THE EFFECTS OF OCCLUSAL FORCES ON COLLAGEN TURNOVER IN DOG MOLAR PERIODONTAL LIGAMENT BY QUANTITATION OF REDUCIBLE COLLAGEN CROSS-LINKS

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ABSTRACT

The influence of eruptive or occlusal forces in modifying collagen turnover in the periodontal ligament of small experimental animals has been studied by autoradiographic techniques. However, histologic demonstration of the incorporation of radioactive label into collagen is open to question because of the lack of sufficient specificity for collagen of the isotope used and the possible recycling of the label from degraded newly synthesized collagenous and non-collagenous proteins. Biochemical methods consisting of measuring over extended time periods the specific activity of hydroxyproline isolated from soluble and insoluble collagen fractions following the injection of isotope into whole animals, shows that periodontal ligament collagen has a higher turnover rate than other connective tissue collagens. There are however no comparative biochemical studies of the turnover rates of ligament collagen of molars with and without antagonists. In addition, no biochemical studies have been done on the effect of physiologic forces on ligament collagen turnover in large animals because of the prohibitive cost of isotope required for such studies.

In order to determine if the metabolism of collagen in molar ligament of large dogs is modified by eruptive or occlusal forces the intermolecular cross-links dehydrodihydroxylysinonorleucine (DHNL) and dehydrohydroxylysinonorleucine (HNL) were quantitated and used as an indication of the rate of new collagen synthesis. During periods of rapid growth and collagen synthesis these cross-links can be detected by treatment of tissue samples with tritium labelled sodium borohydride. This compound reduces the cross-links to dihydroxylysinonorleucine (DHLNL) and

of DHLNL and HLNL in experimental and control specimens do not show a significant difference and therefore does not support previous suggestions that the presence of occlusal forces or an increased eruptive force modifies collagen turnover. Instead the data suggests that increased turnover of periodontal ligament collagen appears to be an innate feature of this tissue.

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INTRODUCTION

The periodontal ligament is a fibrous connective tissue situated within the periodontal ligament space and like connective tissues in general, has as its major component the protein collagen. As seen in the electron and light microscopes, this protein appears as longitudinally arranged fibrils, fibers and fiber bundles which pass from the cementum of the tooth root to the compact bone lining the alveolus.[1] The collagen fibers provide the important property of tensile strength to the periodontal ligament and thus allows it to have a dual function of support and at the same time, enable the tooth to move in response to physiologic forces of mastication, deglutition and speech applied to adult teeth which clinically are stable in a bony socket. These two functions are dependent upon the structural stability of the fibrils brought about by a unique molecular arrangement composed of a highly specific alignment of tropocollagen molecules held together by intermolecular covalent cross-links which confer on the fibrils the high tensile strength.[2]

The collagen fibril in different parts of the body as demonstrated by animal studies, has the ability to turnover at a high rate during growth of the animal[3,4] and in wound healing.[5] However, in most body parts of the adult animal, the collagen fibrils are relatively stable with very little turnover, except to a slight degree in the non-pregnant uterus[6] and a much greater degree in the postpartum uterus.[7]

It has also been claimed that the collagen of periodontal ligament in adult animals with clinically normal gingival tissue, turns over more rapidly than in other connective tissues. The classical method for determining protein turnover times consists of the injection of a

radioactive amino acid into a number of animals followed by a measurement of the amount of labelled protein which remains at subsequent time periods. The earlier evidence suggesting a high turnover rate of collagen in the periodontal ligament came from autoradiographic grain counting experiments over extended time periods after injection of labelled amino acids.[8-14]

The results obtained by this procedure are open to question because tritiated proline, the most commonly used label for studies of collagen metabolism, is not completely specific for collagen as shown by oral connective tissue studies[15,16,17] where half or most of the [H^3]-proline was incorporated into non-collagenous proteins. Hence distinguishing the label in collagen from that in non-collagenous proteins is impossible by autoradiographic techniques alone. In order to differentiate the label in collagen from non-collagenous proteins, highly specific collagenase has been used which demonstrated that positive radiographic labelling was eliminated or markedly reduced.[8,13,15] However, as the proline containing non-collagenous proteins such as proteoglycans and glycoproteins are closely associated and firmly bound to collagen fibrils, any reduction of radioactive label due to removal of collagen, would also be due in part to removal of non-collagenous protein.[18] In addition, in the interpretation of turnover rates of labelled amino acids in time extended autoradiography studies, consideration must be given to the recycling of [H^3]-proline due to localized proteolysis of newly synthesized labelled collagen and non-collagenous labelled proteins.[19] Uncertainty of results could also be produced by the technique itself for according to Rogers,[20] quantitation can be affected by the fixative used, incomplete removal of paraffin embedment, variation in thickness of emulsion if used rather

than stripping film, dirt particles mistaken for silver grains and error in grain density counting due to examiner variability and fatigue.

A more precise method of studying the metabolism of collagen in oral tissues is by the isolation of hydroxyproline and measuring its specific radioactivity following the injection of [H^3]-proline into experimental animals.[16,17,21,22] Although these studies confirm autoradiographic observations that the metabolism of collagen in gingiva and periodontal ligament is much more rapid than in skin and other connective tissues, only one study[22] measured the hydroxyproline radioactivity of soluble and insoluble collagen which allows a more accurate estimate of collagen synthesis and degradation. However, enhancement of these estimates requires consideration of the possibility of recycling of proline label and the measuring of [H^3]-proline uptake by non-collagenous proteins associated with the soluble and insoluble collagen fractions. When the activity of labelled proline in non-collagenous protein was measured, unfractioned samples of collagen were analyzed.[16,17]

Sodek[23] published the first study based on the measurement of specific radioactivity of hydroxyproline and proline isolated from soluble and insoluble collagen fractions from skin, gingiva and molar periodontal ligament of adult rats injected with [H^3]-proline. This author used a microassay technique over short time periods (½ to 27 hrs.) He claimed that measuring the rate of radioactive proline incorporation into newly synthesized collagen and its conversion into mature collagen fractions over the short time period, minimized label recycling problems. The results of the study indicated that circulating proline was rapidly metabolized as demonstrated by a peak of serum activity 20 to 30 minutes

after intraperitoneal injection of isotope which then rapidly declined. In addition, except for skin collagen, the radioactive profile of hydroxyproline in the salt-soluble pool of periodontal ligament and gingiva showed during the first three hours after injection of the isotope, a rapid decrease in activity after an initial high activity. Over the same time period, the profile for the insoluble collagen fractions showed a significant increase in specific radioactivity. From these results it was suggested that there is a much more efficient conversion of soluble collagen to insoluble collagen in periodontal ligament than in skin. Sodek's rationale was that assuming zero growth, the rate of decrease of hydroxyproline specific activity from the salt-soluble compartment must equal the rate of increase of hydroxyproline specific activity in mature insoluble collagen, plus the rate of degradation of the newly synthesized collagen.

More recently Sodek[24] using the same method studied radioactive proline incorporation into collagenous and non-collagenous proteins as well as the recycling of proline label in rat skin, gingiva, alveolar bone and molar periodontal ligament. As in the earlier study,[23] the rate of loss of hydroxyproline radioactivity from the salt-soluble collagen fraction was compared with the increasing rate of activity in the insoluble collagen fraction. This was done to determine the efficiency of conversion of newly synthesized collagen into mature insoluble collagen for each tissue sample. As with the previous study,[23] the radioactivity of the salt-soluble collagen fraction of periodontal ligament showed a rapid drop after an initial peak indicating that the newly synthesized collagen was rapidly removed from the salt-soluble pool. It is possible that recycling of proline label could occur from degradation of newly

synthesized collagen by collagenolytic enzymes. The short time period of the experiment after administration of the isotope was thought to preclude this because it was estimated that it would take at least one hour for synthesis then an additional time period to degrade the newly synthesized collagen to amino acids, plus an even longer time for degradation of insoluble collagen before recycled label is available for incorporation into more recently synthesized tropocollagen molecules. The rapid decline in activity in the periodontal ligament salt-soluble collagen fraction would also indicate that recycling did not occur in this compartment for if it had taken place over the experimental time period, then the specific activity would have remained high for a longer period. Significant recycling of proline label from non-collagenous protein associated with newly synthesized collagen is also negated by a constant low activity relative to the specific activity of hydroxyproline throughout the time period studied.

Comparing the hydroxyproline radioactivities for each tissue sample after correction for differences in the salt-soluble pool size indicated that mature collagen of periodontal ligament turned over at a rate five times faster than that of gingiva, almost six times faster than that of alveolar bone and 15 times faster than the mature collagen of skin.

Although Sodek's results demonstrate that compared to the other tissues studied, there is a higher turnover rate of periodontal ligament collagen in the rat molar which histologically is similar to the periodontium of the human,[25] and even though he claims that the sensitivity of the method reduces the number of animals and amount of isotope compared to other conventional biochemical techniques, he makes

no mention of the number of animals used nor gives a statistical analysis. However, according to Page and Ammons,[22] the amounts of radioactive precursor amino acids necessary to study collagen turnover in intact animals precludes the utilization of large animals, or large number of small animals.

Therefore the use of a whole animal labelling technique may not be appropriate if one wishes to study in large animals, the effect of an experimental variable such as a dental procedure which might influence collagen turnover of the periodontal ligament. Although the use of a very large animal would lend itself more easily to such a procedure, the cost would be prohibitive due to the enormous doses of injectable labelled precursors required.

Even though the collagen of the periodontal ligament in the adult animal turns over at a higher rate than that of connective tissues in other body sites, the reasons are unclear. It has been postulated that it could be due to a persistant eruptive force which can be exaggerated experimentally by extraction of the opposing tooth thus demonstrating active remodelling predominantly in that part of the ligament adjacent to the alveolar bone surrounding the molar of a mature rat.[26] Some support for this theory is suggested by the observation that vertical tooth movement which compensates for occlusal attritional wear is also accompanied by bone apposition at the interradicular crest and fundus with minimal growth of cementum at the root apex.[27] Higher rates of bone growth than that necessary to keep the molars in occlusion is prevented by masticatory forces.[28] However, the rate of [H^3] -proline incorporation into insoluable periodontal ligament collagen of fully erupted rat

molar teeth is three times faster than that for ligament of the continuous erupting rat incisor,[29] thus suggesting that an eruptive mechanism is not responsible for the increased turnover rate or that there is a less efficient conversion of newly-synthesized collagen to mature collagen in incisor ligament.

It has also been postulated that the more rapid collagen synthesis and breakdown in periodontal ligament is the result of mechanical stresses derived from intermittant occlusal forces that cause micro-trauma in the periodontal ligament.[30] Rippen[14] however, demonstrated that the crestal fibrils of rat molar periodontal ligament exhibited an increase in turnover rate in the over-erupting hypofunctional tooth thus supporting his own hypothesis,[13] that turnover is more dependent upon tooth movement and that added functional stress within physiologic limits is not important in determining such turnover rates. As previously stated movement would occur slowly throughout life in a vertical direction as a result of occlusal attrition,[27] as well as migration in a mesial direction due to wear of proximal contact areas. The interproximal wear is a result of occlusal forces giving rise to an anterior component of force which produces a minute reciprocating movement of the teeth within their sockets from their long axis to a mesial position. By the age of 40 years in the human, the overall effect of the flattening of the proximal contacts, is a reduction of 0.5 cm in the length of the dental arch from the third molars to the midline.[31] Another example of minute reciprocating movement of adult teeth which are clinically stable in their bony sockets, is that occurring in a buccal-lingual direction during the functional activities of mastication, deglutition and speech which would also contribute to interproximal wear.

Thus it could be interpreted that movement of adult teeth is the reason for the higher rate of periodontal ligament collagen turnover compared to other connective tissues. This movement is itself a result of functional forces within physiologic limits which initially produce a reciprocating movement in young adult teeth in a mesio-distal and bucco-lingual direction without actual root positional changes. With time this also results in a bodily movement of the teeth in a mesial and occlusal direction accompanied by remodeling of the bony alveolus due to attritional wear on occlusal and interproximal surfaces and rather than being termed a persistant eruptive force it should be called "physiologic drift." We reasoned that because this is such a slow procedure, the measuring of turnover rates of periodontal ligament collagen as a result of physiologic drift must be done within an experimental animal model consisting of unilateral removal of an antagonist tooth. This then allows comparison of the non-functional with the contralateral functional side.

As pointed out previously, biochemical studies of the influences of clinical dental procedures on the turnover rate of periodontal ligament collagen of intact large animals using precursor radioactive labelled amino acids have not been done, because of the prohibitive cost resulting from the large amount of injectable isotope required for adequate incorporation to provide sufficient pre-label for analysis.

PURPOSE

The purpose of the study reported here was to determine whether molar periodontal ligament collagen in large dogs could be shown to be metabolically more active than that of other tissue, i.e., skin collagen; and secondly, whether occlusal non-function (i.e., molar teeth without antagonists) would affect this activity through comparison of collagen turnover rates in ligament of these molars with that of molars functioning under normal physiologic forces during occluding cycles.

We chose to use a novel method to study collagen aging that is, the quantification of collagen cross-links which are reducible with tritiated sodium borohydride as first described by Tanzer[32] and later refined by Mechanic[33] and use this as an indication of the rate of new collagen synthesis.

