Technical Report

January 1, 1986-December 31, 1986

NASA Grant NAG 2-181

Principal Investigator: Gerald L. Mechanic, Ph.D.

Title: Biochemical Changes in Bone in a Model of Weightlessness

The paper concerned with the amounts of nonmineralized and mineralized collagen in bone from, control, immobliized, and immoblized reambulated (various periods of time) monkeys was published. The scientific response to the paper has been positive and many requests have been made for reprints.

In order to understand structure function relationships of bone collagen and the responses of a variety of conditions on control of the three dimensional structure of the collagen fibril, we have been studying the stereochemistry of the cross-linking reactions as well as the stereospecific packing of the collagen molecules. We have chosen, as models, skin, periodontal ligament since the former has a different form of stable cross-link and the latter does not contain stable cross-links. However, the latter upon incubation in vitro forms pyridinoline, the same cross-link that exists in bone collagen. As a model for the phosphoprotein interaction with hard tissue mineralized collagen we have chosen dentin because of its relative

simplicity when compared with bone. The former does not turn over while the latter is always remodeling. Investigations of this sort will allow us to explain the reactions taking place on a molecular and biochemical level. By knowing the latter we shall derive valuable information on the mineralization of bone, where the crystallites reside and their size. Progress along these lines are summarized below.

In 1982 we isolated as an apparently homogeneous peptide, a three-chained peptide containing the stable non-reducible trifunctional cross-link, which was then known at the time as hydroxyaldolhistidine (HAH) (13). Based on the structure proposed by Housley et al. (64) we attempted to specifically cleave the peptide by cleaving the cross-link's putative C-C enamine double bond with $OsO_4/NaIO_4$. From the reaction mixture we obtained a two-chained peptide whose structure after amino acid and sequence analysis was deduced as α1CB4-5(76-90)[Hyl-87] X α1CB6(5^c-18^c)[Lys-16^c]. Designations of cross-linked peptides used here and in the remainder of the proposal are based on the CNBr peptides derived from collagen; the numbers in parentheses represent the residue numbers in the sequence; the residue and its number that are in brackets represents the cross-linking residue in the peptide; and the X denotes the cross-linking moieties. The structure and the identity of the third chain eluded us as it did Becker et al., (1975). Recently we have identified the third chain as α2CB4(76-126)[His-92]. The structures of the tryptic peptides isolated were α1CB4-5(76-90)[Hyl-87] X α1CB6(993-22^c)[Lys-16^c] X α2CB4(76-126)[His-92] and α1CB4-5(76-90)[Hyl-87] X α1CB6(993-22^c)[Lys-16^c] X α2CB4(91-126)[His-92]. The peptides isolated from the latter two peptides after treatment with bacterial collagenase were α1CB4-5(85-90)[Hyl-87] X α1CB6(1012-22^c)[Lys-16^c] X α2CB4(85-99)[His-92] and α1CB4-5(85-90)[Hyl-87] X α1CB6(1012-22^c)[Lys-16^c] X α2CB4(91-99)[His-92]. Measurements from the C-ß of His to the C-ß of Lys obtained from a space filling model of the cross-link and modeling performed by Dr. Elton P. Katz strongly suggested that the trifunctional cross-link linked three molecules of collagen together in the fibril. The data also suggested that skin collagen molecules are packed in fibrils differently from those of periodontal ligament. In the course of other work we noted a precursor-

product relationship between dehydro-hydroxylysinonorleucine and this trifunctional cross-link. We therefore prepared a large amount of the cross-link and subjected it to Fast Atom Bombardment Mass Spectrometry, ^{1}H- and ^{13}C- Nuclear Magnetic Resonance. The structure found was consistent with histidinohydroxylysinonorleucine (HHL). Studies of the concentration of the cross-link in human and bovine skin collagen indicated it constantly increased with chronological age of the organism, approaching one residue per mole of collagen (0.9 residues per mole collagen in 82 year-old human skin). This is quite different from the trifunctional stable cross-link, pyridinoline, which increases during the growth period of the organism and then decreases in abundance with aging. This cross-link, HHL, is the first demonstration of a collagen cross-link that shows a continuous increase with chronological age of organism. Based on glycosylation data it was proposed that the function of specific glycosylated Hyl residues was to prevent cross-links in tissue collagen from maturing to stable cross-links so tissue turnover could occur more readily. This was borne out by our studies on the periodontal ligament which is the fastest turning over collagen in the body.

