Short Communications

Bone Collagen Metabolism in Vitamin D Deficiency

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Radioisotope studies have shown that collagen synthesized in the shafts of bones from rachitic rats and chicks is similar in chain composition to that normally synthesized. However, the extent of lysine hydroxylation in both α 1- and α 2-chains is increased, by approx. 15–30% in the rat and by 50% in the chick.

The aim of this work was to ascertain whether vitamin metabolites might influence bone formation through an effect on the organic matrix of bone in addition to or as part of their known involvement in mineral metabolism (DeLuca, 1967, 1971; Wasserman *et al.*, 1971; Kodicek, 1972).

In the growth of long bones by endochondral ossification, the cartilage is intimately involved in the mineralization process. Calcification occurs initially in the hypertrophic zone of the epiphyseal cartilage plate. This calcified area is then invaded by capillaries and osteogenic cells and becomes replaced by bone; the hypertrophic cells in the calcified cartilage degenerate, while concomitantly the invading osteoblasts produce, in apposition to the calcified cartilage, bone matrix (osteoid), which then undergoes rapid mineralization. The whole process is repeated as further cartilage cells mature to replace the dying cells of the calcified hypertrophic zone (Ham, 1969).

In rickets this process is disrupted, since calcification is largely absent. The epiphyseal plate enlarges as cartilage cells continue to proliferate and mature, and the hypertrophic zone fails to undergo the degeneration consequent to calcification. Osteoblasts continue to produce osteoid, which accumulates as uncalcified seams in the metaphyseal region of the growth zone and elsewhere (Ham, 1969). It is not clear to what extent this material may differ from that normally synthesized (Nicolaysen & Eeg-Larsen, 1953; Kodicek, 1956; Engfeldt *et al.*, 1966; Fourman *et al.*, 1968; Rasmussen, 1969, 1970; Hjertquist *et al.*, 1970). There is evidence, however, of an effect of vitamin D on the metabolism of bone collagen, the major constituent of osteoid (see Paterson & Fourman, 1968; Canas *et al.*, 1969; Baylink *et al.*, 1970; Kivirikko, 1970; see also Oliver *et al.*, 1972). The present study was undertaken to examine the nature of the collagen synthesized in the bone of rachitic animals and in particular to ascertain whether its chain composition resembled that either of the collagen from cartilage consisting of three identical α -chains [of α 1-type and designated α 1 (II) (see Miller, 1971)] or of the collagen normally synthesized in the diaphysis and con-

sisting of two identical β -chains of α 1 type and designated α 1 (I) and a third, distinct in amino acid composition from the other two, and designated α 2 (Miller *et al.*, 1967). It was also decided to examine the relative contents of lysine and hydroxylysine in the α -chains, since the degree of lysine hydroxylation is known to be one parameter that shows considerable variation between different collagens (see, e.g., Piez & Likins, 1960).

These studies were undertaken in both rats and chicks fed on a vitamin D-free diet. Collagen was isolated after the administration of [3 H]lysine and its chain composition was determined by separation of α -chains on CM-cellulose and comparison of the radioactivity in the eluate attributable to chains of α 1- and α 2-type. The degree of hydroxylation of lysine was determined by measuring the distribution of radioactivity between lysine and hydroxylysine by ion-exchange chromatography after acid hydrolysis of the α -chains. The results, a preliminary account of part of which has already been given (Barnes, 1972), indicated that collagen synthesized by rachitic bone was of the same chain composition as that normally found in bone. However, the degree of lysine hydroxylation was increased.

