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EFFECT OF VITAMIN D ON THE CONTENT OF THE STABLE CROSSLINK, PYRIDINOLINE, IN CHICK BONE COLLAGEN

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SUMMARY The effect of vitamin D on the content of a crosslink, pyridinoline, in chick bone collagen was studied. One-day-old chicks were fed a synthetic diet with or without vitamin D for 6 weeks. No significant difference was observed between vitamin D-deficient and -supplemented chicks till 4 weeks, but at 5 and 6 weeks, the pyridinoline content in vitamin D-deficient chicks markedly increased as compared with that in vitamin D-supplememted chicks. Concomitantly, the collagen fiber of vitamin D-deficient chicks became less susceptible to proteolytic enzymes such as pepsin and papain. A possible relationship of these observations to the disturbance of bone remodeling in vitamin D deficiency was discussed.

It is well known that vitamin D deficiency disturbs mineralization and remodeling of bone (1). The major organic constituent of bone is collagen and the fiber of collagen is stabilized by intermolecular covalent crosslinks. It may be conceivable that crosslinks in bone collagen are implicated in the formation of rickets. However, the effect of vitamin D deficiency on the content and chemical nature of crosslinks in bone collagen has not yet been thoroughly investigated.

In the previous study, we have isolated a new collagen crosslink, which is a 3-hydroxypyridinium derivative with three amino and three carboxyl groups and has been named pyridinoline (2,3,4). This crosslink differs from other known crosslinks in that it is stable and non-reducible (3) and abounds in collagens of mature animals (5). It has been suggested that pyridinoline may serve as an interesting index for the maturation and aging of collagen (5). In the present study, we investigated the effect of vitamin D on the content of pyridinoline in chick bone collagen. Vitamin D deficiency markedly increased the pyridinoline content, which appeared to be related to the disturbance of bone remodeling.

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MATERIALS AND METHODS

One-day-old White Leghorn cockerels were devided into three groups. One group was fed a normal corn mash meal (normal chicks). The second group was fed a vitamin D deficient diet (vitamin D-deficient chicks) (6). The third group was fed the same vitamin D deficient diet, but was given 20 units of cholecalciferol per day orally (vitamin D-supplemented chicks). Chicks were killed by decapitation and blood and tibiae were removed. Serum calcium was determined by atomic absorption spectrophotometry. Serum phosphate was determined by the method of Fiske and Subbarow (7). 25-Hydroxycholecalciferol was determined by a competitive protein binding assay as previously reported (8).

The diaphysis of the tibiae was cleaned of marrow, cut into small pieces, washed with 0.5M NaCl and subjected to demineralization in 0.5M sodium EDTA, pH 7.5, at 4°C for 4 days. The demineralized bone (about 0.4g wet weight) was hydrolyzed in 6N HCl (5 ml) at 110°C for 24 hr in a sealed tube. The content of pyridinoline in the hydrolysate was determined as described previously (3). Hydroxyproline was determined according to the method of Kivirikko et al (9).

For enzymic solubilization of bone collagen, demineralized bone (0.4 g wet weight) was finely minced and incubated with 1 mg of pepsin (Seikagaku Kogyo, Tokyo, 3 x crystallized) in 2 ml of 0.5M acetic acid or 1 mg of papain (Midorijuji, Osaka, 1200 units/g) in 2 ml of 0.1M phosphate buffer-0.01M EDTA-2 mg/ml cysteine, pH 7.4. The incubation was performed with stirring at 4°C for 16 hr. Then, the suspension was centrifuged at 10,000 x g for 15 min and the supernatant was collected. An aliquot of the supernatant was mixed with an equal volume of conc.HCl and heated at 110°C for 24 hr in a sealed tube. The amount of collagen was estimated from the hydroxyproline content in the hydrolysate, assuming that hydroxyproline content is 100 residues / 1000 total residues.

RESULTS AND DISCUSSION

Figure 1 shows changes in the content of pyridinoline in bone collagen from normal chicks as a function of age. The pyridinoline content was

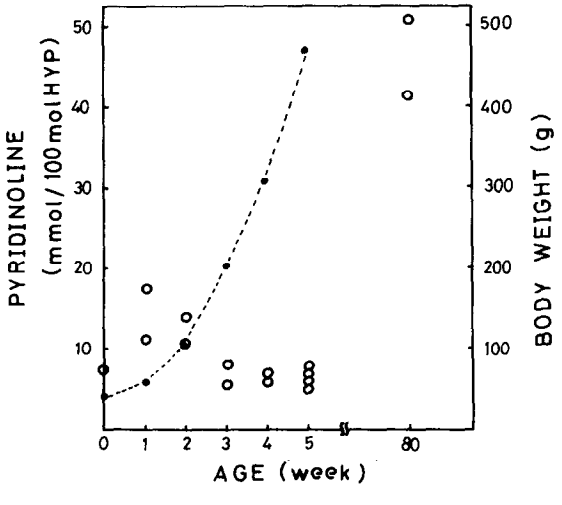

Fig.1 Changes in pyridinoline content of bone collagen (o) and in body weight (●----●) of normal chicks as a function of age.

