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Effects of Dietary Vitamin D and Calcium on Lysyl Oxidase Activity in Chick Bone Metaphyses (39233)

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(Introduced by M. S. Silverman)

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Previous studies from this laboratory have shown that the vitamin D status of chicks can influence the proportions of the major reducible crosslinks, dihydroxylysinonorleucine (DHLNL), and hydroxylysinonorleucine (HLNL), and thus affect the rate of maturation of bone collagen (1, 2). It has also been shown that vitamin D deficiency results in increased hydroxylation of lysine residues in chick bone collagen (2, 3, 4). Whether or not this change can be attributed to the hypocalcemia associated with vitamin D deficiency rather than the absence of vitamin D per se is still unclear (2, 5).

The increased hydroxylation of lysine residues could result in increased synthesis of DHLNL and could thus increase DHLNL/HLNL ratios as seen in rachitic bone collagen. However, previous data suggested that the increased DHLNL/HLNL ratio was due to decreased amounts of HLNL, possibly because of decreased formation of α-amino-adipic-δ-semialdehyde, the aldehydic precursor for HLNL (1). Therefore, it was postulated that the underlying defect in rachitic bone collagen is a decreased activity of lysyl oxidase, the metalloenzyme that oxidatively deaminates peptide-bound lysine residues to yield α-amino-adipic-δ-semialdehyde (6). The purpose of the present study was to test this postulate by examining the effects of different dietary levels of vitamin D and calcium in lysyl oxidase activity in tibial metaphyses.

Materials and methods. Single-comb White Leghorn cockerels were obtained as day-old chicks and were immediately placed on one of the following five dietary regimens: (1) D-deficient: a basal, rachitogenic (vegetable protein) diet containing by analysis 1.4% Ca and 1.1% P (General Biochemicals, diet #170650); (2) control-D: basal diet supplemented with 1.4 I.U. cholecalciferol (Sigma Chemical Company)/g of diet; (3) high-D: basal diet supplemented with 70 I.U. cholecalciferol/g of diet; (4) D-deficient high-Ca: basal diet supplemented with $CaCO_3$ to give a calcium level of 2.8%; or (5) control-D high-Ca: basal diet supplemented with 1.4 I.U. cholecalciferol/g of diet and $CaCO_3$ to give a calcium level of 2.8%. Food and water were supplied *ad libitum*. The animals were maintained in brooders at constant temperature and humidity. Light was supplied by yellow or red incandescent bulbs to eliminate uv radiation.

At the end of 2 weeks, six to eight animals from each group were killed. Blood samples were obtained by cardiac puncture for plasma Ca and inorganic phosphorus determinations. Tibiae were removed and frozen prior to analysis of lysyl oxidase levels. Aortas were removed to test for tissue specificity of the effect of vitamin D.

Preparation of biologically labeled substrate for lysyl oxidase. Tibiae from 11-day-old chick embryos were cultured in chemically defined culture medium (7) with lysine omitted. Tritiated 4,5-lysine was added to the medium (20 μCi/ml medium). Cultures were maintained in spinner bottles (50 bones/60 ml of medium) for 48 hr. The bones were harvested and were homogenized in 0.15 *M* NaCl, 0.1 *M* potassium phosphate, pH 7.65, buffer (PBS) in a Polytron homogenizer. The homogenates were centrifuged and resuspended in PBS two additional times. They were then centrifuged, resuspended in distilled water, and boiled for 8 minutes to destroy endogenous

lysyl oxidase activity. The substrate was then centrifuged and either resuspended in PBS and stored frozen or lyophilized until used.

