Hyp,Gly,Leu,Hse)										
Pig	Gly-Pro-Asn-Gly-Asp-Ser-Gly-Arg-Hyp-Gly-Glu-(Hyp,Gly,Leu,Hse)										
Rat	(Arg,Pro,Asn,Gly,Asp,Ala,Gly,Arg,Hyp,Gly,Glu,Hyp,Gly,Leu,Hse)										
Chick	Gly-Pro-Asn-Gly-Asp-Ala-Gly-Arg-Hyp-Gly-Glu-Hyp-Gly-Leu-Hse										

1. Sequence of α2-CB2 is from Fietzek et al[132].

TABLE 5

AMINO ACID COMPOSITIONS OF CNBr-CLEAVED CHAIN FRAGMENTS DERIVED FROM BOVINE SOLUBLE [illegible] CHAIN, AND [illegible] CARBOXYL GROUPS[1]

	α1-(CB3)			α1-CB(4,5)		
	I	II	III	I	II	III
4-Hydroxyproline	15	16	19	9(9.1)	11	12
Aspartic acid	6(5.8)	4(4.1)	8(7.9)	6(5.9)	4(3.7)	4(3.8)
Threonine	0	3(2.9)	4(3.9)	1(1.0)	1(1.1)	1(0.8)
Serine	3(2.8)	3(3.1)	1(0.8)	2(1.9)	3(2.7)	4(3.9)
Glutamic acid	15	14	12	6(6.1)	6(5.9)	6(6.3)
Proline	15	17	19	8(8.2)	7(7.3)	7(6.8)
Glycine	49	49	49	28	28	28
Alanine	21	14	15	7(7.2)	6(6.0)	9(9.0)
Valine	4(4.2)	2(2.2)	1(1.0)	0	2(2.2)	1(1.0)
Methionine	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	1(0.9)
Leucine	3(3.1)	6(6.3)	5(4.9)	3(3.0)	2(2.1)	1(0.9)
Tyrosine	0	0	0	0	1(1.1)	0
Phenylalanine	3(3.0)	3(3.1)	1(1.3)	1(1.0)	1(0.9)	1(0.9)
Hydroxylysine[4]	0.2	2.5	0.3	1.3	2.9	0.2
Lysine	4.8	4.2	7.0	3.7	2.9	1.6
Histidine	0	0	0	1(1.2)	1(1.0)	1(0.9)
Arginine	6(6.1)	7(6.8)	4(3.8)	5(5.0)	4(4.3)	5(5.1)
Homoserine	1(0.8)	1(0.8)	1(0.8)	2(1.8)	1(0.8)	1(0.8)
Total	146	146	146	84	84	84

1. Table is taken from Miller et al[4].

Table 5 continued:

2. Residues per peptide rounded off to the nearest whole number. Actual values are listed where less than 10 residues are found. A value of zero indicates less than 0.2 residue.

3. In α1(I) the sequence homologous to the region represented by α1-CB(4,5) in α1(II) and α1(III) contains a methionyl residue giving rise α1-CB4 and α1-CB5. The analysis shown is the sum of both.

4. Values for lysine and hydroxylysine have not been rounded off since there is evidence and partial hydroxylation giving rise to nonintegeer values.

TABLE 6

AMINO ACID COMPOSITION OF GLOMERULAR BASEMENT MEMBRANES[1]

Amino Acid	Human	Canine	Ovine	Rat
Hydroxylysine	24.5	25.0	22.5	21.8
Lysine	26.4	26.0	27.0	40.0
Histidine	18.7	14.4	18.5	21.0
Arginine	48.3	48.2	50.5	52.6
3-Hydroxyproline	12.0	8.5	7.0	7.0
4-Hydroxyproline	53.0	56.5	56.0	50.0
Aspartic	70.0	70.0	72.0	70.0
Threonine	40.3	40.5	41.5	45.5
Serine	54.2	49.0	54.5	62.8
Glutamic	101.3	97.0	98.0	100.0
Proline	64.1	69.8	66.0	62.0
Glycine	225.2	229.0	220.0	200.0
Alanine	58.6	65.0	60.0	67.0
Half-Cystine	22.0	22.7	20.0	20.4
Valine	36.0	36.0	38.0	43.0
Methionine	7.0	5.0	7.5	11.0
Isoleucine	28.6	28.1	29.5	30.0
Leucine	60.3	60.2	59.0	60.0
Tyrosine	20.5	22.0	23.0	17.0
Phenylalanine	28.3	26.8	28.0	19.0

1. Table is taken from Kefalides, N.A.[149]

TABLE 7

PHOSPHORUS AND CALCIUM VALUES[1] OF THE PREDENTINE AND CEMENTUM FRACTIONS

Predentine Fraction

	P	Ca
Predentine	0.37	0.4
EDTA extract	0.07	---
Tris-NaCl Extract	1.7	Trace
Predentine Matrix	N.D.	N.D.