RATIONALE

Collagen cross-links that can be stabilized by reduction with borobydride for purposes of isolation and identification, are noted to be numerous in growing tissues but with maturity they decrease until in the aged connective tissues, they are virtually no longer detectable.[34] It has been suggested that this decrease of borhydride reducible cross-links is due to stabilization by in vivo reduction.[35] However, Robbins and co-workers[34] could find no evidence for stabilization by reduction in vivo by the use of three different analytical techniques. It has also been hypothesized from preliminary but as yet not fully confirmed results, that in vivo stabilization of collagen cross-links is by oxidation.[36]

It is nevertheless clear that the number of detectable cross-links is lower in tissues from mature animals which are known to have a low rate of collagen synthesis, than in younger animals.

We postulated that an increase in the amount of reducible cross-links can only mean that new collagen is being laid down in that tissue. If the tissue is not growing then the new synthesis must be balanced by an equal amount of breakdown, i.e. turnover.

Since collagen cross-links can be detected in a few milligrams of tissue we proposed that their quantification should provide information regarding relative collagen turnover rates in control and experimental tissues.

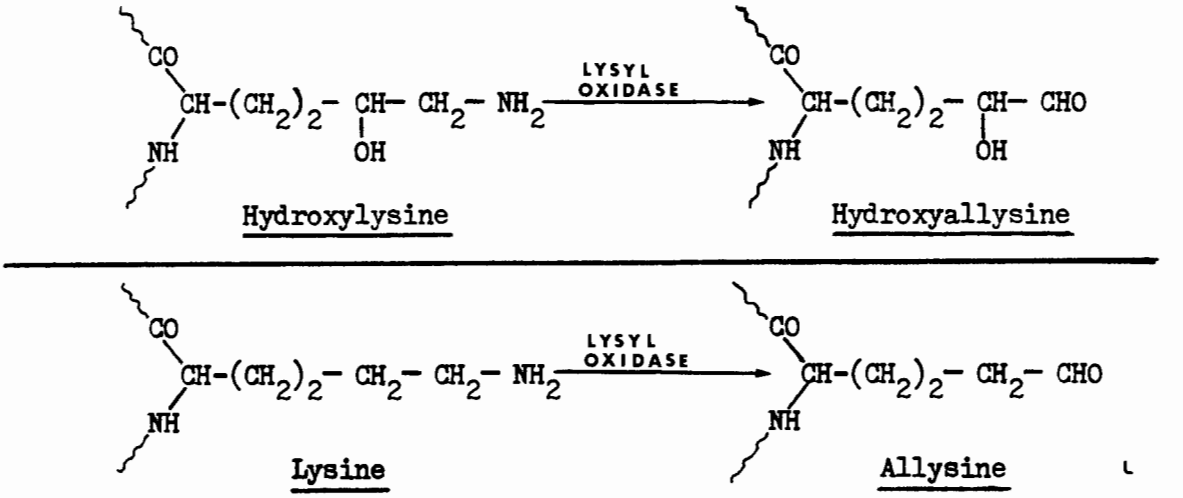
Preliminary studies revealed that adult mongrel dogs sacrificed for other experimental purposes and whose weights ranged from 25 to 36 kilograms, yielded dessicated weights of 5 to 20 mgs. of periodontal ligament scraped from the roots and alveolar sockets of mandibular molars. In order to assure adequate tissue for study it was concluded that dogs weighing more than 34 kilograms would be required for this investigation.

BACKGROUND ON COLLAGEN CROSS-LINKS

The cross-links involved in stabilizing collagen fibrils have been recently reviewed by Bentley.[37] These cross-links have as their origin the amino acid lysine which is incorporated into nascent collagen during synthesis within the cell. Before extrusion of the collagen molecule into the extracellular compartment, hydroxylation of some of the lysine residues occurs to form a product that contains both lysyl and hydroxylysyl residues. Further reaction of some of these latter residues

occurs outside the cell in the form of oxidative deamination by the enzyme lysyl oxidase, resulting in compounds with the trivial names of allysine and hydroxyallysine. Two functional groups are thus present in the collagen molecule; the amino groups of lysine and hydroxylysine and the aldehyde groups of allysine and hydroxyallysine (Fig.1). Intramolecular cross-links are formed by condensation of two allysine residues near the N-terminal region (Fig.2), and are referred to as aldol condensation products whereas, intermolecular cross-links are the result of the reaction of the amino and aldehyde functional groups to form Schiff bases. Theoretically four different Schiff base combinations are possible (Fig. 1); the combination of course depends upon which lysyl and hydroxylysyl residues are oxidized and upon their relative positions on neighbouring molecules. The initial formation of the cross-link by the condensation of hydroxylysine and hydroxyallysine for example, would give rise to an aldimine compound with double bonds between carbon 6 of one partner and the nitrogen on the carbon 6 of the other (Fig.3). However, the aldimine form lacks stability and through the Amadori rearrangement that occurs in most tissues, a more stable keto form of the cross-link is produced (Fig.4). In fact, the hydroxylysine-hydroxyallysine cross-link only exists in the keto form which also is the predominant form of the initial Schiff base formation between lysine and hydroxyallysine whereas the Schiff based formed by hydroxylysine and allysine appears as the aldimine as it cannot exist in the keto form (Fig.5).

The presence of these cross-links can be demonstrated by treating intact tissue with tritium labelled potassium or sodium borohydride which reduces the double bond in the cross-link and results in the addition of the tritium label (Fig.6). When the tissue is subsequently hydrolyzed



POSSIBLE SCHIFF BASE BETWEEN

Hydroxylysine & Hydroxyallysine

Hydroxylysine & Allysine

Lysine & Allysine

Lysine & Hydroxyallysine

Figure 1. The origin of Allysine and Hydroxyallysine and the possible Schiff base pairing giving rise to cross-links.

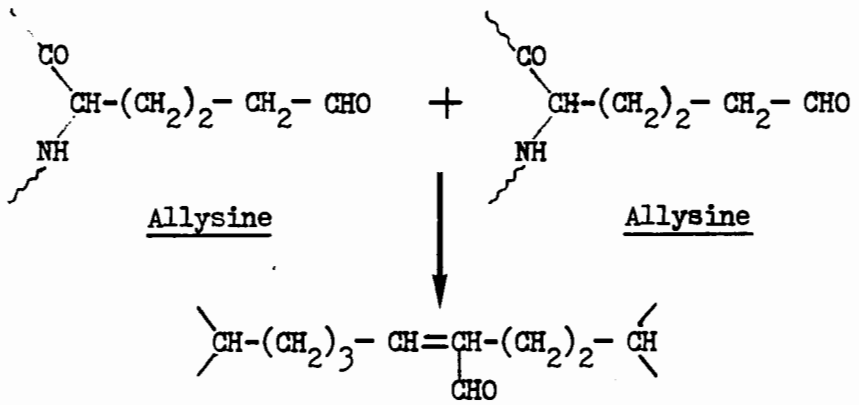


Figure 2. The Intramolecular Crosslink. Internal stabilization of the collagen molecule is by the condensation of two Allysine residues near the N-terminal region which gives rise to an Aldol.

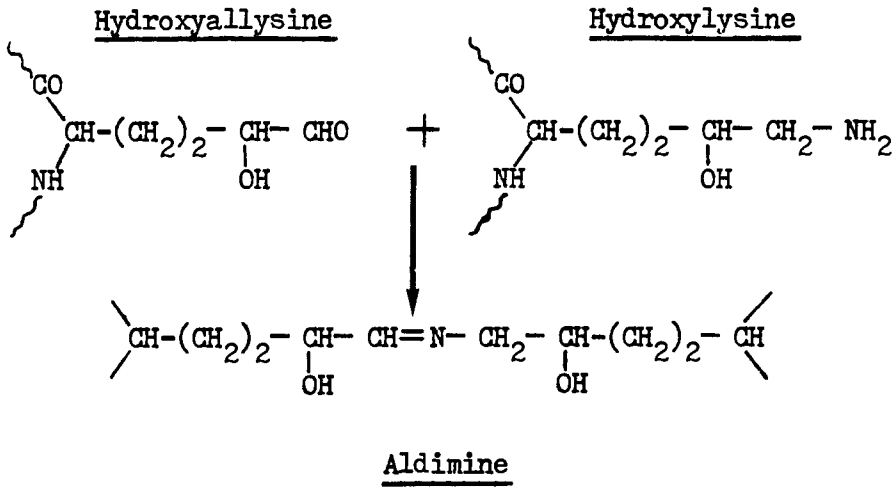


Figure 3. The Intermolecular Crosslink. The initial formation of the Schiff base by the condensation of Hydroxylysine and Hydroxyallysine gives rise to the unstable Aldimine with a double bond between carbon 6 of Hydroxyallysine and the nitrogen on carbon 6 of Hydroxylysine.

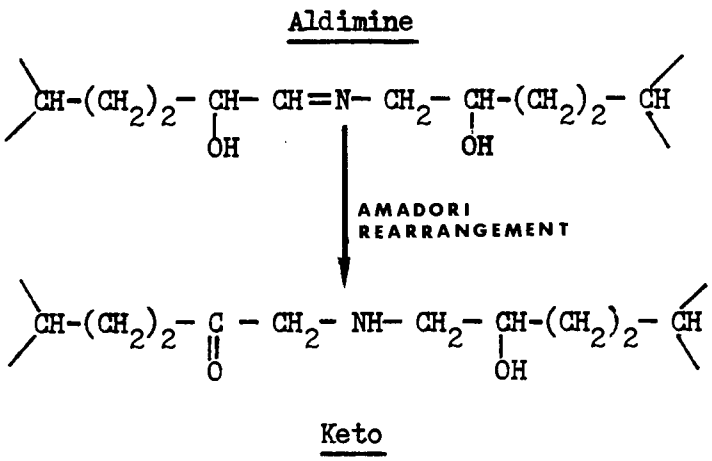


Figure 4. The secondary formation to the more stable Keto form is a result of the Amadori rearrangement of the Aldimine whereby the double bond between carbon 6 of hydroxyallysine and the nitrogen on carbon 6 of hydroxylysine migrates to replace the hydroxyl group with an oxygen molecule on carbon 5 of hydroxyallysine.

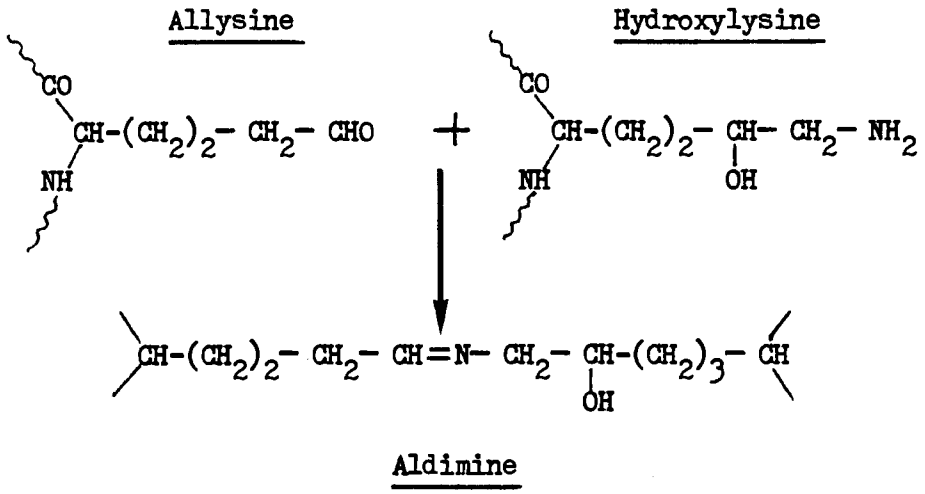


Figure 5. The crosslink formed between Allysine and Hydroxylysine resulting in the formation of the Aldimine dehydrohydroxylysinonorleucine, which cannot exist in the Keto form.

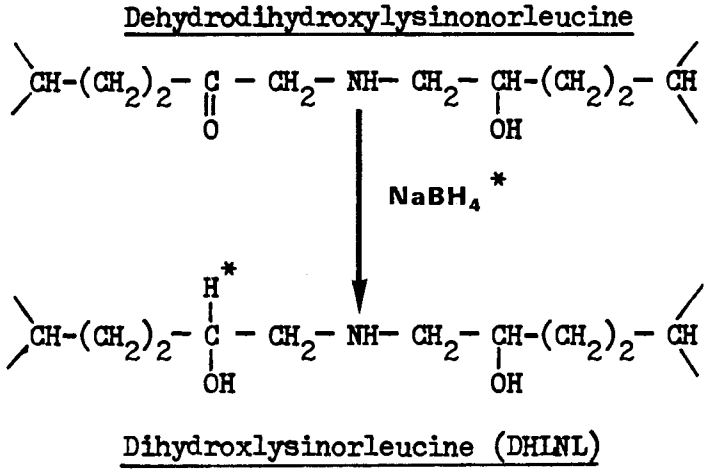


Figure 6. The reduction of the double bond in the dehydrodihydroxylysinonorleucine Keto of the Hydroxyallysine-Hydroxylysine Schiff base pair which results in the formation of the reduced crosslink dihydroxylysinonorleucine. The asterisk represents a tritium atom that would occur in DHLNL after reduction with tritium-labelled Sodium Borohydride.