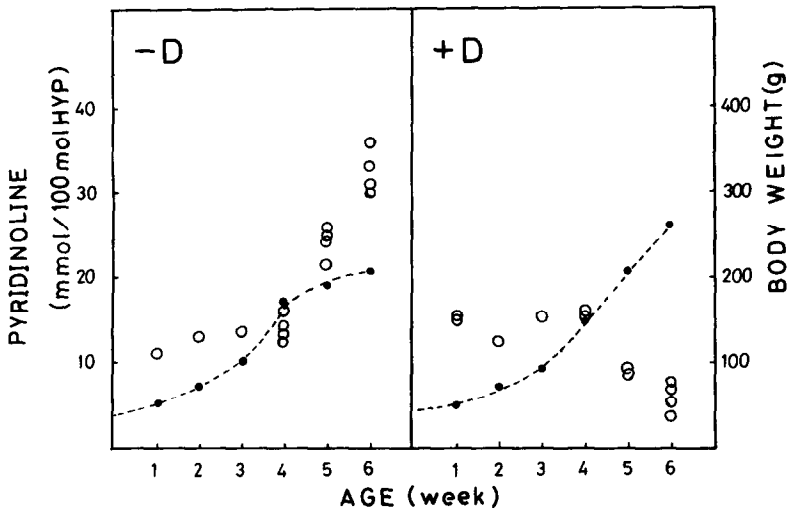

Fig.2 Changes in pyridinoline content of bone collagen (o) and in body weight (●----●) of vitamin D-deficient and -supplemented chicks. - D = vitamin D-deficient; + D = vitamin D-supplemented.

significantly lower at 3-5 weeks than at 1-2 weeks. The content in adult hens (about 1.5 years of age) was very high, as compared with that in young chicks. The pyridinoline content was considered to be inversely related to the growth rate of chicks.

The growth rate of the chicks fed the synthetic diet (both vitamin D-deficient and -supplemented chicks) was lower than that of the normal chicks (Fig. 2). The pyridinoline content in bone collagen was almost identical in both groups till 4 weeks. At 5 weeks, the pyridinoline content decreased in vitamin D-supplemented chicks, whereas it increased appreciably in vitamin D-deficient chicks.

Table 1 summarizes the pyridinoline content in bone collagen and the serum concentrations of calcium, phosphate and 25-hydroxycholecalciferol in chicks fed the synthetic diet with or without vitamin D for 6 weeks. In the vitamin D-deficient chicks, the concentrations of calcium and 25-hydroxy-cholecalciferol decreased significantly, and the pyridinoline content was about 5 times higher than that of the vitamin D-supplemented chicks.

Collagen fibers are solubilized by the action of proteolytic enzymes at low temperatures (10). As shown in Table 2, the bone collagen of the vitamin

TABLE 1

Effect of vitamin D on the pyridinoline content in bone collagen and serum concentrations of Ca, P and 25-hydroxycholecalciferol

	Pyridinoline content in bone collagen (m mol/ 100 mol hydroxyproline)	Serum concentrations		
		Ca (mg/dl)	P (mg/dl)	Hydroxychole-calciferol (ng/ml)
Vitamin D-deficient	32.3 ± 1.2	6.1 ± 0.7	8.5 ± 0.8	5.2 ± 0.6
Vitamin D-supplemented	5.8 ± 0.7	10.7 ± 0.3	8.0 ± 0.4	13.4 ± 1.7

Animals were maintained on a synthetic diet for 6 weeks with or without vitamin D. Numbers shown are means ± standard errors from 4 chicks.

D-deficient chicks was found to be less susceptible to the action of pepsin or papain than that of the vitamin D-supplemented chicks. It may be conceivable that the bone collagen in vitamin D-deficient animals is also less susceptible to collagenase and other proteases which are responsible for the resorption of bone *in vivo*.

It has been shown that vitamin D deficiency results in an elevation of hydroxylysine content (11) and an increased ratio of a crosslink, dehydro-dihydroxylysinonorleucine to another crosslink, dehydrohydroxylysinonorleucine (12) in bone collagen. Our results may be compatible with these observations, since pyridinoline arises most probably from three residues of hydroxylysine (4) and dehydrodihydroxylysinonorleucine may be an intermediate of pyridinoline formation (3).

TABLE 2

Effect of vitamin D on the susceptibility of bone collagen to proteases

	Collagen solubilized (mg/ml)	
	Pepsin	Papain
Vitamin D-deficient	0.28	0.40
Vitamin D-supplemented	2.10	1.86

Animals were maintained on a synthetic diet with or without vitamin D for 5 weeks. Bone collagen was incubated with pepsin or papain at 4°C for 16 hr and the amount of solubilized collagen was measured. Details of the procedures are described in the text.

It has been reported that lysyl hydroxylase is inhibited by calcium ion (13). Thus, a possible interpretation of our observations is that vitamin D deficiency causes hypocalcaemia, which accelerates hydroxylation of lysine residues in collagen and results in the formation of an excess amount of hydroxylysine-derived crosslinks including pyridinoline. High contents of these crosslinks stabilize collagen fiber of bone and prevent the resorption of bone, which results in the disturbance of bone remodeling.

An alternative or additional possibility is that vitamin D deficiency suppresses the induction of collagen-degrading cells or enzymes, which results in an increase in the proportion of old collagen relative to newly synthesized collagen. Old collagen has more pyridinoline than new collagen and, thus, vitamin D deficiency causes the increase in the content of pyridinoline.

In any case, the analysis of pyridinoline may provide an interesting indication of a bone disturbance due to vitamin D deficiency.

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