Cementum Fraction

	P	Ca
Cementum	11.4	26.3
EDTA Extract	0.14	---
Tris NaCl Extract	1.76	Trace
Cementum Matrix	N.D.	N.D.

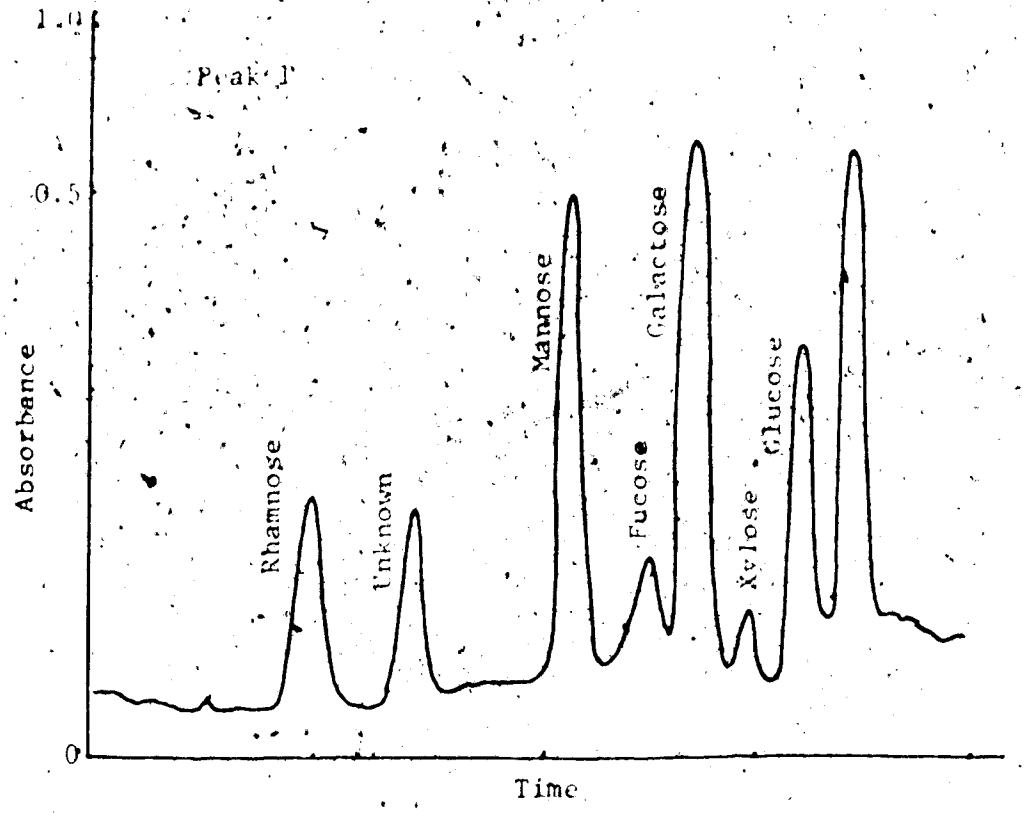
1. Values are expressed in terms of percentage of the total weight of each fraction.