SCHIFF BASE PAIR	NON-REDUCED CROSSLINK	REDUCED FORM MEASURED
HYD-ALLYS & LYS	DEHYDROHYDROXYLYSINONORLEUCINE	HLNL
HYLYS & ALLYS	DEHYDROHYDROXYLYSINONORLEUCINE	HLNL
LYS & ALLYS	DEHYDROLYSINONORLEUCINE	LNL
HYD-ALLYS & HYLYS	DEHYDRODIHYDROXYLYSINONORLEUCINE	DHLNL

Figure 7: Nomenclature of the reduced Schiff base crosslinks: HLNL - hydroxylysinonorleucine; LNL - lysinonorleucine; DHLNL - dihydroxylysinonorleucine.

in acid this radioactively labelled cross-link compound survives and can be separated from the amino acids by the use of a modified amino acid analyzer and quantified.

The three major reduced forms of the intermolecular cross-links that can be measured by this method are shown in figure 7 as hydroxylysinonorleucine (HLNL), lysinonorleucine (LNL), and dihydroxylysinonorleucine (DHLNL). The only cross-links that can be measured therefore, are reducible cross-links and the form in which they are isolated is different from that in which they existed in tissue.

DHLNL which is predominant in collagen of embryonic skin,[38] tendon,[39] bone,[40] and healing wounds[41] is also the major detectable reduced cross-link in bovine periodontal ligament of unerupted, partially erupted and fully erupted functioning molar teeth, while HLNL forms the minor constituent.[42] However, unlike fetal and mature bovine skin collagen where DHLNL falls to a low level after birth and HLNL rises to a maximum at about one year of age[38] along with a decrease in the ratio of DHLNL to HLNL with age,[34,43] these two cross-links were observed by Pearson et al.[42] to remain relatively constant in amount and proportion in bovine periodontal ligament over the different stages of tooth eruption. This would suggest that unlike developing skin, the type and ratios of intermolecular cross-links of periodontal ligament collagen remain constant from the early developmental stage to the fully erupted and functional stage.

METHODS

I. Tissue Collection and Preparation

Three adult male dogs, one a Rhodesian Ridgeback (dog #1), another a German Shepherd (dog #2), and the third a Siberian Husky (dog #3), were used in this experiment. The initial weights of the animals were 36.1, 37.9, and 35.7 kilograms respectively. Management of the animals was under the Principles of Laboratory Animal Care according to the American Association of Laboratory Animal Science.

Under general anesthesia induced by a mixture of Ethrane-N_2O and O_2 via tracheal intubation, the right and left mandibular molars and premolars of each dog received a clinical examination for assessment and recording of plaque and gingival indices,[44] and for gingival sulci depths using a Michigan periodontal probe with Williams markings. The crowns of the posterior teeth on the right and left mandibular sides were cleaned of debris and stains as well as a planing of roots to the base of the gingival sulcus. This procedure was again repeated 2 to 3 weeks later and at the same time, a full thickness flap was raised to gain better access and the maxillary fourth premolar was extracted and the normally occluding mesial marginal ridge of the maxillary first molar and the distal cusp of the mandibular first molar were ground to eliminate tooth contacts on the right side of dogs #1 and #3 and the left side of dog #2. The alveolus was then reduced in height and contoured to allow complete wound closure with 4-0 chromic sutures. Following the surgery the animals were given 2,400,000 units of procaine penicillin G. intramuscularly and a 3 gram acetylsalicylic acid suppository. Up to this time the animals were fed a standard diet of kibbled dog chow but after the extraction of

the maxillary fourth premolars and up to the end of the experiment, the diet was switched to a softer one of canned dog food.

After an interval of two weeks dog #1 was again given a general anesthetic, plaque and gingival scores and sulci depths were first recorded then right and left mandibular molars were extracted after the raising of full thickness flaps and removal of all ligament tissue coronal to the bony crests by vigorous scraping with a periodontal curet. The molars were placed in individual labelled petri dishes and the roots moistened with 0.02 M phosphate buffer (pH 7.4) before covering the dishes. The wounds were closed and postoperative medication given as previously described.

The ligament tissue was obtained from each molar by gently scraping the roots with a Ward's double-ended hoe and spoon and immediately placed in separate labelled 10 ml. Sorval centrifuge tubes. The centrifuge tubes had been previously weighed and were then handled with clean metal tongs in order to maintain the known weights of the tubes. The tissues were covered with approximately 5 ml of 0.02 M.phosphate buffer (pH 7.4) and stirred overnight in the cold in order to extract non-collagenous material. The samples were centrifuged, the supernate discarded and the residue washed by briefly stirring in three portions of distilled water. Following the final centrifuging and drawing off of the supernate, the tissue samples were frozen and stored until the other ligament tissue samples were collected and treated in a similar manner, from dog #2 at four weeks and dog #3 at six weeks. A sample of dermis was also collected by careful dissection from a small area in the anterior throat region of the third animal.

The frozen samples in the Sorval tubes were lyophilized overnight, the tubes weighed immediately as they were taken off of the lyophilizer and dry tissue weights calculated. Each sample was then resuspended in 5 ml. of 0.2 M phosphate buffer (pH 7.4), for four hours in the cold, to render the tissues more permeable for the reduction with sodium borohydride.

Tritiated sodium borohydride (Amersham) with a specific activity of 555 mCi/mmoles (14.6 mCi/mg) was prepared so as to have a final suspension of 3 mg/ml with a specific activity of 250 mCi/mmoles. From previous studies in this laboratory it was found that a volume of 150 µl of ^{3}H-borohydride with this specific activity was required for reduction of 40 mg of collagen. Therefore using these figures as a standard, the volume required to reduce the weights of each tissue sample was calculated. Reduction of each sample in phosphate buffer was accomplished on a magnetic stirrer in the fume hood by three individual additions of the calculated volume of the ^{3}H-borohydride. The radioactive samples were then carefully transferred to dialysis bags and dialyzed overnight in a cold room against running water in order to remove excess salts and radioactive material. After removal from the dialysis bags and placing in 20 ml bottles, the samples were frozen in dry ice, lyophilized to dryness and transferred to hydrolysis vials. Hydrolysis was with 3N p-toluenesulfonic acid after sealing under vacuum and heating at 107°C for 24 hours. Upon completion of hydrolysis, the liquified samples were filtered into screw cap vials by passage through glass wool packed in a Pasteur pipette.

II. Chromatography on the Long Column

From each sample of the hydrolyzate a 25 µℓ aliquot was drawn, placed in a 7 ml scintillation vial to which 5 ml of Aquasol was added and the vials were then placed in a Packard Ticarb Liquid Scintillation Counter for determination of total counts per minute (cpm) in order to calculate the volume which would give 2×10^6 cpm/ml to apply to the column. In more than half of the samples, this volume was greater than 50% of the total sample. Hence in order to insure sufficient volume of sample for a second analysis in the case of a mishap, the volume needed for 1×10^6 cpm/ml was determined and for standardization this amount was applied to the column with appropriate adjustments made in the radioactive counting time to compensate for the lowering of the cpm/ml.

Chromatography of each aliquot of the reduced-aldehydic cross-link precursors and cross-links was through a 62 x 0.9 cm column packed to the 58 cm mark with a spherical cation exchange resin of 9-12 microns (Mark Instruments Co.) as described by Mechanic.[33] Elution was performed with a complex gradient generated by a 9 chambered Technicon Autograd gradient device using a starting buffer of pH 3.15 sodium citrate, 0.25 M in Na^+ and containing 6% isopropanol and a limiting buffer of 0.4 M sodium citrate (pH 9.1).

The procedure was initiated by drawing off the starting buffer from the top of the column resin and gently layering on top of the resin the required volume of the reduced hydrolyzate to which had been added 0.2 ml of a 1 mg/ml concentration of amino acid "spike" consisting of glycine, phenylalanine, tyrosine, hydroxylysine and lysine dissolved in 0.01 N HCl. Starting buffer was then used to rinse the vial that tempo-

rarily held the sample and the "spike" and this amount plus whatever additional amount was required to fill the column to the top, was gently layered over the sample followed by attaching the fitting to the top of the column.

The column effluent was led through a 1/16" teflon tubing to a Gilson Escargot Fraction Collector which was set to dispense a fraction every 1.5 minutes into three racks of 80 tubes each for a total of 240 numbered tubes. The complete fractionation took slightly more than 6 hours at a flow rate of 70 ml/hr with a back pressure on the column of 315/240 psi.

III. Radioassay Technique

Previous studies[33,35,45,46] showed that the major reducible cross-links are not eluted in the early fractions collected. Therefore a 200 µℓ aliquot was taken from even numbered tubes up to tube number 60 and henceforth from every tube and placed into 7 ml mini scintillation vials. In order to prevent cloudiness, 200 µℓ of distilled water was added to the vials containing the aliquots taken from the first rack of 80 tubes, 400 µℓ to the aliquots from the second rack of 80 tubes and 600 µℓ to the third set of aliquots. A 5 ml volume of Aquasol was then added to the vials and each vial was counted three times in a Packard Tri-Carb scintillation spectometer for a period of 2 minutes. A second 200 µℓ aliquot was taken for a qualitative ninhydrin reaction to determine the positions of the amino acids used as standards. The position of the amino acids and the radioactive values obtained in cmp's for the tritium labelled reducible cross-links were plotted against their locations in each numbered fraction (Figs. 8-14). Counts per minute were converted

to disintegrations per minute by the use of automatic external standardization of the Tri-Carb.

IV. Chromatography on the Short Column

To increase resolution of radioactive peaks eluting from the long column in the region of the basic amino acids (see Fig. 8) and to verify the identity of a presumptive reduced cross-link by comparison with a standard (fetal dentine), a 30 x 0.9 cm column packed with the same cation resin to the 28 cm mark was used. In this column, elution was performed using a pH 5.28 sodium citrate buffer 0.35 M in Na^+ ion containing 0.55% benzyl alcohol with an average flow rate of 72 ml/hr and a column back pressure of 200-250 psi.

Samples for rechromatography were obtained by pooling a group of 5-7 fractions representing each radioactive peak eluted from the long column and the volume to apply to the short column that would give 10,000 cpm/ml was determined by counting a 25 µl aliquot from each pooled sample. To this volume was added 0.05 ml of an amino acid "spike" containing cysteic acid, glycine, phenylalanine, hydroxylysine and lysine in a concentration of 5 mg/ml of 0.01 N HCl. The "spike" and the sample were then acidified to a pH of 1-2 with 6 N HCl and applied to the top of the resin. Sodium citrate buffer was gently layered on top of the sample and nitrogen pressure applied until the fluid at the top of the resin became introduced into the column to a point level with the top of the resin. The column was then carefully filled to the top with buffer and the top fitting placed. As with the long column, the effluent was passed into the Fraction Collector which was set to dispense a fraction every 1.0 minute into each of 80 numbered tubes.

The radioactivities of the fractions were counted by transferring a 200 µℓ aliquot from even numbered tubes into mini scintillation vials, adding distilled water to prevent cloudiness and then a 5 ml volume of Aquasol. Counts were made for one minute and then in the region of the peaks, recounts for five minutes were made for every fraction. A qualitative ninhydrin reaction was also performed on a 200 µℓ aliquot of every fraction in order to determine the position of the added amino acids in relation to the radioactive peak. Plots were constructed for each of the peaks representing a reduced cross-link (Figs. 15-21). A sample of fetal dentine with known positions of DHLNL and HLNL was likewise eluted through the short column and a plot made of the radioactive counts obtained (Fig. 22).

V. Hydroxyproline Analysis[47]

Dilutions of 1/125 were made on a 200 µℓ aliquot drawn from each of the six reduced hydrolyzates of periodontal ligament and a 1/250 dilution on a similar aliquot from the skin sample in order to determine hydroxyproline values. From the results obtained in this analysis the concentration of collagen in the reduced hydrolyzed sample and the percentage of collagen content of the original dry weight of each sample were calculated. In calculating the results it is assumed that: (collagen = hydroxyproline x 7.46).[48] The figures obtained for the collagen concentration was used to calculate the amount of collagen in each sample applied to the long column. This then allowed an accurate expression of specific radioactivities in disintegrations per minute/unit weight of collagen for comparison of reducible cross-links in each sample.

RESULTS

Although plaque scores (Table I) at the time of extraction of the antagonist maxillary fourth premolars were only slightly reduced from those recorded two weeks earlier, the gingival inflammation indices showed greater reduction. However, from this period and up to the time reported for collection of experimental and control tissues, no further removal of debris or plaque was attempted and as a result, the gingval indices had increased significantly. As expected, the gingival inflammation and plaque indices increased progressively in severity with the length of the experiment (Table I), but sulci depths (not shown) were increased by less than 1 mm in isolated areas particularly at the six week period. There also appeared to be no real differences in plaque and gingival indices of experimental and control sides, hence the figures given in Table I represent combined recordings for both sides. At the time of collection of ligament tissue from each animal, it was confirmed with carbon articulating paper that no occlusal contact existed between the posterior grinding element (talonid) of the experimental mandibular molar and the maxillary molar.

Dry weights of tissue samples after removal of non-collagenous material with phosphate buffer followed by lyophilizing are shown in Table II. For each pair of periodontal ligament samples, weights are almost identical except for the six week specimens. This difference is a result of fracturing and a loss of a significant portion of the coronal third of the mesial root of the experimental tooth during the extraction. Hence less periodontal containing root surface was available.