A trypsin digest of denatured $NaB^{3}H_{4}$-reduced native bovine periodontal ligament was prepared and fractionated by gel filtration and cellulose ion-exchange column chromatography. Prior to trypsin digestion a complete acid hydrolysate was subjected to analyses for non-reducible stable and reducible intermolecular cross-links. Minute amounts of the former and significant amounts of the reduced cross-links, dihydroxy-lysinonorleucine (1.1 moles/mole collagen), hydroxylysinonorleucine (0.9 mole/mole collagen) and histidinohydroxymerodesmosine (0.6 mole/mole collagen) were found. The covalent intermolecular cross-linked two-chained peptides that were isolated were subjected to amino acid and sequence analyses. The analyses indicated that the structures for the different two-chained linked peptides were α1CB4-5(76-90)[Hyl-87] X α1CB6(993-22^{c})[Lys^{a,d}-16^{c}], α1CB4-5(76-90)[Hyl-87] X α1CB6(993-22^{c})[Hyl^{a,d}-16^{c}], α2CB4(76-90)[Hyl-87] X α1CB6(993-22^{c})[Lys^{a,d}-16^{c}], and α2CB4(76-90)[Hyl-87] X α1CB6(993-22^{c})[Hyl^{a,d}-16^{c}]. All the cross-link peptides isolated were solely from Type I collagen. This is the first direct evidence to demonstrate involvement of an α2 chain in the cross-linking of collagen. Alkaline hydrolysis indicated that all the cross-links in the peptides were completely glycosylated. Quantitative determination of the amounts of the various two-chained peptides present in the collagen coupled with their molecular locations

indicated a stoichiometric conversion of residue 16^c aldehyde to intermolecular cross-link in each of the COOH-terminal non-helical peptide regions of both α1 chains in a molecule of Type I collagen. Analyses from our data of the ratio of α1 to α2 intermolecularly cross-linked chains involved at the latter locus (residue 16^c) showed it to be 3.3:1. This strongly indicates that the three dimensional molecular packing of Type I collagen molecules on a hexagonal lattice in bovine periodontal ligament is not random but stereospecific in nature and also indicates that the molecules are in a specific azimuthal (angular) orientation with each other. This is the first time chemical evidence has been obtained to indicate the conclusions concerning the three dimensional packing of collagen molecules in fibrils. This paper was published in Biochemistry this year. In addition we found the cross-links completely in their glycosylated form (Table 3 of the paper). We proposed as above that gycosylation of specific Hyl residues might be necessary to prevent stable non-reducible mature cross-link formation for virtually no stable non-reducible mature cross-links were found in the periodontal ligament collagen.

Embryonic bovine dentin (8-8.5 month-old embryo) was separated into reduced-nonmineralized fraction (Red-NMF), mineralized fraction-EDTA extract (MF-ED) and reduced-mineralized fraction (Red-MF) portions as described in the methods below. The yield (weight) was approximately 1 to 0.1 to 1.1 for each fraction respectively. This ratio is significantly different from normal bovine bone in which case the ratio was 1 to 0.1 to 3 (7 month-old embryo) and 1 to 0.1 to 4 (2 year-old bovine bone). Cross-link analyses indicated the major reduced cross-link was dihydroxylysino-norleucine in each case. Quantification has not been accomplished as yet.

The collagen content in each fraction was approximately 60% in Red-NMF, 10% in MF-ED and 95 % in Red-MF based on a value of 300 residues of Hyp per collagen molecule.

Digestion of MF-ED and Red-MF with trypsin almost completely solubilized each of these fractions. Equal collagen aliquots (based on Hyp analysis) of the tryptic digests of Red-NMF (see above), Red-MF and 1/5 the amount of MF-ED by weight (limited quantities of this fraction are obtained and very small amount of collagen was present) were each subjected to molecular sieve column chromatography using Sephadex G50 SF (see methods and ref 9 in appendix). The MF-ED profile contained one large peak in close to the void volume of