Extraction and assay of lysyl oxidase. A 2- to 3-mm transverse slice of trabecular bone from the metaphyseal region was removed for enzyme extraction. Metaphyseal bone was selected in order to ensure sample homogeneity and to minimize contamination by cartilage. The tissues were homogenized in PBS buffer using a Polytron homogenizer and the insoluble material was centrifuged from the solution. This process was then repeated. The lysyl oxidase was extracted from the pellet by suspension in 4 *M* urea buffered with 0.01 *M* potassium phosphate, 0.015 *M* NaCl, pH 7.65, and was allowed to remain in an ice bath for 2 hr with intermittent shaking (8, 9). This procedure has been shown to extract approximately 10 to 15% of the total activity in the PBS and approximately 80 to 85% in the urea solution. The urea suspension was centrifuged at 15,000*g* for 15 min, and the pellet was resuspended in a second solution containing 0.01 *M* potassium phosphate, 0.015 *M* NaCl, and 4 *M* urea. The second urea suspension was then centrifuged, and the two supernatant solutions were combined and dialysed extensively against PBS buffer, pH 7.65. Protein content of the dialysed urea supernatant solutions was estimated by the method of Lowry (10).

Aliquots of 0.1–0.2 ml of the urea extract were tested for enzyme activity in an assay containing 125,000 cpm of the tritium-labeled collagen substrate made to a total volume of 0.5 ml with PBS. Incubation time was 4 hr at 37°. The assay mixture was vacuum-distilled, and an aliquot of the tritiated water was counted in a scintillation counter (Packard model 3375). Tritium release in this assay system is linear for at least 8 hr and is proportional to the amount of added enzyme extract. Activity was calculated as total activity minus a β-amino propionitrile-inhibited control (11), and was expressed as cpm tritium released per milligram of protein. Significance of difference between treatment groups was determined by Student's *t* test.

Results. Overall growth, as shown by body weight (Table I) was slightly, but significantly, depressed at 2 weeks in the D-deficient animals. The addition of calcium to the D-deficient diet completely prevented the decrease in growth at 2 weeks. Ca addition to the control-D diet caused a slight, but significant, decrease in growth rate while there was no effect of the high-D diet.

Hypocalcemia associated with vitamin D deficiency was seen in the 2-week-old chicks. Addition of extra Ca to the D-deficient diet partially prevented the decrease in plasma Ca levels, although values were significantly lower than in control-D animals. There were no alterations in plasma Ca lev-

TABLE I. BODY WEIGHT, PLASMA Ca AND PLASMA INORGANIC PHOSPHORUS (P) LEVELS AND ACTIVITY OF LYSYL OXIDASE FROM METAPHYSIS AND AORTA OF CHICKS MAINTAINED ON DIETS CONTAINING DIFFERENT LEVELS OF VITAMIN D AND CALCIUM.[a]

	Body weight (g)	Plasma Ca (mg/100 ml)	Plasma P (mg/100 ml)	Lysyl oxidase (cpm/mg Prot.)	
				Metaphysis	Aorta
D-Deficient	136*	7.64*	7.04*	23,000*	4100
D-Deficient High-Ca	146	9.82*	6.13*	23,500*	5290
Control-D	147	10.91	5.50	12,400	5330
Control-D High-Ca	131*	10.50	6.21*	12,400	6920
High-D	145	10.91	5.27	13,900	4680
SE	±3	±0.19	±0.17	±1900	±1280

[a] Chicks were maintained from hatching on diets containing 1.4% Ca and either no vitamin D (D-deficient), 1.4 I.U. vitamin D_3 per g of diet (control-D), 70 I.U. vitamin D_3 per g of diet (high-D), no vitamin D plus calcium carbonate to give 2.8% Ca (D-deficient high-Ca), or 1.4 I.U. vitamin D_3 per g of diet plus calcium carbonate to give 2.8% Ca (control-D high-Ca). The chicks were weighed, and blood and tissue samples were collected after 2 weeks. Lysyl oxidase was extracted from tibial metaphyses using 4 *M* urea. The urea extract was dialyzed and enzyme activity was estimated by tritium released from labeled, embryonic chick-bone collagen as substrate.

* $P < 0.01$, compared to control-D group.

els in either the control-D high-Ca or the high-D groups.

Vitamin D-deficient, control-Ca animals were markedly hyperphosphatemic, an effect noted previously in animals on this dietary regimen (12). High dietary Ca levels reduced the hyperphosphatemia in the D-deficient group. Slight, but significant, hyperphosphatemia was seen in the control-D high-Ca group. There was no significant effect of the high-D diet on plasma phosphorus.

