QUANTITATIVE CHANGES OF BONE COLLAGEN CROSSLINKS AND PRECURSORS IN VITAMIN D DEFICIENCY

Gerald L. Mechanic, Svein U. Toverud and Warren K. Ramp

Dental Research Center and the Departments of Biochemistry
and of Pharmacology
University of North Carolina
Chapel Hill, North Carolina 27514

Received April 12, 1972

SUMMARY

The crosslinks from NaB^3H_4-reduced bone collagen of 4-week-old rachitic and normal chicks were compared. The ratio of the reduced crosslink δ,δ'-dihydroxylysinonorleucine (DHLNL) to δ-hydroxylysinonorleucine (HLNL) was significantly increased in vitamin D deficiency. The ratio of ε-hydroxynorleucine (HNL) (reduced crosslink precursor of HLNL) to the reduced unknown peak 1 was decreased while that of HLNL to HNL was unaffected. The data indicate that vitamin D affects the quantitative relationships between crosslinks and aldehydic precursors that are present in mineralizing macromolecular matrices.

INTRODUCTION

The time-dependent formation of aldehydic crosslink precursors and their respective crosslinks has been shown to stabilize the fibrillar macromolecular matrix of collagen (1,2). Aldehydes are formed in the protein soon after its synthesis (3,4); Schiff base crosslinking ensues when aggregation into a polymeric matrix occurs (2,5). Differences in types and relative abundance of NaB^3H_4-reducible compounds have been demonstrated in tissues having different collagen architectures and in various states of maturation (6,7,8).

The major reducible crosslinks in bovine mineralized tissues are δ,δ'-dihydroxylysinonorleucine and δ-hydroxylysinonorleucine (7). Furthermore, deuterium labelling studies indicate that in vivo reduction of the Schiff bases $\Delta^{6,7}$ dehydrodihydroxylysinonorleucine and $\Delta^{6,7}$ dehydrohydroxylysinonorleucine is quantitatively important in dentine (24% of the former) and in calf bone collagen (25%-50% of both) (7).

In bovine foetal bone, which is comparatively less mineralized than

mature bone, δ,δ'-dihydroxylysinonorleucine represents 57% of the radioactivity in the elution profile while 11% is due to δ-hydroxylysinonorleucine (7). Simultaneously, markedly smaller amounts of reduced aldehydic precursors are in evidence. However, more mature bone, which contains more mineral, possesses much larger quantities of the crosslink precursors and a lower ratio of δ,δ'-dihydroxylysinonorleucine to δ-hydroxylysinonorleucine.

This report concerns the effect of vitamin D on the reducible crosslinks in the macromolecular matrix of bone collagen. The data indicate that the same relationships that are present between foetal and mature bone also exist between vitamin D deficient and normal chick bone collagen.

MATERIALS AND METHODS

One-day-old male chicks were housed in a windowless room and fed a rachitogenic chick diet containing 1.4% Ca and 1.1% P (General Biochemicals, Inc., Diet # 170650). One half of the chicks (+D group) received this diet to which was added vitamin D_3, dissolved in corn oil, to provide 2.2 I.U. vitamin D per g of diet. The remaining chicks (-D group) received the diet with only corn oil added.

The chicks were weighed twice a week. At the end of 4 weeks, 8 chicks selected at random from both groups were sacrificed by decapitation for the purpose of removing:

(1) a blood sample for serum Ca determination by atomic absorption spectrometry.

(2) one tibia for dry weight (105°C) and ash (600°C) determinations

(3) the other tibia for fixation in neutral formalin, decalcification and staining of sections with hematoxylin and eosin.

The tibias from 3 additional chicks from both groups were removed. The diaphysis was cleaned of marrow and subjected to demineralization, NaB^3H_4 reduction, and analytical chromatography of the bone collagen as described (1,6,7).

