Collagen Heterogeneity Within Different Growth Regions of Long Bones of Rachitic and Non-Rachitic Chicks

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The different anatomical regions involved in osteogenesis in the chick long bone have been examined for heterogeneities in collagen structure that might relate to the mechanism of ossification. Experimentally induced lathyrisms was employed to enhance collagen solubility, and vitamin D deficiency to allow accumulation of osteoid, the precursor of bone matrix. The extractable lathyritic collagens of the cartilaginous and osseous regions of growing long bones from rachitic and non-rachitic chicks were examined for α -chain type and amino acid composition. In both groups of animals the growth plate and cartilaginous regions of the epiphysis gave collagen molecules of the constitution $[\alpha 1(\text{II})]_2$, whereas the ossifying regions contained $[\alpha 1(\text{I})]_2$. The degree of hydroxylation of the lysine moieties was increased by approximately 50% in the $\alpha 1(\text{I})$ -chain and $\alpha 2$ -chain of rachitic bone collagen. Since uncalcified osteoid is greatly enriched in rachitic bone, it is concluded that the collagen of osteoid has the configuration $[\alpha 1(\text{I})]_2$, similar to that of bone matrix, but has an elevated hydroxylysine content. The possible relationship of this difference to the mechanism of calcification is discussed.

Since the recent discovery of molecular heterogeneity of the primary and secondary structure of collagen, questions concerning possible roles of collagen in developmental and physiological processes have become more cogent. It is now established that cartilage collagen, both sternal and epiphyseal, differs from that found in skin and bone both in amino acid sequence of the α -chains and in their distribution within the triple helical collagen molecule (Miller & Matukas, 1969; Trelstad *et al.*, 1970; Miller *et al.*, 1971; Miller, 1971). There is also increasing evidence for the presence of other types of α -chains in embryonic chick skin (Trelstad *et al.*, 1971) and in human foetal skin (Miller *et al.*, 1971), which have not been detected in the mature tissue. Thus there is the distinct possibility of transitions of one type of collagen to another in developmental processes, perhaps akin to that in the haemoglobin system (see Wilt, 1967).

The present study aimed to examine the types of collagen in various anatomical regions of growing long bones in normal animals as compared with that in animals exhibiting an experimental abnormality of ossification (rickets), for the purpose of understanding better the biochemical role of collagen in the mechanism of ossification.

Endochondral bone formation at the growth plate and at the secondary centres of ossification in the epiphysis involves initial calcification of a cartilaginous matrix followed by bone deposition and cartilage resorption. The newly formed organic matrix of bone

is uncalcified osteoid, which is rapidly transformed into or replaced by bone matrix and then calcified. The structural protein of all the involved matrices consists mainly of collagen. Although the collagens of cartilage and bone are different from each other in primary structure, nothing is known about the detailed composition and structure of osteoid. In growing animals made rachitic by vitamin D deficiency, the cartilaginous growth plate ossifies at a retarded rate and is greatly enlarged, and osteoid accumulates along the newly forming bony trabeculae at the endochondral, endosteal and periosteal sites of ossification (Park, 1939; Engfeld & Zetterstrom, 1955; Sheldon & Robinson, 1961; Baylink *et al.*, 1970; Simmons & Kunin, 1970) (see also Plate 1).

Major questions concerning the structure, chemistry and function of collagen are raised by the above changes of the organic matrix during osteogenesis in the normal and rachitic states. (1) Is the distribution of different types of collagen in cartilage and bone conserved in rickets; in particular, has the collagen of the enlarged and disorganized rachitic growth plate the same composition as normal? (2) Are there primary or secondary structural differences between the collagens of osteoid and bone matrix and if so can they be revealed by examining rachitic bone that is enriched in osteoid? (3) In what way might the different types of collagen be involved in the mechanisms of calcification and ossification?

As a first approach to these questions we have examined the extractable collagens from separate

anatomical regions of the growing long bones of rachitic and non-rachitic chicks for α -chain type and amino acid composition. Both groups of animals were concurrently made lathyrctic to block cross-linking, thus greatly enhancing the yields of soluble collagen. In this manner it was expected that the extractable collagen of the rachitic animals would be in greater part derived from osteoid than that from the non-rachitic animals.