TABLE I: Plaque and Gingival Indices Recorded From Mandibular Right and Left Molars and Bicuspids at Initial Examination,[a] at Time of Extraction of Antagonist Teeth[b] and Collection of Experimental and Control Tissues.[c]

Dog No.	Plaque Index	Gingival Index
1	1.12 1.08 1.33 (1)	1.00 [a] 0.75 [b] 0.91 [c]
2	1.31 1.28 1.50 (2)	0.91 [a] 0.72 [b] 1.16 [c]
3	1.34 1.25 1.90 (3)	1.03 [a] 0.78 [b] 1.25 [c]

(1) - two weeks after removal of antagonist

(2) - four weeks after removal of antagonist

(3) - six weeks after removal of antagonist

Elution profiles from the 62 cm column of the 3N p-toluenesulfonic acid hydrolyzates of 3H-sodium borohydride reduced periodontal ligament connective tissue from non-functioning (experimental) and functioning (control) mandibular first molars are shown in Figures 8 to 14. The positions of the amino acids in relation to the radioactive peaks as indicated by the qualitative ninhydrin reaction, are shown in each of the profiles. As in other studies,[33,35,45,46] the peak for DHNL falls between aspartic acid and threonine, the peak for HNL lies between proline and glycine, the peak for DHLNL is between phenylalanine and hydroxylysine and the peak for HLNL lies between hydroxylysine and histidine. The radioactive peaks given in cpm's show that in dog periodontal ligament, DHLNL is the major reduced cross-link and HLNL is the minor reduced cross-link whereas in dog skin, HLNL is the major and DHLNL is the minor reduced cross-link (Fig. 14). This is in agreement with previously published information of adult bovine skin[34] and bovine periodontal ligament.[42]

It is not possible to compare the data directly as shown in figures 8 to 14, since the concentrations of collagen and hence the amount applied to the column varied from specimen to specimen (Table II).

The homogeneity of each cross-link was checked by rechromatography on the 30 cm (short column) and identified by the characteristic elution position between phenylalanine and hydroxylysine. As can be seen in figures 15 to 21, the average position for the observed maximum peak of DHLNL is fraction number 32 and for HLNL fraction number 39. This compares favorably with fraction number 30 and 37 for the observed peaks of DHLNL and HLNL in fetal dentine (Fig. 22). The profiles also indicate that DHLNL eluted completely in 4-5 fractions while HLNL eluted

TABLE II: Specific Radioactivities of Each Sample Calculated From mg/ml of Collagen Applied to the Long Column.

Dog No.	Specimen Number	Dry Weight (mgs.)	Total Collagen (mgs.)(1)	Mgs. Collagen Per Mg. Dry Weight(2)	Mgs. Collagen Applied To Column(3)	DPM/Peak	DPM Per Mg. of Collagen	Ratio DHLNL/HLNL
1 (2 wks)	R-10 (Exper.)	11.05	4.136	0.374	1.30	60,304	405,011 a	2.8
						21,267	142,831 b	
	R-11 (Cont.)	11.27	4.660	0.413	1.28	70,944	484,399 a	2.7
						26,286	179,477 b	
2 (4 wks)	S-12 (Exper.)	18.30	8.552	0.467	1.62	86,778	467,300 a	3.4
						25,268	136,059 b	
	S-13 (Cont.)	18.40	8.658	0.471	1.49	76,778	449,268 a	3.5
						20,238	128,568 b	
3 (6 wks)	H-14 (Exper.)	12.39	6.136	0.495	1.47	67,884	403,347 a	3.2
						21,533	127,943 b	
	H-15 (Cont.)	20.20	9.954	0.493	1.89	94,242	436,014 a	2.6
						36,470	168,731 b	
Dog Skin Dermis	H-16	43.06	19.504	0.453	2.05	14,386	61,466 a	0.66
						21,697	92,703 b	

(1)(2)-accuracy uncertain because of variable percentages of collagen in sample and loss in transfer of dry tissue.

(3)-determined from hydroxyproline analysis of hydrolysate.

a DHLNL

b HLNL

in 6-7 fractions. It is also obvious that the short column profiles of periodontal ligament (Figs. 15 to 20) demonstrate that no other significant activity is present in eluted fractions except in those fractions representing the radioactive peaks for DHLNL and HLNL. This indicates a lack of contamination with other material. There was however, an additional peak of low radioactive magnitude eluted in fractions 16 to 22 of the skin dermis collagen sample (Fig. 21) which was not identified.

The hydroxyproline assays on the hydrolysate of the total dried ligament and of the aliquot applied to the column permitted us to calculate the concentration of cross-links either in terms of specific activity, e.g. dpm in DHLNL/mg of collagen or as total activity, e.g. total dpm in DHLNL/tooth.

The data shown in Tables II and III suggest that the cross-link specific activities and total activities of all periodontal ligament specimens were four to five times higher than those from dermal collagen, which is in agreement with earlier biochemical studies in rodents.[16,21,22,23,24] This shows that this tissue does indeed exhibit a higher collagen turnover than skin. The data also confirms that the novel analytical method used can indeed give information regarding collagen turnover in minute pieces of tissue and has potential in studies of biopsy specimens of human periodontal tissues exhibiting periodontal disease or excessive occlusal forces.

No significant differences were seen between the ratios of DHLNL to HLNL in ligaments from control (functional) or experimental (non-functional) teeth. These ratios were all relatively close to 3:1 similar to that shown in bovine periodontal ligament.[42]

TABLE III: Composite Averages of Disintegrations Per Minute of DHLNL and HLNL Per Mg of Collagen and the Resulting Ratios of DHLNL to HLNL in Experimental and Control Samples of Dog Periodontal Ligament and Skin Connective Tissues.

	DHLNL	HLNL	DHLNL/HLNL
Experiment.	$425{,}220 \pm 10^4$	$135{,}615 \pm 10^4$	3.1
Control	$456{,}560 \pm 10^4$	$158{,}925 \pm 10^4$	2.9
Skin	$61{,}470 \pm 10^3$	$92{,}700 \pm 10^3$	0.66
Exp/Skin	6.9	1.5	
Cont/Skin	7.4	1.7	

FIGURE 8: Chromatographic patterns of the radioactive components of a 3N p-toluenesulfonic acid hydrolysate of tritium labelled sodium borohydride reduced dog periodontal ligament collagen from the two week non-functioning (experimental) mandibular first molar. The order of elution is dihydroxynorluecine (DHNL), fractions 34-42; hydroxynorluecine (HNL), fractions 50-54; dihydroxylysinonorluecine (DHLNL), fractions 171-179; hydroxylysinonorluecine (HLNL), fractions 190-196. Note that the major reducible cross-link is DHLNL. Elution was through a single column 62 x 0.9 cm packed to the 58 cm mark with a spherical cation exchange resin of 9-12 microns.

FIGURE 8: SPECIMEN R-10 (Experimental)

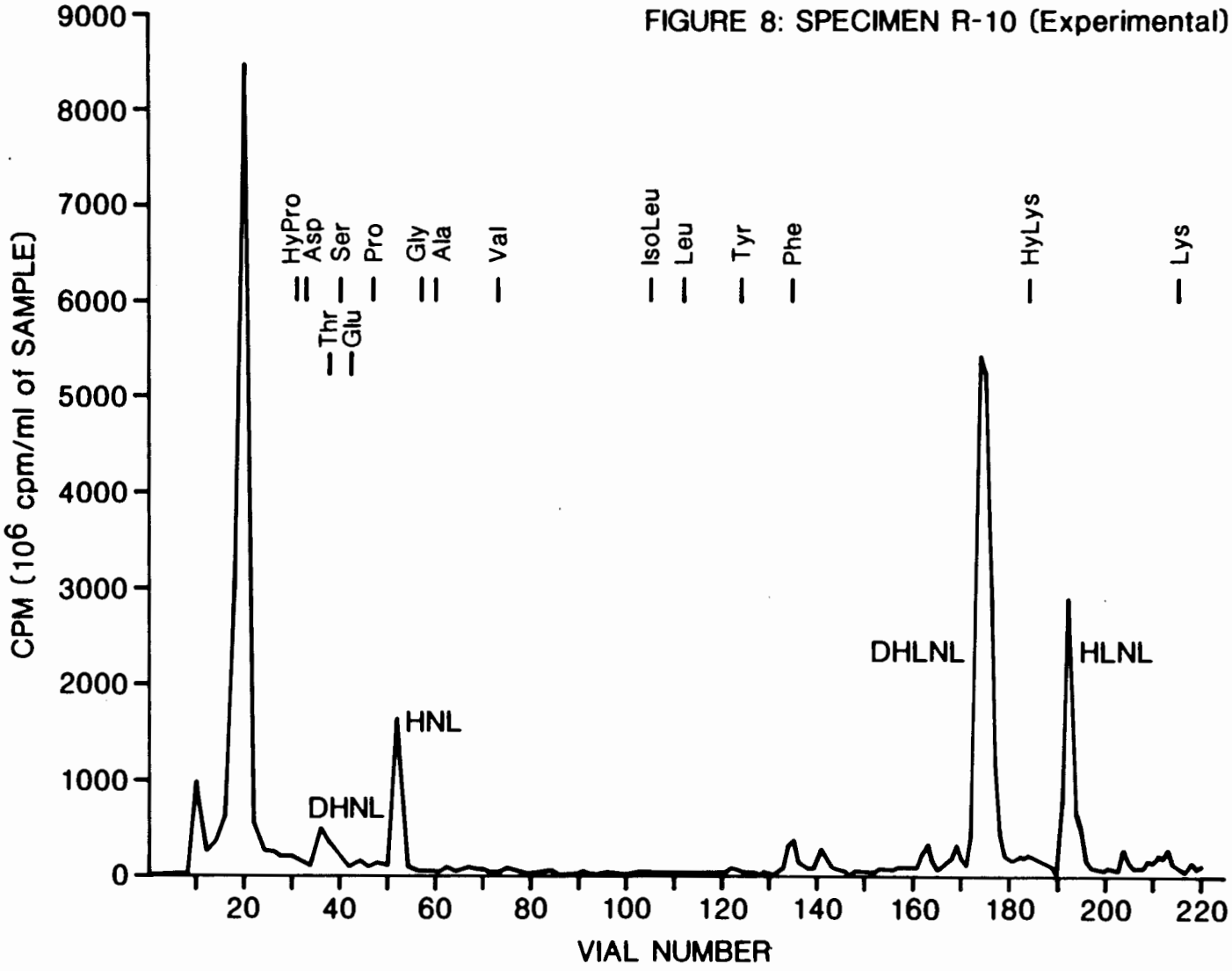


FIGURE 9: Chromatographic patterns of the acid hydrolysate of tritium sodium borohydride reduced ligament collagen from the functioning (control) mandibular first molar on the opposite side of the two week experimental animal. Note that DHLNL is also the major reducible cross-link.

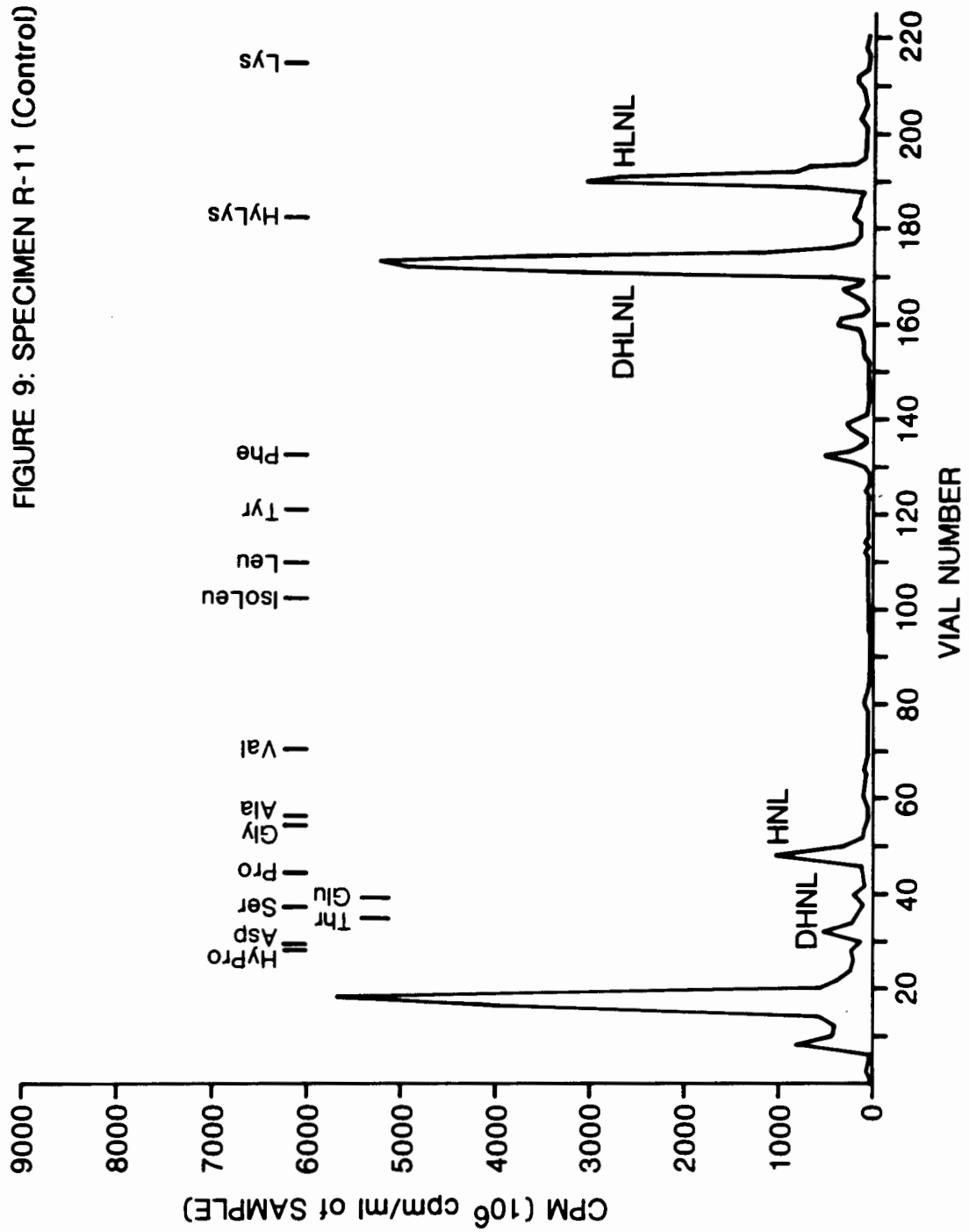


FIGURE 9: SPECIMEN R-11 (Control)
CPM (10⁶ cpm/ml of SAMPLE)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
20
40
60
80
100
120
140
160
180
200
220
VIAL NUMBER
HyPro
Asp
Thr
Glu
Ser
Pro
Gly
Ala
Val
IsoLeu
Leu
Tyr
Phe
HyLys
Lys
DHNL
HNL
DHLNL
HLNL

Figure 10: Chromatographic patterns of the radioactive components of a 3N p-toluenesulfonic acid hydrolysate of tritium labelled sodium borohydride reduced dog periodontal ligament collagen from the four week non-functioning (experimental) mandibular first molar. Note that the major reducible cross-link is still DHLNL.