the column (>20,000 dalton). No radioactivity nor fluorescence were detected. In the case of Red-NMF and Red-MF, the most prominent radioactive peak appeared in the region of the standard cross-linked peptide α1CB6(993-22^{c})[Hyl-16^{c}] X α1CB4-5(76-90)[Hyl-87] obtained from bovine periodontal ligament. This fraction was designated as peak VII in our recent report. The minor radioactive peak was observed in the position where the standard cross-linked peptide α1CB1-2(1^{N}-9)[Lys-9^{N}] X α1CB6(928-933)[Hyl-930] eluted from the column. This fraction was designated as peak X in the same report. Three distinct flourescent peptide peaks were also observed in both profiles. The most prominent flourescent peak in each case eluted at the same position as α1CB6(993-22^{c})[Hyl-16^{c}] X α1CB6(993-22^{c})[Hyl-16^{c}] X α1CB4-5(76-90)[Hyl-87] which we have isolated from bovine tendon and bone collagen as well as 3 months old osteogenic osteoblastoma chick bone collagen and have previously isolated from dentin. The second flourescent peak in the profile eluted in the approximate molecular weight range of 8000 daltons and its structure is as yet unknown. The third flourescent peak contains a leading edge which contains a flourescent peptide whose structure is still not known while the main peak contains the cross-linked peptide α1CB1-2(1^{N}-9)[Hyl-9^{N}] X α1CB1-2(1^{N}-9)[Hyl-9^{N}] X α1CB6(928-933)[Hyl-930] which we have also isolated from bovine tendon. Although the overall elution patterns of radioactivity and fluorescence showed some similarity between Red-NMF and Red-MF, the ratio of the major fluorescent peak to the major radioactive peak is less in Red-MF than in Red-NMF, indicating significantly more pyridinoline cross-link containing peptides in NMF than in MF.

In our examination of the nonmineralized and mineralized collagen in bone we have noted that a small amount of phosphoprotein is bound covalently to collagen only in the mineralized fraction. Similar studies using dentin fractions described above as a model were performed. Organic phosphorus (P) assays were carried out on each fraction obtained from the Sephadex G50SF gel filtration column. The major P containing peak from Red-NMF eluted in the 3,000 dalton range while those from MF-ED and Red-MF appeared in the

high molecular weight range from the column (>20,000 dalton). DEAE-cellulose chromotograpy of the P containing peak from Red-NMF provided 5 sharp peaks with only the 4th containing P which eluted at 0.15 M NaCl. Amino acid analysis indicated it was 1/4 Pro. This could represent a fragment of a proteoglycan since its amino acid composition was not characteristic of phosphoprotein. lt is known that proteoglycan is present in predentin. The P containing fraction from MF-ED separated into three peaks on the same column. The first peak was broad and amino acid analysis indicated it was somewhat collagenous in nature, it contained no P. The second peak eluted at 0.4 M NaCl. It contained a large amount of P and it's amino acid composition indicated that 85% of the amino acids were Asp and Ser. The amino acid profile also contained a peak at the elution position of phospho-Ser. The latter three characteristics are typical of dentin phosphoprotein. The third peak eluted at 0.55 M NaCl and also contained P. Its amino acid composition was non-descript. The P containing fraction from the G50SF column derived from Red-MF contained two approximately equal sized peaks in the DEAE-cellulose elution profile. The first was broad and heterogeneous eluting between 0.05 and 0.15 M NaCl and did not contain P, however, the second peak eluted as a single symmetrical peak at 0.4 M NaCl. Amino acid analysis demonstrated

that 80% of the total amino acids consisted of Asp and Ser. In addition a peak typical of Ser phosphate was present at the void volume of the amino acid profile. The latter two chacteristics are typical of a phosphoprotein. This DEAE-cellulose fraction also contained significant amounts of Hyp and Hyl. Additionally there was present a significant quantity of histidinoalanine. The latter is absent from the P containing fraction from Red-NMF. These results indicate that only the P containing fraction from the mineralized portion of the dentin contains the histidinoalanine, a cross-link that has been suggested to be associated with dentin phosphoprotein previously. The fact that Hyp and Hyl were also present in the same protein peak could very well mean that the collagen portion is covalently linked to the phosphoprotein via the histidinoalanine present. Phosphoprotein linked to collagen peptides have been proposed previously. Further work will be needed to demonstrate whether or not this protein peak is homogeneous and to characterize this peptide. Its structure will be identified in future work.

In addition we have analysed bones of rats that were aboard Shuttle Spacelab 3 in collaboration with Dr. Sara Arnaud. We found that the concentration of collagen in the bone was increased. The results are now in press in the American Journal of Physiology.