Materials and Methods

Preparation of collagens

White Rock chicks (1 day old) were fed either with normal corn mash meal or on a rachitogenic diet (General Biochemical) for 3 weeks. During the period 7–17 days of age both sets of animals were given 0.02% β -aminopropionitrile fumarate in their drinking water to induce lathyrism. The dose of lathyrigen was increased to 0.03–0.04% for 3 more days and then the chicks were killed by decapitation. The long bones (femurs and tibiae) were removed from the animals, cleaned and dissected into four separate regions: the epiphysis, growth plate, junction and diaphysis [Plate 1(A)]. The epiphysis included the articular cartilage, the cartilage of the epiphysis and the secondary centres of ossification, which have begun to develop in chicks of this age. Complete separation of the cartilaginous growth plate and the bony diaphysis was ensured by removing a section between the two. This section, termed the 'junction', was roughly equivalent to the metaphysis, and contained the zone of provisional calcification and a small portion of the diaphysis. The diaphysis was cleaned of periosteum by scraping with a scalpel and the marrow was removed by the use of a pipe-cleaner.

The minced tissues were extracted by shaking overnight at 4°C in phosphate buffer, pH 7.6, 1.0 M. Extractions were repeated twice more with the phosphate buffer and then once by dialysis against large volumes of 0.2 M-EDTA, pH 7.6. The extracts were clarified by centrifugation and the collagen was precipitated by adding cold 30% NaCl soln. to give a final concentration of 20% NaCl. The precipitate was collected by centrifugation, redissolved in the phosphate buffer and dialysed against a large volume of cold 0.01 M- Na_2HPO_4 over 2 days. The collagen that precipitated was redissolved in 0.1 M-acetic acid and reprecipitated by adding NaCl to a final concentration of 7.5%. It was then dialysed free of salts against 0.1% acetic acid and freeze-dried.

Carboxymethylcellulose chromatography

Between 3 and 10 mg of various collagen samples were solubilized in 5 ml of cold 0.1 M-acetic acid and dialysed against 0.06 M-sodium acetate buffer, pH 4.8.

After thermal denaturation (45°C for 15 min) the samples were fractionated in 0.9 cm $\times$ 5 cm columns of carboxymethylcellulose equilibrated with the same buffer at 42°C as described by Piez *et al.* (1963). A linear gradient of NaCl from 0 to 0.1 M over a total volume of 200 ml was superimposed and the effluent was monitored at 230 nm continuously in a Gilford spectrophotometer equipped with a flow cell. Meticulous attention was paid to keep the conditions of chromatography constant in all cases in order that the resulting chromatograms could be compared directly for the distribution of the α 1-chain and α 2-chain. The α 1-chain/ α 2-chain ratios were determined from measurement of the areas under the curves by the use of a planimeter.

Amino acid analysis

The samples were hydrolysed in constant-boiling HCl at 108°C for 24 h in tubes sealed under an atmosphere of N_2 . Analyses were performed by a single-column method with stepwise gradient as described by Kang (1972) on an automatic analyser (JEOLCO 5 AH). Correction factors for the loss of labile amino acids (threonine, serine, methionine and tyrosine) and for the incomplete release of valine were used as previously determined (Piez *et al.*, 1960).

Results

The terminology of Miller & Matukas (1969) is used here to distinguish the different collagen types of cartilage and bone. The collagen of sternal cartilage has been shown to contain three apparently identical α -chains, which are termed α 1 type II or α 1(II). These α 1(II)-chains show similar chromatographic behaviour on carboxymethylcellulose to that of the α 1-chains derived from bone and skin collagens, termed α 1 type I or α 1(I). However, their amino acid compositions are clearly distinguishable. Thus the α -chain constitution of cartilage collagen has been shown to be [α 1(II)]₃ (Miller & Matukas, 1969; Trelstad *et al.*, 1970) whereas that of bone is [α 1(I)]₂ α 2 (Miller *et al.*, 1967).