Weanling rats (Black and White hooded) were divided into three groups of six. One group (group I) was fed on the high-calcium low-phosphorus rachitogenic diet described by Numerof *et al.* (1955), a second group (group II) on the vitamin D-free diet described by DeLuca *et al.* (1961) with a Ca/P ratio approx. 1:1 and a third group, the vitamin D-dosed control group (group III), received the same diet as group I but each animal also received orally 5 μ g of ergocalciferol at 72 h and 12.5 μ g at 24 h before being killed. After 3 weeks on the diet each rat received by intraperitoneal administration 0.5 ml of 20% (w/v) 3-aminopropionitrile fumarate in 0.9% NaCl (to inhibit the cross-linking of collagen α -chains to β -dimers) and 1 h later 1 mCi of L-[4,5- 3 H]lysine monohydrochloride (The Radiochemical Centre, Amersham, Bucks., U.K.) in 1 ml of water. Animals were killed 4–5 h later. The mean body weight for each group before the animals were killed was as

follows: group I, 81 g; group II, 92 g; group III, 77 g. Tibiae and femora were removed, dissected free of extraneous tissue and then severed in two along the longitudinal axis to facilitate the removal of the epiphysis (at the junction of the epiphysal plate and metaphysal region of the diaphysis) and to allow dispersion of the marrow by rinsing in 0.9% NaCl. The shafts were then disintegrated in a stainless-steel mortar after being frozen in liquid N₂. The extraction of collagen from the crushed bone by prolonged dialysis against 0.5 M-acetic acid, its precipitation and the separation of α -chains from denatured collagen solutions by CM-cellulose chromatography were as described previously (Barnes *et al.*, 1971a). The elution of α 1- and α 2-chains from CM-cellulose columns was detected by counting the radioactivity in the eluate (Barnes *et al.*, 1971a). Total radioactivity in α 1- and α 2-chains in the control group (group III) was approximately double that in either those of group I or of group II (see Table 1). This may reflect a stimulation of collagen synthesis in the control group after dosage with vitamin D, in accord with the findings of Canas *et al.* (1969). The α 1-chain/ α 2-chain radioactivity ratios are given in Table 1.

Bone collagen of chain composition [α (I)]₂ α 2 would be expected to yield a ratio slightly greater than 2:1 [from a consideration of the lysine+hydroxylysine contents of α 1- and α 2-chains, the content in the former being approx. 10% higher than in the latter (see, e.g., Miller *et al.*, (1967)]. The ratios obtained thus suggest the synthesis of this type of collagen in rachitic bone. A preferential synthesis of cartilage collagen would have given rise to a ratio much in excess of 2:1. The slightly higher ratio in the control group (III) might reflect the persistence in this case of some cartilage matrix in the metaphysal

region during the process of endochondral ossification (Ham, 1969).

Lysine and hydroxylysine radioactivities were determined by the procedure described previously (Barnes *et al.*, 1971a). The degree of lysine hydroxylation calculated from these determinations is indicated in Table 1. In both α 1- and α 2-chains there was an increase of about 15–30% in the extent of hydroxylation in the two vitamin D-deficient groups, the increase being somewhat larger in group I than in group II.

These results were confirmed in the chick. A group of six 1-day-old chicks (from the National Institute for Research in Dairying, Shinfield, Reading, Berks., U.K.) were fed on the rachitogenic diet described by Lawson *et al.* (1969) until they were 3 weeks of age. A second group of six, which served as controls, received the same diet but also received orally once weekly 2.5 μ g of cholecalciferol. After 3 weeks all chicks received intracardially 0.5 ml of 20% (w/v) 3-aminopropionitrile fumarate in 0.9% NaCl and about 1 h later 0.4 mCi of L-[4,5-³H]lysine monohydrochloride/100g body wt. by intraperitoneal administration. Chicks were killed 4–5 h later. The mean body weight in the control group was 225 g and in the rachitic group was 180 g. Collagen was extracted from the shafts of the tibiae and femora as described for the rats. The α -chain composition was examined by CM-cellulose chromatography as described above. Total incorporations of radioactivity into α -chains were similar in the two groups, in contrast with the situation in the rat (see Table 1). This may reflect the fact that the control chicks were receiving vitamin D for some considerable time before administration of the [³H]lysine (see Canas *et al.*, 1969). The α 1-chain/ α 2-chain radioactivity ratio, given in Table 1, indicated, as before, that collagen synthesized in the