N.D. Not detected

--- Not determined

TABLE 8

DIAGRAMMATIC ILLUSTRATIONS OF TYPICAL CHROMATOGRAMS FOR NEUTRAL SUGAR ANALYSIS OF G-25 PEAK 1 AND PEAK 2



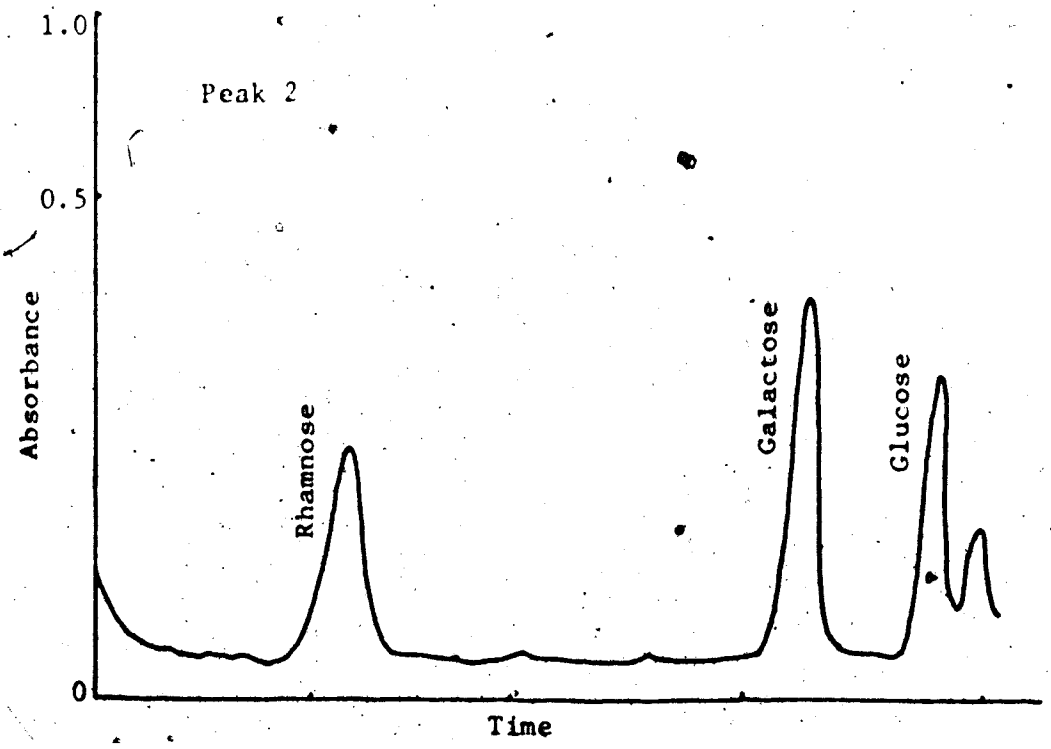
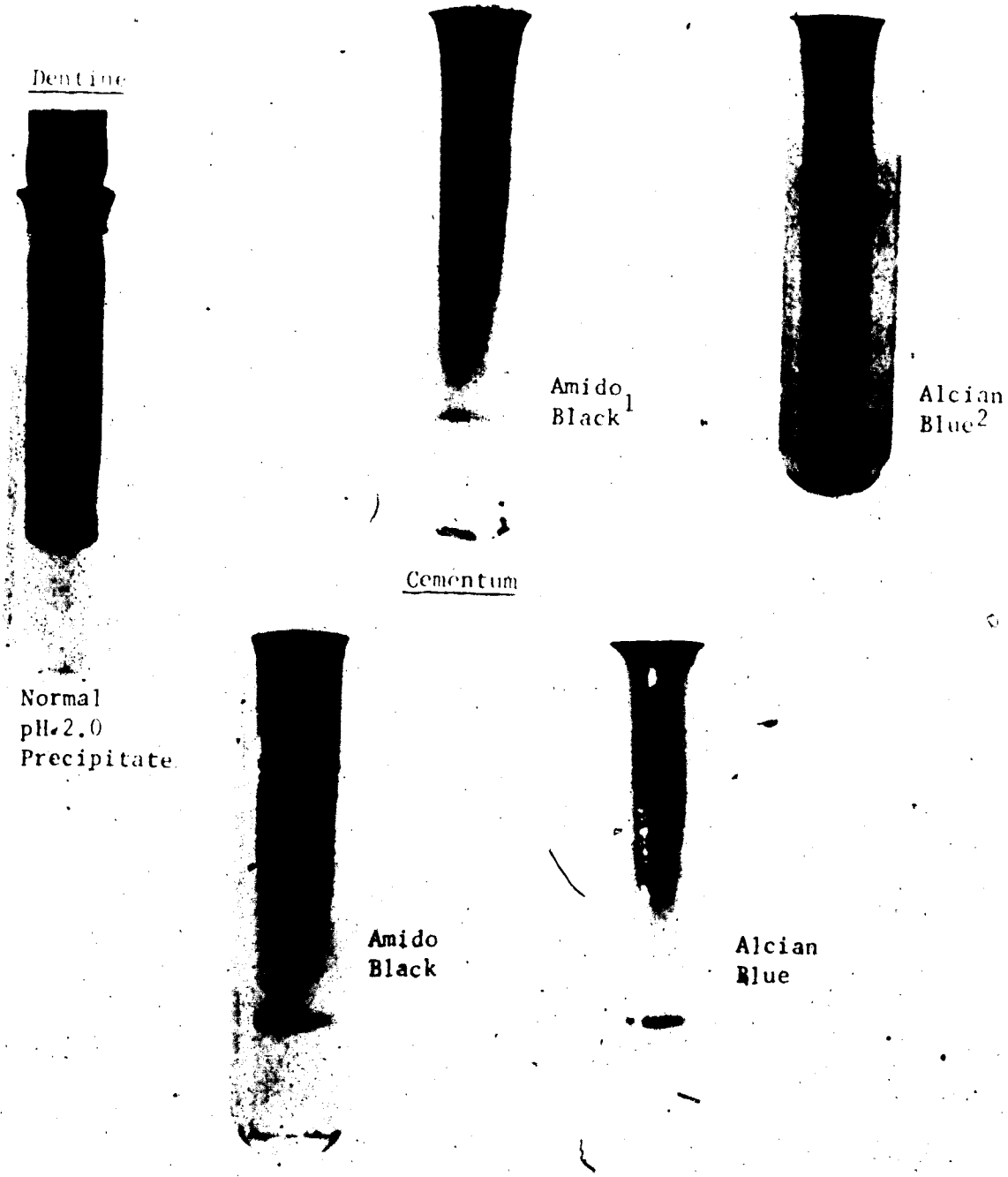