Distribution of soluble collagens in non-rachitic bones

Representative carboxymethylcellulose chromatograms of denatured collagen samples from neutral phosphate buffer extracts of epiphysis, growth plate, junction and diaphysis are shown in Fig. 1. The α 1/ α 2 ratios in the diaphysis and junction collagens were always close to 2:1 (range 1.9–2.5:1), consistent with the composition [α 1(I)]₂ α 2. The growth-plate collagen gave ratios of 10–15:1, suggesting that 80% or more was [α 1(II)]₃. For the epiphysis the ratio was

Fig. 1. Carboxymethylcellulose chromatograms of denatured lathyritic collagen preparations from non-rachitic chicks

(a) Growth plate; (b) epiphysis (after removal of secondary centres of ossification); (c) junction; (d) diaphysis.

4–6:1, i.e. approx. 50% $[\alpha 1(\text{II})]_3$. However, 3-week chicks have well-advanced secondary centres of ossification in the epiphysis, which would be expected to contribute $[\alpha 1(\text{I})]_2 \alpha 2$ to the extracts. In one experiment, these secondary centres of ossification were removed and the collagen obtained from the remaining cartilage gave a ratio of 35:1 (Fig. 1), i.e. greater than 90% $[\alpha 1(\text{II})]_3$. The ratios given above changed very little between successive extracts within a given experiment, suggesting that the populations of soluble collagen molecules within each tissue type were homogeneous.

Amino acid analyses given in Table I for $\alpha 1$ -chain and $\alpha 2$ -chain are identical with those obtained by other investigators for $[\alpha 1(\text{I})]_2 \alpha 2$ (Miller *et al.*, 1967) and $[\alpha 1(\text{II})]_3$ (Trelstad *et al.*, 1970). Thus the major soluble collagen of the growth plate and the cartilaginous portion of the epiphysis is $[\alpha 1(\text{II})]_3$ whereas that of the diaphysis is $[\alpha 1(\text{I})]_2 \alpha 2$.

Table 1. Amino acid composition of α -chains from soluble collagen samples from rachitic and non-rachitic cartilage and bone

Values have been rounded off to the nearest whole number when greater than 10 residues per 1000. R, Rachitic; N-R, non-rachitic.

Composition (residues/1000 residues)											
	Epiphysis			Growth plate		Diaphysis			Diaphysis		
	$\alpha 1$		$\alpha 1(\text{II})^*$	$\alpha 1$		$\alpha 1$		$\alpha 1(\text{I})^\dagger$	$\alpha 2$		$\alpha 2^\dagger$
	R	N-R		R	N-R	R	N-R		R	N-R	
3-Hyp	—	0.8	—	0.2	1.6	0.9	1.1	—	0.8	1.0	1.1
4-Hyp	100	104	100	97	101	102	104	102	100	92	93
Asp	44	42	44	45	42	42	42	42	48	48	47
Thr	25	24	25	26	25	19	19	19	19	19	19
Ser	26	27	26	31	26	29	26	30	29	30	30
Glu	85	83	87	97	84	78	79	81	67	70	67
Pro	113	118	111	111	111	118	117	122	118	117	116
Gly	336	333	332	322	337	329	330	329	329	337	336
Ala	105	106	111	104	104	129	120	120	102	102	100
Val	19	19	20	20	20	14	16	15	28	30	30
Met	11	10	11	12	12	8.6	8.8	9.3	6.0	4.0	4.6
Ile	7.6	7.4	7.3	8.3	7.8	6.1	6.7	6.9	18	18	18
Leu	25	24	24	25	25	20	20	20	32	31	30
Tyr	1.5	0.9	1.7	2.6	1.9	2.1	3.3	3.0	2.0	2.6	2.8
Phe	14	13	13	15	13	14	12	12	14	13	13
Hyl	22	19	21	22	22	5.5	6.6	10	8.0	10	7.5
Lys	16	17	18	13	15	29	29	25	24	20	22
His	2.3	2.4	2.2	2.3	2.0	2.8	3.0	2.8	7.1	6.9	7.4
Arg	49	50	48	47	50	51	49	48	49	50	50
Hydroxylation (%)											
Pro	46.9	46.8	47.7	46.6	47.7	46.4	47.0	46.7	45.9	44.2	44.4
Lys	57.9	52.6	53.9	54.7	60.0	15.9	18.5	28.7	25.0	34.1	25.0

* Trelstad *et al.* (1970). † Miller *et al.* (1967).