There were no significant differences in lysyl oxidase activity (counts per minute per milligram of protein) from aortas between any of the groups. In contrast, the activity in bone from vitamin D-deficient animals was almost twice that of animals on the control-D diet. Ca addition to the D-deficient diet did not alter the effect of the vitamin D deficiency. Neither the control-D high-Ca or the high-D diet had any effect on lysyl oxidase activity.

Discussion. The present results represent the first demonstration of an effect of vitamin D on an enzyme system involved in maturation of bone matrix. The twofold increase in lysyl oxidase activity after consumption of a vitamin D-free diet for 2 weeks may be specific for bone, since there were no similar changes in lysyl oxidase activity in aorta. It also appears that the increase in enzyme activity is not due primarily to an effect of lowered circulating Ca or lowered body growth rate, since Ca addition to the D-deficient diet counteracted the hypocalcemia and the growth impairment, but did not counteract the increase in lysyl oxidase activity. The lack of a relationship between growth rate and lysyl oxidase activity is also apparent when chicks on the D-deficient and control-D high-Ca diets are compared; both groups showed a similar reduction in growth rate, while enzyme activity was increased only in the D-deficient group.

Previous reports have shown that both bone DHLNL/HLNL ratios (13, 2) and cartilage lysyl oxidase activities (14) decrease as the tissue matures. Our previous data have indicated that vitamin D may be necessary for such maturation as far as the normal decrease in DHLNL/HLNL ratios is concerned. The results of the present study do not support our hypothesis that the increased DHLNL/HLNL ratios of rachitic bone collagen are due to decreased lysyl oxidase activities. Rather, they suggest that vitamin D may have an effect on lysyl oxidase activity during normal maturation similar to that for the crosslink ratios, and that bone lysyl oxidase activity may remain high in the rachitic chick as a reflection of the persistent immature state of the bone.

Summary. Activity of lysyl oxidase, an enzyme responsible for production of aldehydic precursors for lysine-derived collagen crosslinks, was measured in tibial metaphyses from chicks receiving different dietary levels of vitamin D and Ca for 2 weeks after hatching. Enzyme activities were increased twofold in D-deficient chicks compared to activities from chicks receiving control levels of vitamin D. Addition of Ca to the D-deficient diet had no effect on lysyl oxidase activity. It is suggested that vitamin D may play a role in the age-related decrease in lysyl oxidase activity that normally occurs in chick bone.

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1. Mechanic, G. L., Toverud, S. U., and Ramp, W. K., Biochem. Biophys. Res. Commun. **47,** 760 (1972).
2. Mechanic, G. L., Toverud, S. U., Ramp, W. K., and Gonnerman, W. A., Biochim. Biophys. Acta **393,** 419 (1975).
3. Toole, B. P., Kang, A. H., Trelstad, R. L., and Gross, J., Biochem. J. **127,** 715 (1972).
4. Barnes, M. J., Constable, B. J., Morton, L. F., and Kodicek, E., Biochem. J. **132,** 113 (1973).
5. Barnes, M. J., Constable, B. J., Morton, L. F., and Kodicek, E., Biochim. Biophys. Acta **328,** 373 (1973).
6. Pinnell, S. R., and Martin, G. R., Proc. Nat. Acad. Sci. USA **61,** 708 (1968).
7. Ramp, W. K., and Neuman, W. F., Amer. J. Physiol. **220,** 270 (1971).
8. Harris, E. D., Gonnerman, W. A., Savage, J. E., and O'Dell, B. L., Biochim. Biophys. Acta **341,** 332 (1974).

9. Narayanan, A. S., Siegel, R. C., and Martin, G. R. Arch. Biochem. Biophys. **162,** 231 (1974).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
11. Narayanan, A. S., Siegel, R. C., and Martin, G. R., Biochem. Biophys. Res. Commun. **46,** 745 (1972).
12. Ramp, W. K., Toverud, S. U., and Gonnerman, W. A., J. Nutrition **104,** 803 (1974).
13. Mechanic, G. L., Gallop, P. M., and Tanzer, M. L., Biochem. Biophys. Res. Commun. **45,** 644 (1971).
14. Siegel, R. C., and Martin, G. R., J. Biol. Chem. **245,** 1953 (1970).

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