TABLE 9

PREDENTINE, CEMENTUM AND DENTIN POLYACRYLAMIDE GEL ELECTROPHORESIS [illegible]



1. Amido Black stains mucopolysaccharide.

2. Alcian Blue stains protein.

TABLE 10

AMINO ACID COMPOSITION OF EDTA, [illegible] EXTRACTED PREDENTINE AND DENTINE[1]

	Predentine	Dentine
Lysine	23.0	18.9
Histidine	7.0	5.2
Arginine	65.4	46.9
Hydroxylysine	12.4	9.2
Hydroxyproline	90.0	98.7
Aspartic Acid	44.1	49.9
Threonine	16.8	17.0
Serine	31.6	38.2
Glutamic Acid	69.8	70.9
Proline	174	117.8
Glycine	281	325.8
Alanine	102	124.2
Valine	20.0	20.7
Methionine	7.9	5.9
Isoleucine	10.3	11.1
Leucine	25.7	24.7
Tyrosine	5.7	4.0
Phenylalanine	14.3	11.9

Results expressed in terms of amino acid residues per 1000 total amino acid residues.

1. Values for dentine collagen are from Veis and Perry[187].

TABLE 11

REDUCED CROSS-LINK AND CROSS-LINK PRECURSOR CONTENT OF DENTINE, PREDENTINE AND CEMENTAL MATRIX COLLAGENS

	Reduced Cross-Link Precursors		Reduced Cross-Link	
	di HNL	HNL	HLHNL	HLNL
Predentine	1.2×10^{-2}	1.2×10^{-2}	4.8×10^{-1}	3.5×10^{-2}
Cementum	3.2×10^{-2}	1.1×10^{-2}	2.6×10^{-1}	5.2×10^{-2}
Dentine[1]	1.3×10^{-2}	1.0×10^{-2}	5.8×10^{-1}	9.0×10^{-3}

Results expressed in terms of lysine derived residues per 1000 total amino acid residues.

di HNL - dihydroxynorleucine

HNL - hydroxynorleucine

HLHNL - hydroxylysinohydroxynorleucine

HLNL - hydroxylysinonorleucine

1. Values for dentine from Carmichael and Wohllebe (unpublished work).

TABLE 12

NEUTRAL SUGAR COMPOSITION OF EDTA, Tris-NaCl EXTRACTED PREDENTINE AND CEMENTUM

g/14g Hypro.

	Peak 1	Peak 2	Peak 1					Peak 2[1]	
			Ga	M	F	Gl	U	Ga	Gl
Predentine	2.2[2]	4.9	33	23	10	20	14	59	41
Cementum	0.9[2]	1.5	40	26	7	16	11	62	38

1. Also detected mannose ($<3\%$ of total hexose of peak 2) and traces of fucose and xylose. Traces of the latter and ribose were detected in peak 1.