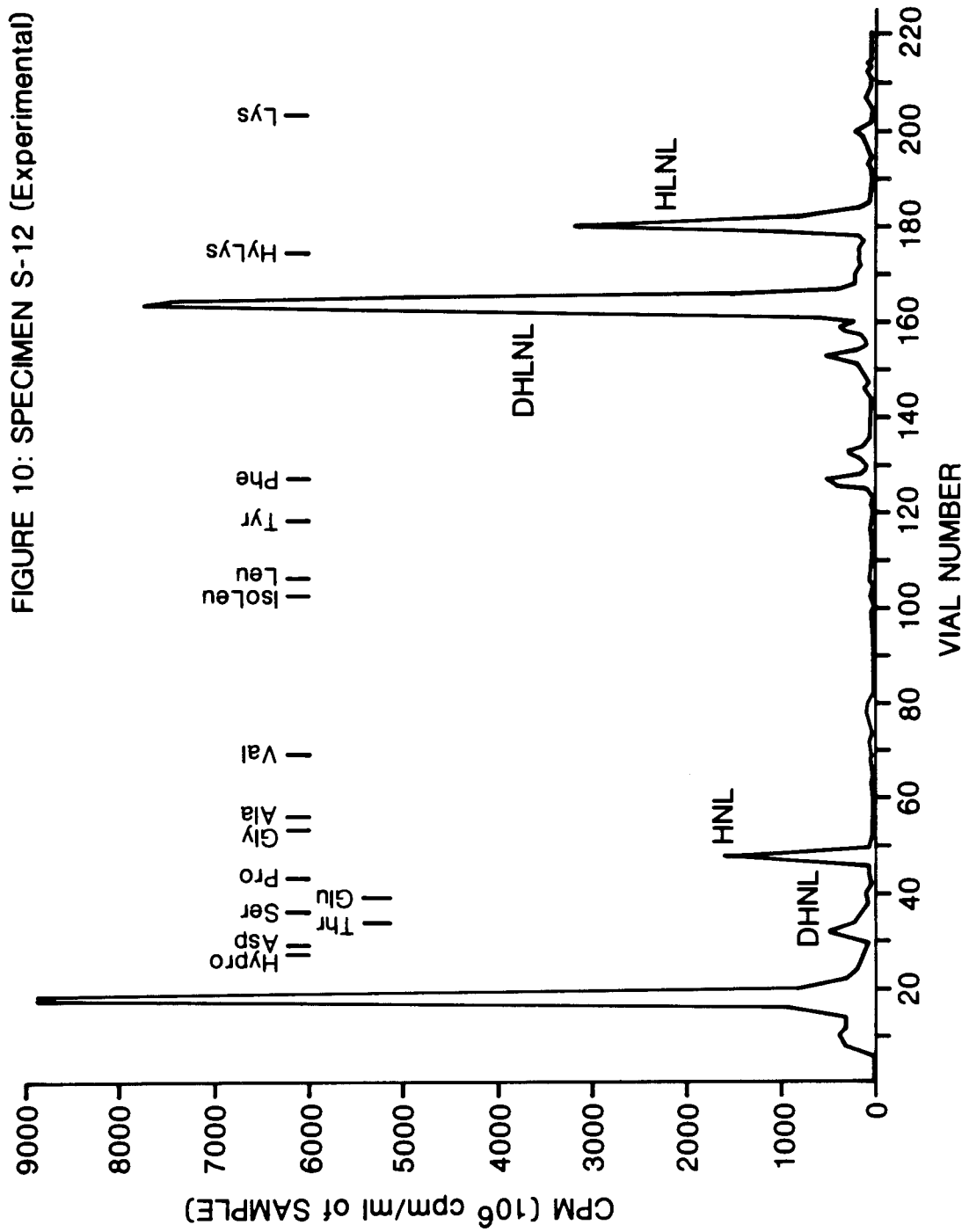


FIGURE 10: SPECIMEN S-12 (Experimental)
CPM (10⁶ cpm/ml of SAMPLE)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
20
40
60
80
100
120
140
160
180
200
220
VIAL NUMBER
HNL
DHNL
DHLNL
HLNL
Hypro
Asp
Thr
Ser
Glu
Pro
Gly
Ala
Val
Isoleu
Leu
Tyr
Phe
Hylys
Lys

Figure 11: Chromatographic patterns of the acid hydrolysate of tritium sodium borohydride reduced ligament from the functioning (control) mandibular first molar on the opposite side of the four week experimental animal. Note that there are no obvious changes in the major and minor reducible cross-links.

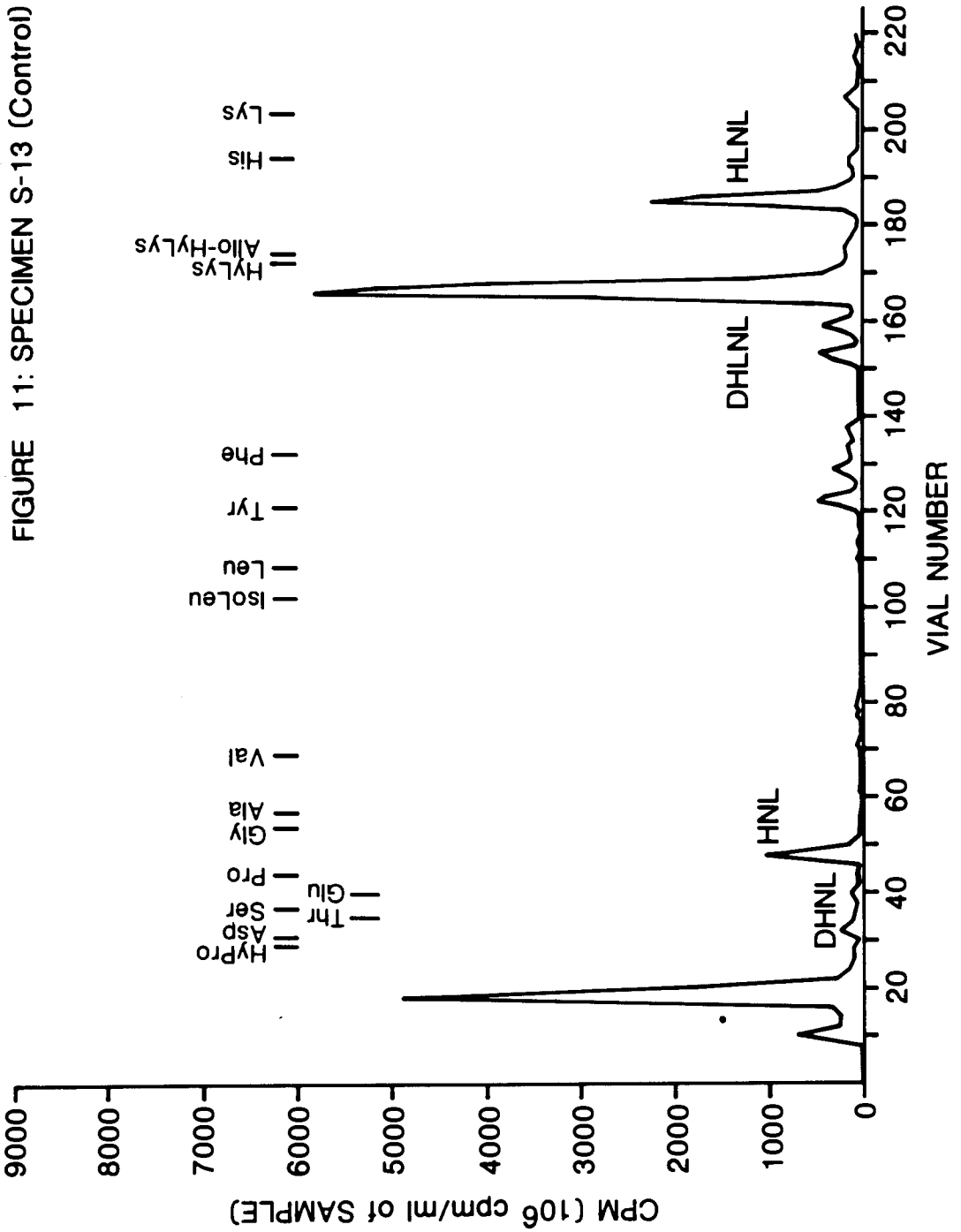


FIGURE 11: SPECIMEN S-13 (Control)
CPM (10⁶ cpm/ml of SAMPLE)
VIAL NUMBER
DHNL
HNL
DHLNL
HLNL
HyPro
Asp
Thr
Ser
Glu
Pro
Gly
Ala
Val
IsoLeu
Leu
Tyr
Phe
HyLys
Allo-HyLys
His
Lys

Figure 12: Chromatographic patterns of the radioactive components of a 3N p-toluenesulfonic acid hydrolysate of tritium labelled sodium borohydride reduced dog periodontal ligament collagen from the six week non-functioning (experimental) mandibular first molar.

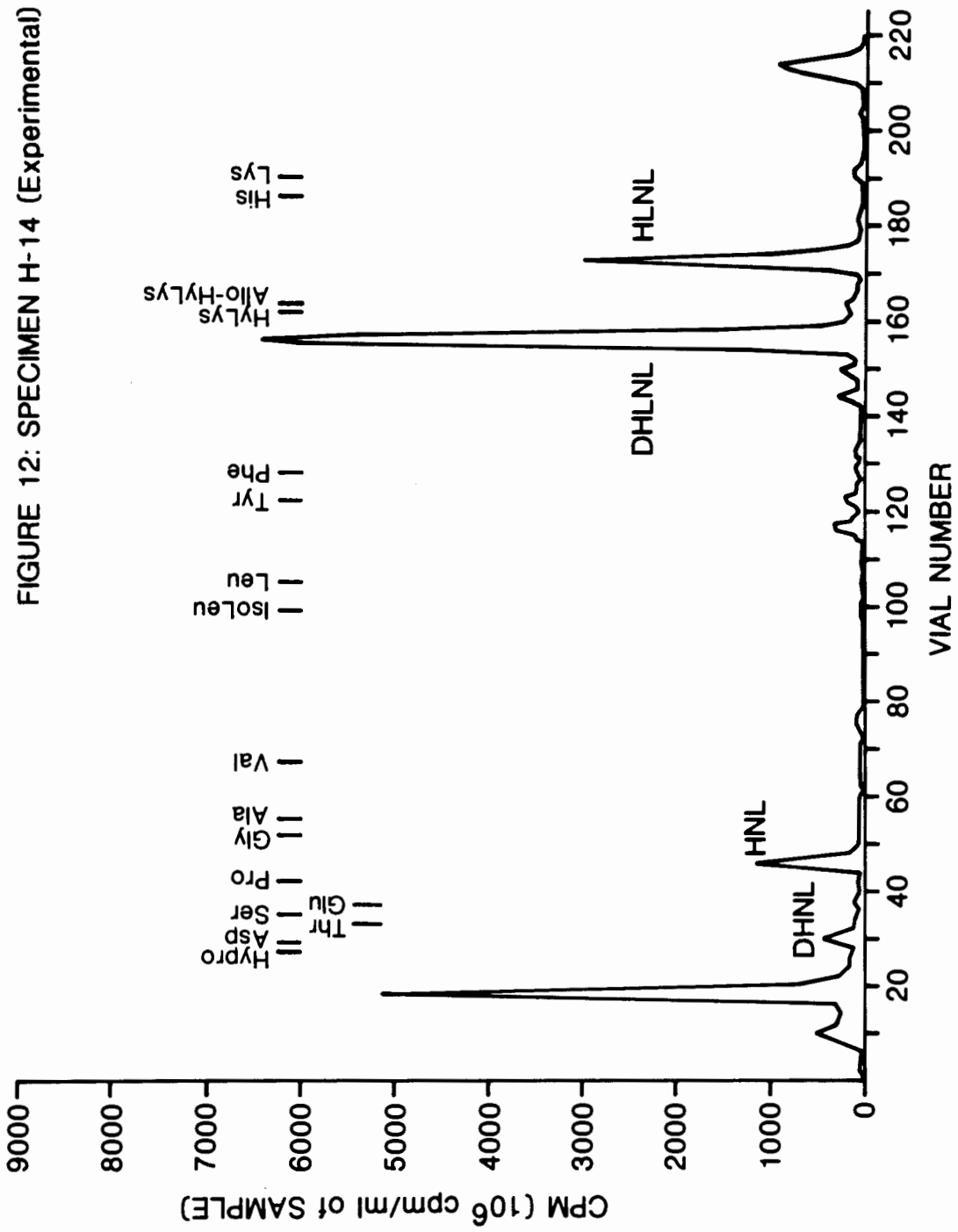


FIGURE 12: SPECIMEN H-14 (Experimental)
CPM (10⁶ cpm/ml of SAMPLE)
VIAL NUMBER
DHNL
HNL
DHLNL
HLNL
Hypro
Asp
Thr
Glu
Ser
Pro
Gly
Ala
Val
IsoLeu
Leu
Tyr
Phe
HyLys
Allo-HyLys
His
Lys

Figure 13: Chromatographic patterns of the acid hydrolysate of tritium sodium borohydride reduced ligament collagen from the functioning (control) mandibular first molar on the opposite side of the six week experimental animal. Note that as in all the previous chromatographs, DHLNL is the major reducible cross-link in periodontal ligament collagen.