TABLE 1

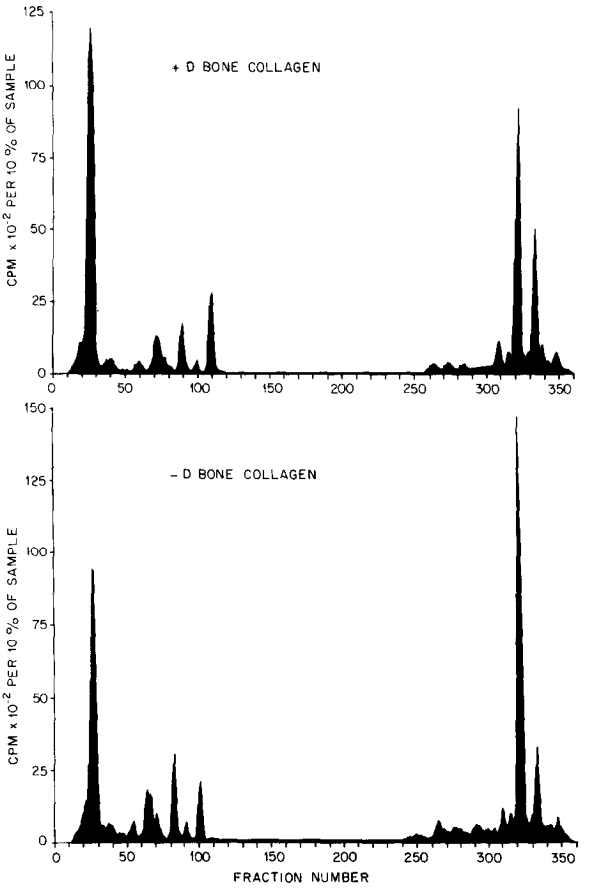
EFFECT ON CHICKS AFTER 4 WEEKS

		Body Weight g	Serum Calcium mg/100 ml	TIBIA	
				Dry Weight g	% Ash of Dry Weight
- Vitamin D	MEAN	237	5.5	0.572	37.1
+ Vitamin D	MEAN	328	10.1	1.029	43.2
	SE	8.6	0.12	0.03	0.63
	P	< 0.001	< 0.001	< 0.001	< 0.001

RESULTS AND DISCUSSION

Table 1 shows that the -D group of chicks demonstrated the usual features of vitamin D deficiency rickets (9): diminished body weight gain, severe hypocalcemia, decreased bone weight and percentage ash. The histological sections of the tibias showed characteristic widening of the epiphyseal cartilage and irregular, wide and markedly shortened trabeculae (10).

Typical elution profiles of NaB^3H_4-reduced normal and vitamin D deficient chick bone collagen are presented in Figure 1. The quantitative chromatographic patterns of the 3 bones from each group were concordant. Note, in rachitic bone, the smaller amounts of δ-hydroxynorleucine and δ-hydroxylysinonorleucine relative to unknown peak 1, while there is an apparent increase in δ,δ'-dihydroxylysinonorleucine. Unknown peak 1 seems to remain constant in both groups and was used as a quantitative reference peak. Dihydroxynorleucine, the reduced aldehydic crosslink precursor of δ,δ'-dihydroxylysinonorleucine, also seems to be slightly more abundant in vitamin D deficient bone. Quantitative data on this compound could not be obtained because of other unknown substances present in this section of



LEGEND TO FIGURE 1

Typical radioactive elution profiles of NaB^3H_4-reduced normal (upper) and vitamin D deficient (lower) chick bone collagens. Dihydroxynorleucine elutes at fractions 70-73 (upper) and 68-71 (lower). Unknown peak 1 elutes at fractions 88-91 (upper) and 80-83 (lower). Equal amounts of radioactivity are present in both unknown peak 1's. ε-Hydroxynorleucine (HNL) elutes at fractions 108-112 (upper) and 99-104 (lower). Dihydroxylysinonorleucine (DHLNL) elutes at fractions 320-323 and hydroxylysinonorleucine (HLNL) elutes at fractions 331-333 for both profiles.

---

the chromatogram. The data were expressed quantitatively by integrating the radioactivity under the curves and calculating the ratios between the areas under the 4 peaks. These are presented in Table 2.