2. Value includes unknown orcinol positive compounds U.

Ga - Galactose M - Mannose F - Fucose Gl - Glucose

TABLE 13

AMINO ACID COMPOSITION OF CEMENTUM, BONE AND DENTINE MATRIX COLLAGENS

	Cementum	Bone[1]	Dentine[2]
Lysine	20.0	26.2	18.9
Histidine	5.2	5.8	5.2
Arginine	57.6	49.0	46.9
Hydroxylysine	10.6	6.4	9.2
Hydroxyproline	81.2	100.8	98.7
Aspartic Acid	46.6	49.8	49.9
Threonine	18.0	19.7	17.0
Serine	33.6	37.8	38.2
Glutamic Acid	72.7	75.8	70.9
Proline	156	118	118
Glycine	306	314	326
Alanine	115	109	124
Valine	21.4	21.2	20.7
Methionine	7.7	5.1	5.9
Isoleucine	9.9	12.3	11.1
Leucine	22.8	27.9	24.7
Tyrosine	3.9	2.9	4.0
Phenylalanine	12.3	16.3	11.9

Results expressed in terms of amino acid residues per 1000 total amino acid residues.

1. Values for bone matrix collagen are from Eastoe, J.E.[6].

2. Values for dentine matrix collagen are from Veis and Perry[187].

TABLE 14

REDUCED CROSS-LINK CONTENT OF DENTINE, CEMENTUM, BONE, CARTILAGE AND SKIN NATIVE COLLAGEN

	HLHNL	HLNL
Dentine[1]	5.8×10^{-1}	9.0×10^{-3}
Cementum	2.6×10^{-1}	5.2×10^{-2}
Bone[2]	2.3×10^{-1}	6.0×10^{-2}
Cartilage[2]	1.3×10^{-1}	6.0×10^{-2}
Skin[2]	1.0×10^{-2}	1.6×10^{-1}

Results are expressed in terms of lysine derived residues per 1000 amino acid residues.

HLHNL - hydroxylysinohydroxynorleucine

HLNL - hydroxylysinonorleucine

1. Values for dentine from Carmichael and Wohllebe (unpublished work).
2. Values from bone, cartilage and skin from Davis, N.R.[248]

TABLE 15

AMINO ACID COMPOSITIONS[1] OF TYPE I, PRECENTINE, TYPE II, CEMENTUM AND TYPE III COLLAGENS

	Type I[2] (Skin)	Precentine	Type II[3] (Cartilage)	Cementum	Type III[4] (Skin)
Lysine	27	23.0	17	20.0	30
Histidine	4.2	7.0	2.4	5.2	6
Arginine	51	65.4	50	57.6	46
Hydroxylysine	6.9	12.4	19	10.6	5
Hydroxyproline	106	90.0	106.2	81.2	125
Aspartic Acid	45	44.1	42	46.6	42
Threonine	19	16.8	24	18.0	13
Serine	29	31.6	27	33.6	39
Glutamic Acid	73	69.8	83	72.7	71
Proline	118	174	118	156	107
Glycine	332	281	333	306	350
Alanine	116	102	106	115	96
Valine	18	20.0	19	21.4	14
Methionine	7.4	7.9	10	7.7	8
Isoleucine	10	10.3	8	9.9	13
Leucine	24	25.7	24	22.8	22
Tyrosine	1.6	5.7	2.2	3.9	3
Phenylalanine	12	14.3	13	12.3	8

1. Results expressed in terms of amino acid residues per 1000 total amino acid residues.
2. Values for Type I collagen are from Kang et al[141].
3. Values for Type II collagen are from Linsenmayer, T.F.[260].
4. Values for Type III collagen are from Chung et al[255].

TABLE 1c

AMINO ACID COMPOSITION[1] OF CEMENTUM

	Bovine Cementum	Coronal Cementum[2]	Bovine Cementum[3]	Mature Bovine Periodontal Ligament[4]
Lysine	20.0	25	21.9	27
Histidine	5.2	--	4.8	5.3
Arginine	57.6	51	46.1	53
Hydroxylysine	10.6	11	9.7	9.7
Hydroxyproline	81.2	106	93.5	105
Aspartic Acid	46.6	50	48.1	45
Threonine	18.0	19	18.8	17
Serine	33.6	39	37.5	29
Glutamic Acid	72.7	80	80.8	75
Proline	156	124	112	134
Glycine	306	307	332	310
Alanine	115	115	111	95
Valine	21.4	21	22.3	22
Methionine	7.7	3	5.5	7.9
Isoleucine	9.9	12	11	13
Leucine	22.8	27	25.4	28
Tyrosine	3.9	3	5.9	5.8
Phenylalanine	12.3	14	12.7	14

1. Results expressed in terms of amino acid residues per 1000 total amino acid.
2. Values for coronal cementum are from Glimcher, M.J. et al[234].
3. Values for bovine cementum are from Berkedal-Hansen, H. et al[246].
4. Values for mature periodontal ligament are from Pearson, C.H. et al[269].

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