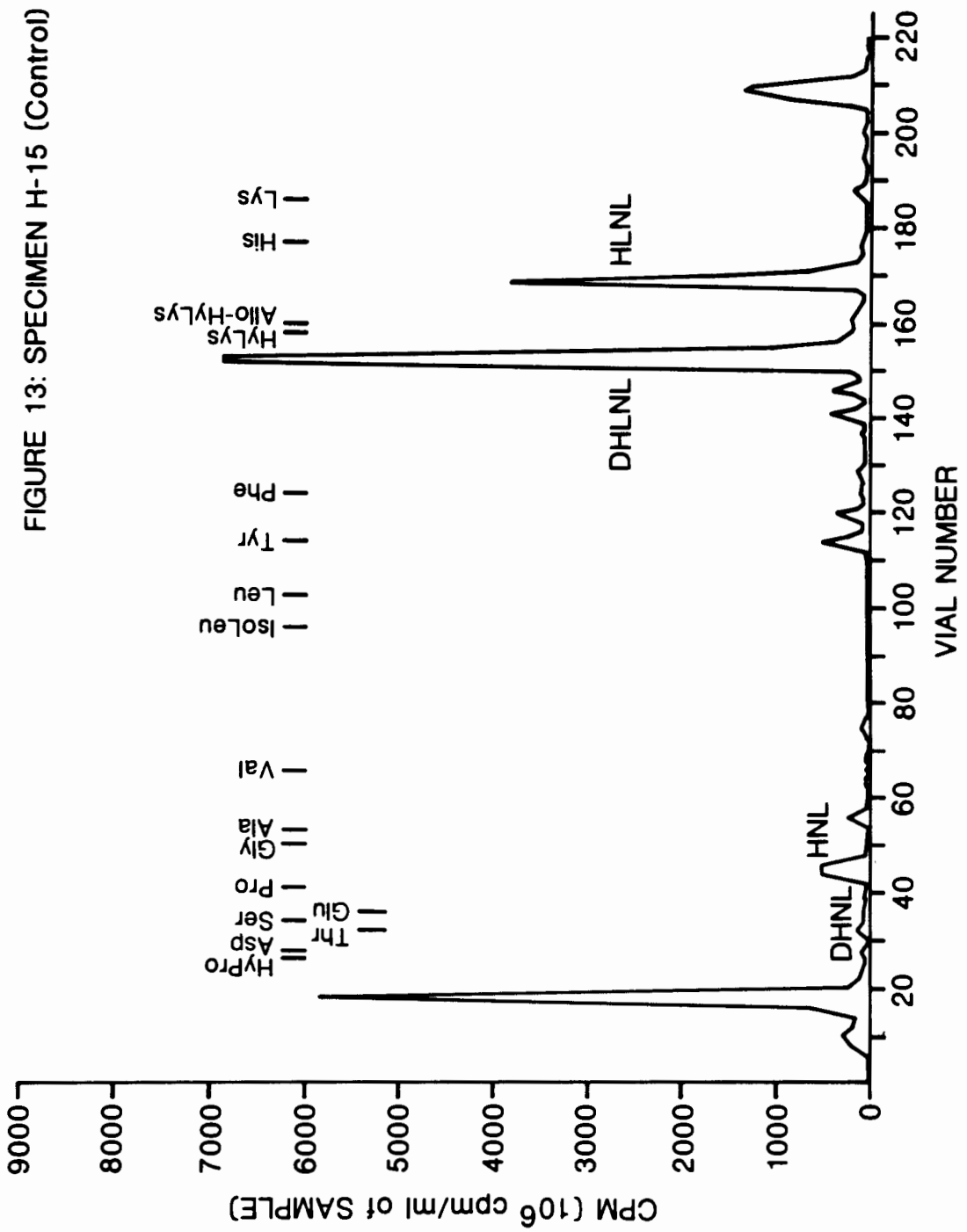


FIGURE 13: SPECIMEN H-15 (Control)
CPM (10⁶ cpm/ml of SAMPLE)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
20
40
60
80
100
120
140
160
180
200
220
VIAL NUMBER
DHNL
HNL
DHLNL
HLNL
HyPro
Asp
Thr
Glu
Ser
Pro
Gly
Ala
Val
IsoLeu
Leu
Tyr
Phe
HyLys
Allo-HyLys
His
Lys

Figure 14: Chromatographic patterns of the acid hydrolysate of tritium sodium borohydride reduced dog subcutaneous collagen. Note the higher peak for HLNL which is the major reducible cross-link in skin collagen.

FIGURE 14: SPECIMEN H-16 (Skin Dermis)

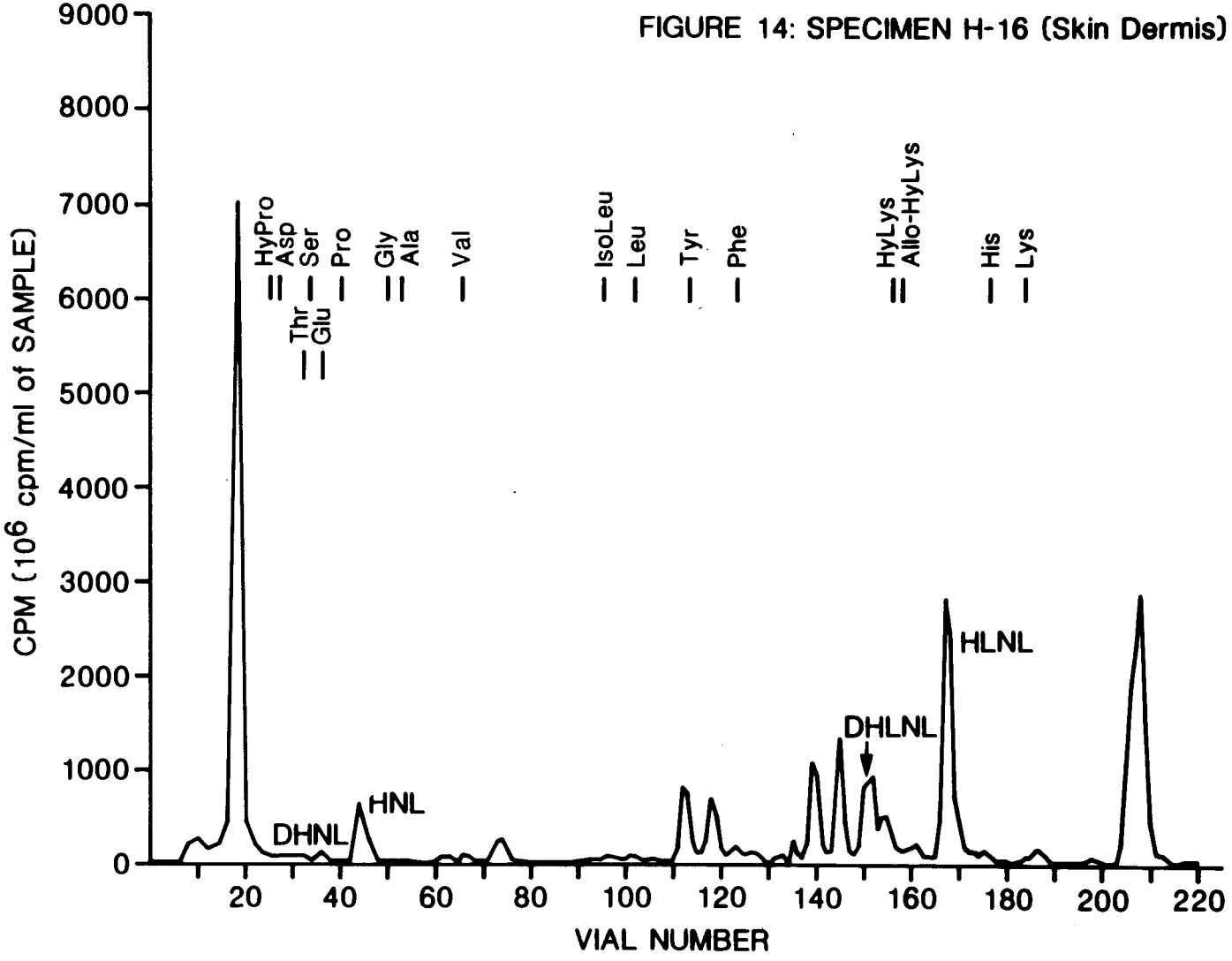


Figure 15 & 16: Rechromatographic fractionation of the reduced crosslinks in ligament collagen from the two week non-functional (Figure 15, experimental) and functional (Figure 16, control) molars that coeluted from the 62 cm column. Individual pooled fractions representing the radioactive peaks of dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL) from the 62 cm column, were eluted through a 30 x 0.9 cm column packed to the 26.5 cm mark with a spherical cation exchange resin of 9-12 microns. The order of elution for both reduced crosslinks in all the experimental and control samples is in the range of fractions 29-35 for DHLNL and fractions 37 to 44 for HLNL.

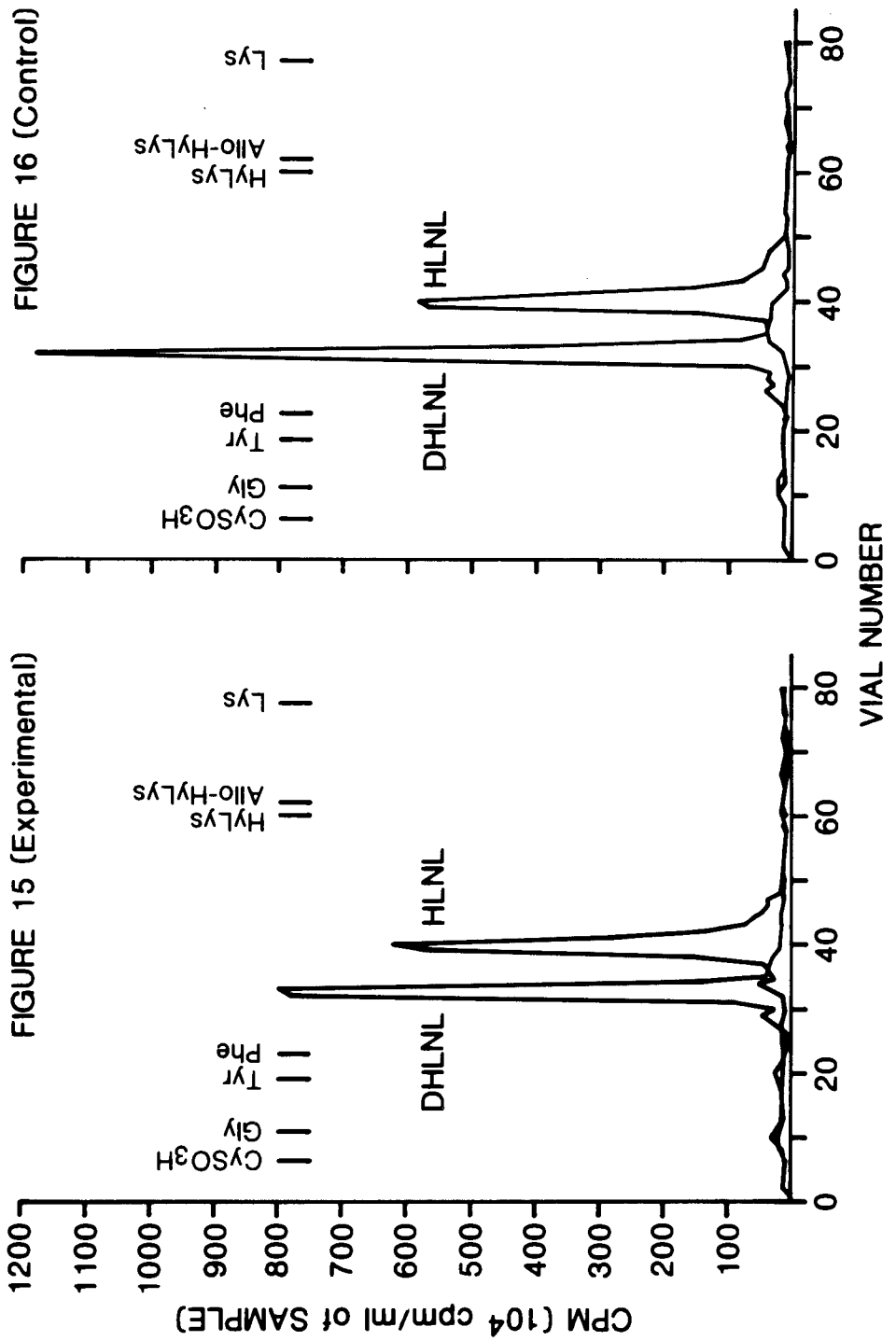


FIGURE 15 (Experimental)
FIGURE 16 (Control)
CPM (10⁴ cpm/ml of SAMPLE)
VIAL NUMBER
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
20
40
60
80
CysO3H
Gly
Tyr
Phe
DHLNL
HLNL
HyLys
Allo-HyLys
Lys

Figure 17 & 18: Rechromatographic fractionation of the reduced cross-links in ligament collagen from the four week non-functional (Figure 17, experimental) and functional (Figure 18, control) molars.