The pattern exhibited by the vitamin D deficient collagen is similar to that for foetal bovine bone while that for the normal chick bone collagen resembles an intermediate stage in development as compared to mature bovine

TABLE 2

Ratios of NaB^3H_4-Reduced Intermolecular Crosslinks and Aldehydic Precursors in Vitamin D Deficient and Normal Chick Bone Collagen

RATIOS	HNL / Unk. Peak 1		DHLNL / HLNL		HLNL / HNL	
Treatment	+D	-D	+D	-D	+D	-D
	1.40	.696	1.71	3.81	1.56	1.74
	1.52	.728	1.88	4.08	1.73	1.78
	1.51	.771	2.48	3.15	2.00	2.17
Mean	1.48	.731	2.02	3.68	1.76	1.90
SE	0.031		0.255		0.132	
P	< 0.001		< 0.02		> 0.4	

-D - Vitamin D deficient rickets bone
+D - Normal bone
HNL - ε-hydroxynorleucine
DHNL - δ,δ'-dihydroxylysinonorleucine
HLNL - δ'-hydroxylysinonorleucine
Unk. Peak 1 - See Figure 1

bone (7). In the present study, the similar ratios for both groups of δ-hydroxylysinonorleucine to ε-hydroxynorleucine indicate that in vitamin D deficiency a normal proportion of the Schiff base, involving α-amino-adipic-δ-semialdehyde (unreduced HNL) and hydroxylysine, is being formed. However, the low ratio of ε-hydroxynorleucine to unknown peak 1 and the high ratio of δ,δ'-dihydroxylysinonorleucine to δ-hydroxylysinonorleucine suggests that insufficient quantities of α-amino-adipic-δ-semialdehyde are being synthesized in rachitic bone to satisfy the crosslinking requirements needed for a normal cohesive mineralizing collagen matrix.

Electron micrographs indicate that rat rachitic collagen has a normal

appearance and does not differ from normal bone collagen (13). Autoradiographic localization of [1α-^{3}H] cholecalciferol suggests that vitamin D is concerned with the biochemical processes leading to the elaboration of a normal collagen matrix (12). On the basis of the present data and the hypotheses of Norman (14) and Wasserman and Taylor (11) that vitamin D acts as an affector of protein or enzyme synthesis, we propose that vitamin D may stimulate the synthesis of lysyl oxidase in order to facilitate the increased conversion of lysyl residues in the collagen molecule to the aldehydic crosslink precursor α-amino-adipic-δ-semialdehyde. In any event, the data strongly suggest a relationship between vitamin D and the crosslinks that stabilize the macromolecular mineralizing collagen matrix. Crosslinking in collagen may play an important role in the formation of a normal tissue architecture.

ACKNOWLEDGEMENTS

We are grateful for the technical assistance of Sam Weddington, Unni Kristoffersen, Judy Knight, Carol Ellison and Catherine Lane. This research was supported by USPHS Grant DE-02668.

REFERENCES

1. Tanzer, M. L. J. Biol. Chem. 243, 4045 (1968).
2. Tanzer, M. L. and Mechanic, G. Biochem. Biophys. Res. Commun. 32, 885 (1968).
3. Pinnell, S. R. and Martin, G. R. Proc. Nat. Acad. Sci. U. S. 61, 708 (1968).
4. Siegal, R. C. and Martin, G. R. J. Biol. Chem. 245, 1953 (1970).
5. Kang, A. H., Faris, B. and Franzblau, C. Biochem. Biophys. Res. Commun. 39, 175 (1970).
6. Mechanic, G. and Tanzer, M. L. Biochem. Biophys. Res. Commun. 41, 1597 (1970).
7. Mechanic, G., Gallop, P. M. and Tanzer, M. L. Biochem. Biophys. Res. Commun. 45, 644 (1971).
8. Mechanic, G. Israel J. Med. Sci. 7, 458 (1971).
9. Migicovsky, B. B. and Belanger, L. F. Radioisotopes and Bone (F. C. McLean, P. Lacroix and A. M. Budy, eds.), Blackwell Sci. Publ., Oxford and Edinburgh, 1962.
10. Yuile, C. L., Chen, P. S. and Bossman, A. B. Arch. Pathol. 83, 241 (1967).
11. Wasserman, R. H. and Taylor, A. N. Science 152, 791 (1966).
12. Kodiecek, E. and Thompson, G. A. In Structure and Function of Connective and Skeletal Tissue, Butterworth, London, pg. 369, 1965.
13. Robinson, R. A. and Sheldon, H. In Calcification in Biological Systems (R. Sognnes, ed.), AAAS, Washington, D. C., pg. 261, 1960.
14. Norman, A. W. Biological Reviews 43, 97 (1968).