Papers published this year that were sponsored in part by NASA Grant NAG 2-181 and that were stimulated by the work to be performed for the research program are:

Mechanic, G.L., Young, D.R., Banes, A.J., and Yamauchi, M. "Non-Mineralized and Mineralized Bone Collagen in Bone of Immobilized Monkeys" Calcif. Tissue Int. 39, 63-68 (1986)

Patterson-Buckendahl, P., Arnaud, S., Mechanic, G.L., Martin, R.B., Grindeland, R.E., & Cann, C.E. "Bone Fragility and Composition of Growing Rats After One Week in Spaceflight" Amer. J. Physiol. In Press (1986)

Graham, L. & Mechanic, G.L. "[^{14}C]Acrylonitrile: Preparation Via a Stable Tosylate Intermediate and Quantitative Reaction With Amine Residues in Collagen" Anal. Biochem. 153, 354-358 (1986)

Yamauchi, M., Katz, E.P., & Mechanic, G.L. "Intermolecular Cross-linking and Stereospecific Molecular Packing in Type I Collagen Fibrils of the Periodontal Ligament" Biochemistry 25, 4907-4913 (1986)

Mechanic, G.L., Katz, E.P., Henmi, M., Noyes, C. & Yamauchi, M. "Locus of a Histidine-Based, Stable, Trifunctional, Helix to Helix Collagen Cross-link: Stereospecific Collagen Molecular Structure In Type I Skin Fibrils" Biochemistry, Submitted for Publication (1986)

Yamauchi, M., London, R.E., Guenat, C., Hashimoto, F., and Mechanic, G.L. "Structure and Formation of a Stable Histidine-based Trifunctional Cross-link in Skin Collagen J. Biol. Chem. In Press (1986)

G.L. Mechanic NAG 2-181

Work to be Performed

January 1, 1987-December 31, 1987

This year we shall be finishing off a bone collagen turnover study performed on 55 rats put on an experimental protocol that lasted for 6 months. The rats were treated as proposed in our grant to model the weightless condition and had miniosmotic pumps implanted subcutaneously containing radioactive proline. In most case the miniosmotic pumps were of the 2 week variety, however, in the short time experimental periods there were pumps implanted that had a duration of 1 week.

We shall also be analyzing monkey bones from an immobilization study performed by Dr. Donald Young from which data was obtained for the nonmineralized and mineralized collagen (see publication above). Phosphoprotein in each of the collagen compartments will be assessed. Pyridinoline cross-link will also be measured. Cross-links between phosphoprotein and collagen will be quantitatively determined in order for it to be fit into a model to be proposed for loss of bone mass due to weightlessness. In addition bones from vitamin D deficient monkeys will be put through the same analytical procedures. The latter is an excellent model for osteopenia and osteomalacia. Using these models we shall be able to evaluate bone collagen structures in specific states of disorder on a molecular and biochemical level.

DETAILED BUDGET NAG 2-181

Budget Period: JANUARY 1, 1987 - DECEMBER 31, 1987

PERSONNEL Name	Position Title	% Effort	SALARY	Fringe Benefits	TOTALS
G. L. Mechanic	Prin. Invest.	20	16,380	3,112	$19,492
M. Yamauchi	Co-Prin. Invest.	40	16,800	3,192	19,992
Gloria Chandler	Res. Tech. II	100	20,496	3,894	24,390
		Totals	$53,676	10,198	63,874
Medical Insurance $766/FTE					1,226
				Total	$65,100

EQUIPMENT

Shimazu HPLC Fluoresence Monitor plus Accessories 7,315

SUPPLIES

Radioactive $NaBH_4$-1,600, [^{3}H]Lysine-1,500, [^{3}H]Proline-1,500 Scintillation chemicals-2,000, Special chemicals(ultra pure) 2,200, Scintillation vials-1,000, General Chemicals(buffers etc.) 1,200, Glassware-850, Chromatographic columns & Resins-3,000, Supplies for HPLC analyzers and sequencing peptides-2,100, Four cases of 0.2μ filters@$300ea.-1,200 18,150

TRAVEL

Two meetings per year per Investigator 2,400

OTHER EXPENSES

Cost of Publications- 900, Art work & Photography-800 Photocopying costs-600, Communications-400, Books-250, Instrument Contract costs-1,500 4,450

TOTAL DIRECT COSTS $97,415

Indirect Costs@25.0% Modified Total Direct Costs as per letter dated February 3, 1986 from Wayne R. Jones, Associate Vice Chancellor for Finance $22,525

(Modified total direct costs=total direct costs less equipment)

TOTAL COST $ 119,940

S. Kent Walker
Contract Specialist

Gerald L. Mechanic, Ph.D.
Principal Investigator