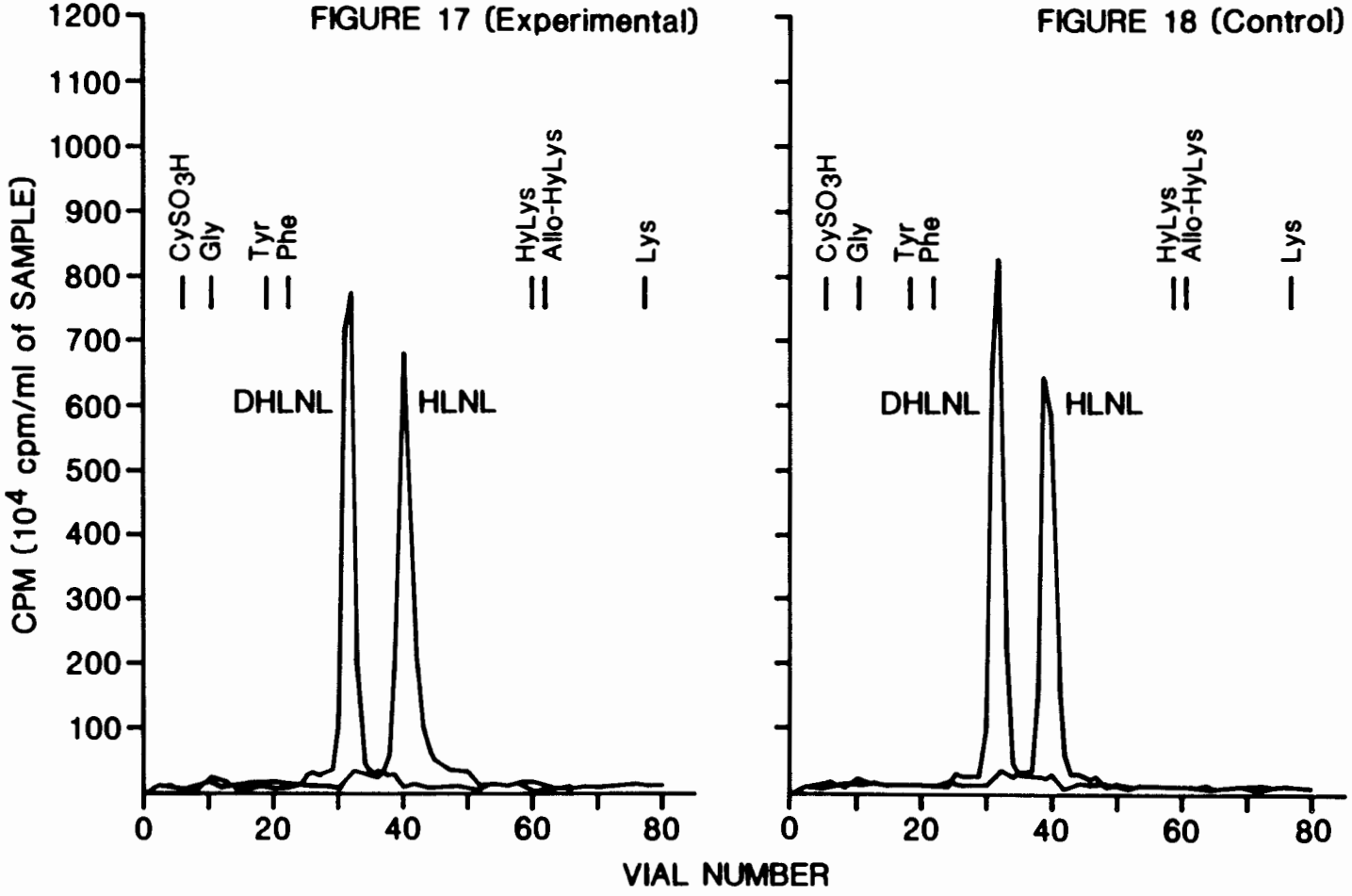


FIGURE 17 (Experimental)
FIGURE 18 (Control)
CPM (10^4 cpm/ml of SAMPLE)
1200
1100
1000
900
800
700
600
500
400
300
200
100
CySO3H
Gly
Tyr
Phe
HyLys
Allo-HyLys
Lys
DHLNL
HLNL
0
20
40
60
80
VIAL NUMBER

Figure 19 & 20: Rechromatographic fractionations of the reduced cross-links in ligament collagen from the six week non-functional (Figure 19, experimental) and functional (Figure 20, control) molars. Note that the radioactive peaks for HLNL in both these samples is slightly higher than that of DHLNL.

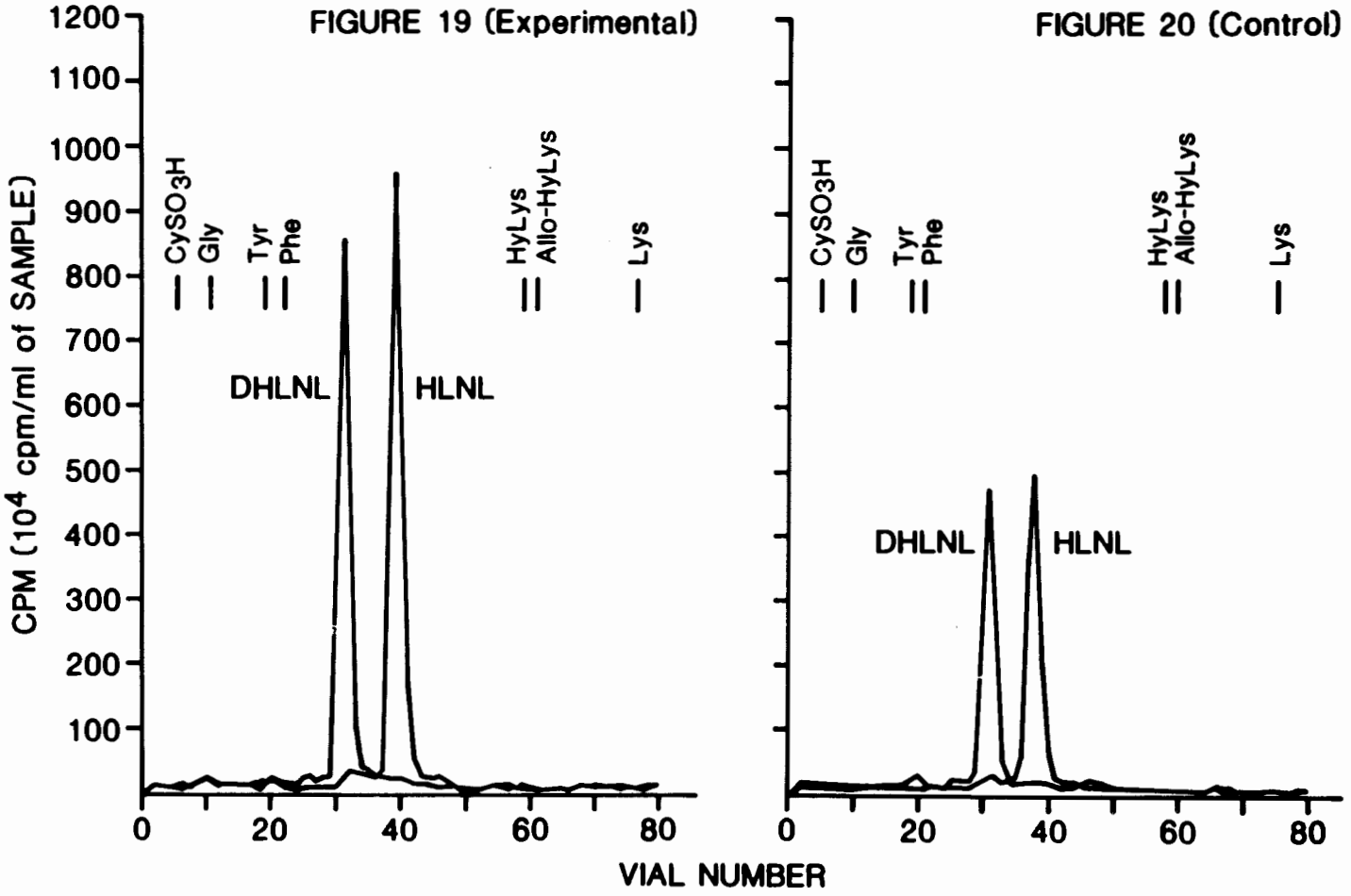


FIGURE 19 (Experimental)
FIGURE 20 (Control)
CPM (10^4 cpm/ml of SAMPLE)
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
20
40
60
80
VIAL NUMBER
$CySO_3H$
Gly
Tyr
Phe
HyLys
Allo-HyLys
Lys
DHLNL
HLNL

Figure 21: Rechromatographic fractionation of the reduced cross-links in subcutaneous collagen that coeluted from the 62 cm column.

Figure 22: Chromatographic fractionation of the reduced cross-links in a sample of fetal dentine. Elution was also through the 30 cm basic column.

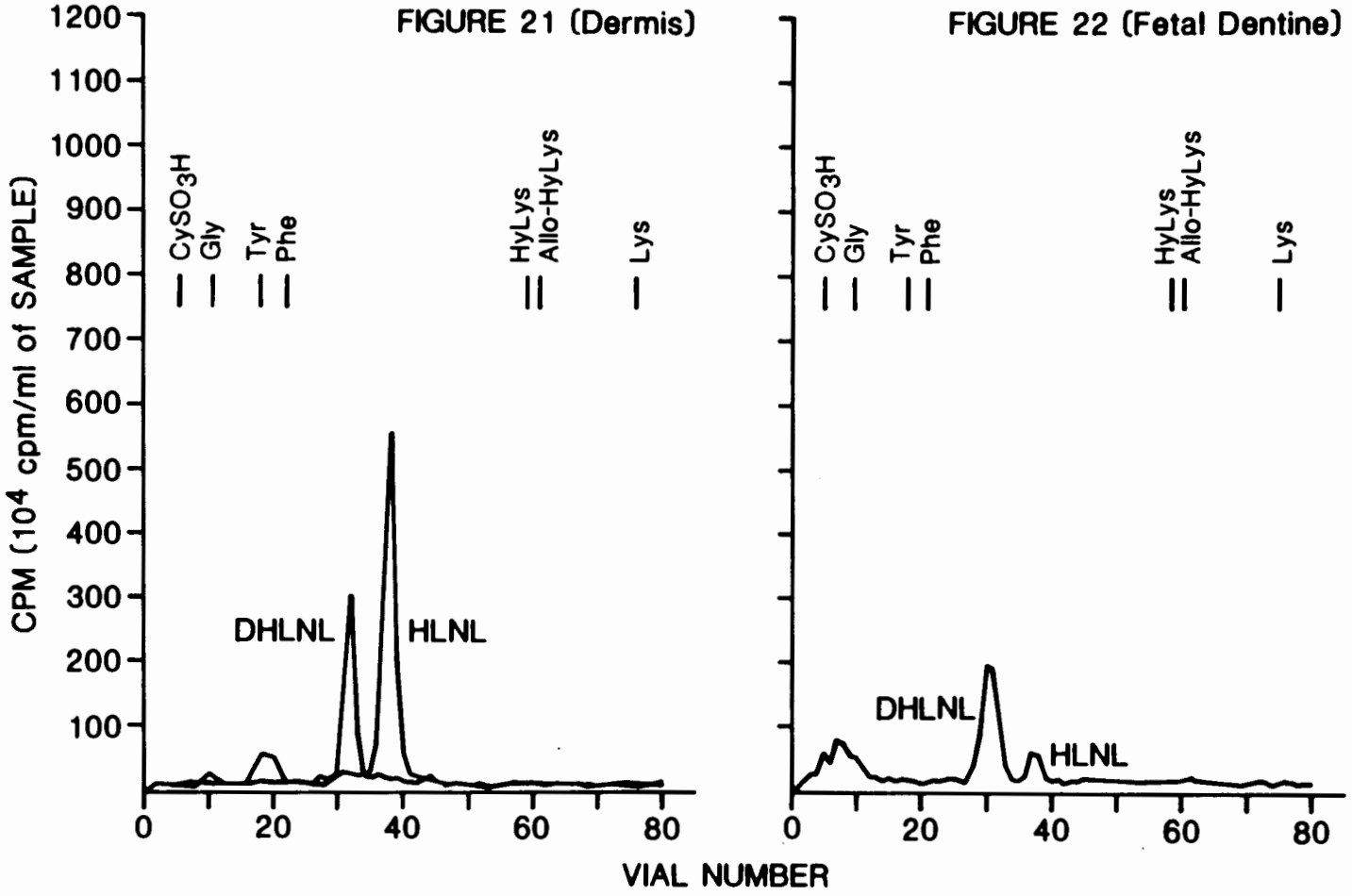


FIGURE 21 (Dermis)
FIGURE 22 (Fetal Dentine)
CPM (10^4 cpm/ml of SAMPLE)
1200
1100
1000
900
800
700
600
500
400
300
200
100
CySO3H
Gly
Tyr
Phe
HyLys
Allo-HyLys
Lys
DHLNL
HLNL
0
20
40
60
80
VIAL NUMBER

DISCUSSION

Previous Studies[11,12,15,16,23,24] using autoradiographic or biochemical assay techniques of radioactive proline incorporation have shown that periodontal ligament collagen is much more metabolically active than skin or other oral connective tissue collagen. However, few of these studies took into account the recycling of proline label, the incorporation of radioactive proline into non-collagenous proteins and the differences in turnover rates of salt-soluble, acid-soluble and insoluble collagen fractions. In those biochemical studies[23,24] which did consider these factors, no quantitative comparison was made of the effect of occlusal forces on the rate of collagen turnover in periodontal ligament.