Table 1. α -Chain composition and the extent of lysine hydroxylation in the α -chains of collagen synthesized in normal and rachitic bone

³H-labelled collagen was isolated from leg bones after the administration of L-[4,5-³H]lysine to chicks and rats as described in the text. Collagen chain composition was determined from the distribution of radioactivity between chains of α 1- and α 2-type separated by CM-cellulose chromatography. The extent of lysine hydroxylation was determined from the distribution of radioactivity between lysine and hydroxylysine after their separation after acid hydrolysis of α -chains. Results have been corrected for a 25% loss of radioactivity occurring when L-[4,5-³H]-lysine in collagen is converted into hydroxylysine (Popenoe *et al.*, 1965). Results are expressed at 30% efficiency of counting.

Species	Group	10 ⁻³ × Total radioactivity in collagen α -chains (c.p.m.)			Extent of lysine hydroxylation (%)	
		α 1-Chain	α 2-Chain	α 1-Chain/ α 2-chain ratio	α 1-Chain	α 2-Chain
Rat	I (vitamin D-deficient)	417	172	2.4	38	48
	II (vitamin D-deficient)	516	220	2.3	33	46
	III (vitamin D-dosed)	1112	336	3.3	29	40
Chick	Vitamin D-deficient	1172	424	2.8	33	49
	Vitamin D-dosed	1064	342	3.1	21	32

rachitic bone is similar with respect to chain composition to that synthesized normally. The general extent of lysine hydroxylation, determined as described above, again revealed an increase (in this instance of about 50%) in both $\alpha 1$ - and $\alpha 2$ -chains in the rachitic group in comparison with controls (see Table 1).

The significance of this increase in the hydroxylation of lysine is as yet unclear. While this work was in progress Toole *et al.* (1972) reported their findings on the nature of collagen synthesized in rachitic chick bone. In their studies the yield of extractable collagen was increased by the continuous administration of 3-aminopropionitrile, which was dissolved in the drinking water. Collagen α -chains were separated by CM-cellulose chromatography and the overall extent of lysine hydroxylation was determined by amino acid analysis. They concluded, as we have in the present study, that collagen synthesized in the rachitic bone was similar to that synthesized normally except for an increased hydroxylation of lysine, and they conjectured that this increase may be important in the calcification process. It was postulated that osteoid may normally undergo a process of maturation whereby the extent of collagen lysine hydroxylation is decreased, this decrease permitting calcification; in rickets this maturation may be inhibited. It seems possible to us that the increased hydroxylation in collagen from rachitic bone may be in some way more akin to the increased extent noted in collagens from embryonic or young tissues (Miller *et al.*, 1967; Barnes *et al.*, 1971b; Barnes, 1972), perhaps related to an altered metabolic activity in the tissue or reflecting an alteration, in response to the deficiency, in the balance between rate of collagen protein synthesis and activity of lysine hydroxylase. There is as yet no interpretable pattern in the variability of lysine hydroxylation between different collagens. Examination of the state of hydroxylation of specific lysine residues may be more useful. It is known, for example, that the extent of hydroxylation of the lysine residues in the *N*-terminal telopeptide region of both $\alpha 1$ - and $\alpha 2$ -chains of collagen varies according to the tissue from which the collagen is derived, and this may be correlated with the type of cross-links present (see Miller *et al.*, 1969; Lane & Miller, 1969; Barnes *et al.*, 1971a,b; Barnes, 1972; Bailey & Robins, 1972). Mechanic *et al.* (1972) have reported an increase in the reduced cross-link dihydroxylysine norleucine relative to the content of monohydroxylysine norleucine in collagen from rachitic bone, and this may reflect increased hydroxylation of the *N*-terminal telopeptide lysine residues.

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