Studies which have attempted to relate physiologic forces as being responsible for the higher turnover rate of periodontal ligament collagen by experimentally inducing eruption or altering occlusal forces,[8,13,14,26,49] have reported conflicting results as to the amount and site of greatest remodelling in the periodontium compared to the controls. Diverse sites of remodelling have also been reported in teeth subjected to orthodontic movement.[9,50,51] Therefore very little conclusive evidence is available about the influences of experimentally induced and physiologic tooth movements on the metabolism of periodontal ligament collagen subjected to these stresses.

Autoradiographic[13,14,49,52] and electron microscopy studies[49,53,54] which considered only the effects of normal masticatory forces on the periodontium have suggested that the most active site of collagen metabolism in the rodent incisor is located at an "intermediate plexus" whereas in molar teeth, collagen turnover appears to be evenly distri-

buted throughout the width of the periodontal ligament with a slightly higher turnover in areas of osteoclastic activity.

As stated earlier, there are no quantitative biochemical studies cited in the literature describing the isolation of hydroxyproline and the measuring of its specific radioactivity following the injection of $[H^3]$-proline in order to determine metabolic differences in periodontal ligament collagen of molar teeth with and without antagonists.

Studies of this nature are difficult to do in small animals because the minimal amount of tissue available often requires pooling of periodontal ligament samples from roots of more than one tooth to provide sufficient material for analysis. Similarly, gingival samples containing supracrestal ligament fibers also require pooling of more than one sample. However, overcoming this problem by using larger animals such as dogs or miniature pigs is not feasible because of the prohibitive cost of the large volume of injectable isotope required for adequate incorporation to provide sufficient pre-label for analysis.

We therefore chose to study the applicability of a novel cross-link analysis procedure for the measurement of collagen turnover in specimens of periodontal ligament obtained from mandibular first molars of dogs. The cross-links which are intermolecular in nature are numerous in metabolically active collagen and their presence can be demonstrated by treating intact tissue with tritium labelled borohydride which reduces the cross-link and results in the addition of the tritium label. Hence a quantiative comparison can be made of collagen turnover in tissue samples of periodontal ligament taken from the roots of molar teeth with and without antagonists. The results show that the method is applicable

as all specimens of periodontal ligament had a four to five times higher specific activity of cross-links per weight of collagen than did skin collagen, thus demonstrating as previously mentioned that periodontal ligament collagen is much more metabolically active than skin or other oral connective tissue collagens. The results also demonstrate that like bovine periodontal ligament,[42] DHLNL is the major reduced cross-link and HLNL is the minor reduced cross-link whereas in dog skin,HLNL is the major and DHLNL is the minor reduced cross-link which is in agreement with bovine skin.[34] In addition, the approximate 3:1 ratio of DHLNL to HLNL in ligaments from both control (functional) and experimental (non-functional) teeth are similar to the ratio shown for bovine periodontal ligament.[42]

A comparison between the specific activity of the reducible cross-links DHLNL and HLNL in the experimental and control specimens obtained at the different time periods, does not show a significant difference and therefore does not support previous suggestions that either tooth movement or mechanical stresses as a result of a persistant eruptive force or intermittant occlusal functional forces within physiologic limits, are responsible for modifying collagen turnover within the periodontal ligament. That is, if masticatory forces are a factor then functioning control specimens should have had a higher specific activity than the non-functioning experimental side. On the other hand if tooth movement is responsible for the more rapid collagen synthesis and breakdown in periodontal ligament, then the experimental side should have shown a higher specific activity. It is possible that six weeks is not a long enough period in the dog for sufficient eruption to take place

in order to significantly affect collagen turnover. However, although extrapolation of data from one animal species to another is difficult because of the differences in dentitions, it seems logical to assume that if mandibular molars of the rat show signs of eruption within two days after removal of the maxillary antagonist[26] then mandibular molars of the dog would surely begin eruption over the time period of our experiment.

It is also possible that there was little difference between the masticatory forces applied to experimental and control sides due to the soft diet fed the animals which would require very little chewing and the absence in the dog of a lateral excursion movement of the lower jaw during masticatory cycles. Nevertheless, this does not explain why periodontal ligament collagen is much more metabolically active than skin or other oral connective tissue collagen.

It could be speculated that the reason for the higher turnover rate of periodontal ligament collagen than skin collagen is the greater amount of type III collagen in ligament. The percentage of type III collagen in dog molar periodontal ligament and skin has not been reported, however, similarly to rapidly growing fetal calf skin,[41] rat molar ligament[55] and bovine incisor ligament[56] have been shown to contain 15 percent and 18 percent respectively of type III collagen whereas, in mature guinea pig skin[41] type III collagen comprises only 4 percent of the total collagen. There are no comparative studies of periodontal ligament and skin of any species as to the rate of maturation of type I and type III collagens. However the results of Sodek and Limeback[55] suggest that rat gingiva and molar ligament have the same rate of maturation of type I and type III

collagen even though gingiva has 5 percent less type III collagen and a slower collagen turnover rate than molar ligament.[23,24] These authors[55] also conclude that the proportion of type III synthesis bears no relationship to the rate of turnover of collagen in these tissues as rat incisor and molar ligament contain similar amounts of type III collagen, yet incisor ligament turns over at only 1/3 the rate of molar ligament.

It therefore appears that according to the present knowledge, differences in turnover rates are not related to differences in the content of type III collagen. Nor does it appear that the absence of occlusal forces or the presence of an increased eruptive force modifies the collagen turnover of periodontal ligament. Instead it appears that the increased turnover of periodontal ligament is an innate feature of this tissue.

Rippen[14] from his autoradiographic observations of a slower turnover rate in healthy crestal fibers of the periodontal ligament of the rat, suggested that this may act as barrier and resist the spread of disease from the gingiva directly into the periodontal ligament. He hypothesized that this barrier function could help to explain the usual route of the disease process which has been shown to be initially via vascular channels at the alveolar crest of non-traumatized teeth.[58-60] As stated earlier, biochemical techniques using labelled precursor amino acids[16,23,24] have shown that non-inflammed gingival collagen in animals, turns over at a slower rate than ligament collagen. Comparative studies of collagen turnover rates in inflammed gingiva and normal controls using a similar biochemical technique, have not been done in animal tissues. However other methods have shown that qualitative and quantitative changes do occur in animal and human connective tissue components of the periodontium,

beginning in the very early stages of inflammatory disease. Histologic, histochemical[61-63] and electron microscopic[64-67] observations have provided information on altered morphologic features of fibroblasts and collagen fibrils while others[63,67-74] comparing the collagens of normal and pathologically altered gingival and periodontal tissues, showed differences in the solubility of salt and acid-soluble fractions, as well as a decrease in the total collagen content of the affected tissues. Paunio[71] concluded from his solubility experiments, that collagen in diseased samples of human periodontal ligament dissolved more readily because it had less developed cross-links than those of control samples. It has also been suggested[75] that because collagen loss occurs within 2 to 4 days after initiating experimental gingivitis the rapidity of breakdown is due initially to an increased degradation by hydrolytic enzymes and at subsequent stages, inhibition of collagen synthesis may then be an important factor. However, recent in vitro studies[76] have shown that fibroblasts harvested from diseased human gingiva, synthesized an abnormal molecule with the chain structure $\alpha 1[I]_3$ which persisted through several cell generations. If the same situation occurs in vivo, altered intermolecular cross-links may also be associated with this chemically different collagen. In fact, Bailey and co-workers[77] found that in contrast to normal subcutaneous collagen of the rat, the major reducible cross-link in experimentally induced granulomas was DHLNL. It was also reported that in this rapidly proliferating granulation tissue, the newly synthesized collagen was of a lower solubility than collagen of normal rat skin. Fetal skin collagens with a high ratio of the reduced cross-link DHLNL have likewise been reported to exhibit a decreased solubility compared to mature skin.[39,57] Collagens of normal

rat gingiva[45], bovine periodontal ligament[42] and dog molar periodontal ligament as shown in this present study, all have in common DHLNL as the major reducible cross-link. There are no reports on the solubility of bovine and dog periodontal ligament collagen, however rat periodontal ligament and gingival collagen like embryonic skin collagen, were shown to be less soluble to salt and acid solutions than mature skin collagen.[25]

However as stated earlier, inflammation has been shown to increase the solubility and decrease the total collagen content of gingival and periodontal ligament tissues. It therefore could be postulated that in the early inflammatory lesion of gingival and ligament connective tissues, degradation of collagen is preceeded by synthesis of a chemically different type of collagen and a change in the nature or number of intermolecular cross-links. Both of these events could render the collagen much more susceptable to degradation by hydrolytic enzymes. These alterations in collagen could occur quite rapidly as Sodek's results[23,24] showed that the newly synthesized collagen in normal rat gingival and periodontal connective tissues turns over within a few hours of its extra-cellular appearance.

The possibility of inflammatory induced changes in the nature or number of intermolecular cross-links presents an interesting application of the method described in this report to a study in human subjects. The object being to quantitate and qualitate the reducible cross-links and thus compare turnover rates in collagen of diseased gingiva and periodontal ligament with that of collagen from healthy tissues. Both the control and experimental samples could be obtained from partially or fully dentated individuals who require extraction of all their teeth for purposes of full denture construction. As reducible cross-links

can be measured in as little as 2 mgs. of connective tissue,[78] sufficient gingival samples could be obtained by excising the interdental papilla to the level of the alveolar crest just prior to extraction and ligament samples, could be obtained by gently scraping the coronal half of the roots immediately after extraction. Control specimens would consist of gingival and periodontal ligament connective tissues without clinical, radiographic and histologic evidence of disease. Experimental specimens from the same mouth would be of three categories. That is, gingival and periodontal connective tissues with clinical, radiographic and histologic evidence of active disease. Secondly, specimens of gingival and periodontal connective tissues which have had the disease process arrested by local therapeutic measures, i.e. closed or open flap root planing and curettage where applicable followed by strict plaque control of these teeth by daily polishing for a pre-selected time period. Thirdly, specimens of gingival connective tissue with induced inflammation by allowing plaque accumulation for varying time periods on teeth which formerly had healthy tissue.

Quantitative and qualitative differences in the reducible cross-links in each gingival specimen and each periodontal ligament specimen as well as, differences in gingival specimens compared to periodontal ligament specimens of the same teeth, would provide an additional understanding of the degree of synthesis of collagen in health and disease. In addition, as quantitative measurements of the reduced aldehydic cross-link precursors of hydroxyallysine (i.e. dihydroxynorleucine - DHNL) and allysine (i.e. hydroxynorleucine - HNL) were not done in this study, their comparison in each of the tissue samples may also prove to be

beneficial in detecting different rates of new collagen synthesis in experimental and control specimens of gingival and periodontal ligament collagen obtained from human subjects.

SUMMARY

1. The reduction of and simultaneous labelling of collagen inter-molecular cross-links with tritiated Na borohydride in order to determine collagen turnover, is applicable to periodontal ligament collagen of dog molars.

2. The results show that molar periodontal ligament had a four to five times higher specific activity of cross-links per weight of collagen than skin dermis.

3. The results also demonstrate that in dog periodontal ligament like bovine periodontal ligament, DHLNL is the major reduced cross-link and HLNL is the minor reduced cross-link whereas in dog skin like bovine skin, HLNL is the major and DHLNL is the minor reduced cross-link.

4. It is concluded from the results that neither tooth movement or mechanical stresses as a result of a persistant eruptive force or intermittant occlusal functional forces within physiologic limits, are responsible for modifying the turnover of periodontal ligament collagen. It is also suggested, that increased turnover of periodontal ligament collagen appears to be an innate feature of this tissue.

5. An application of the method is outlined for a comparative study of collagen turnover rates in the presence and absence of disease surrounding human teeth.

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