Table 2. *Yields of extractable collagen samples from different anatomical regions of rachitic and non-rachitic bones*

Yields are the sum of amounts obtained in three extractions with phosphate buffer, 1.0.4, and one extraction with 0.2M-EDTA, pH7.6. All animals were made lathyritic to increase the yield of soluble collagen.

Bone type	Extractable collagen (mg/100g wet wt. of tissue)		
	Epiphysis	Growth plate	Diaphysis
Rachitic	650	84	190
Non-rachitic	910	54	200

Distribution of soluble collagens in rachitic bones

The rachitic condition of the chicks was indicated by greatly thickened cartilaginous growth plates, disorganization of the zone of provisional calcification and the accumulation of osteoid observed histologically (see Plate 1). The animals continued to grow throughout the 3 weeks of feeding but at a slower rate than the controls. For example, in one experiment the control animals increased in weight from a mean of 35g at 1 day to 170g at 20 days, whereas the rachitic animals were 130g at 20 days. However, the different rate of growth did not substantially affect the yields of soluble collagen. Table 2 summarizes the yields as a percentage of wet weight of tissue obtained in a typical experiment. Approximately twice the amount of growth-plate tissue was obtained from the rachitic animals as from non-rachitic, thus giving rise to a comparable increase in total yield of soluble collagen.

Carboxymethylcellulose chromatograms of collagen samples prepared from extracts of the rachitic tissues, shown in Fig. 2, were very similar to those obtained from collagen samples of the non-rachitic control bones. Collagen samples from rachitic diaphysis and junction gave ratios α_1/α_2 close to 2:1 whereas those from the growth plate were 10–12:1. The ratios obtained for the rachitic epiphysis varied from 5:1 to 30:1, in some cases being much greater than the controls. This was probably due to retarded ossification of the secondary centres in the rachitic epiphysis. As was the case for the non-rachitic animals the variation from extract to extract for a given experiment was small.

With the exception of the significant differences in the hydroxylysine and lysine contents of the diaphysis collagen discussed below, and the elevated glutamate content of the rachitic growth-plate collagen, the amino acid analyses of the rachitic α -chains were, within experimental error, identical with the controls (Table 1). It is apparent then that, with the exceptions given above, the composition and distribution of $[\alpha_1(\text{II})]_3$ in cartilage and $[\alpha_1(\text{I})]_2\alpha_2$ in ossified tissue are similar for rachitic and non-rachitic animals.

Fig. 2. *Carboxymethylcellulose chromatograms of denatured lathyritic collagen preparations from rachitic chicks*

(a) Growth plate; (b) epiphysis; (c) junction; (d) diaphysis.

Hydroxylysine content of soluble collagen samples from bone

Although the sum of lysine and hydroxylysine in the soluble collagen of the diaphysis was constant for rachitic and non-rachitic animals, the proportion of hydroxylysine was elevated in the rachitic tissue for both α_1 -chain and α_2 -chain. Table 3 lists the percentage hydroxylation of lysine in collagen samples from control and rachitic bone obtained in three separate experiments. The hydroxylysine content of the α_1 -chain was increased by about 50% in the rachitic tissue. No corresponding differences were observed in the degree of hydroxylation of proline. The content of hydroxylysine might have been elevated if these preparations were contaminated with $[\alpha_1(\text{II})]_3$. However, this possibility was precluded by the normal ratio of α_1/α_2 in these preparations and by the elevated hydroxylysine content in the α_2 -chains. α -Chains isolated from the skin collagens of rachitic animals contained the same amount of hydroxylysine as controls.

(A)

(B)

(C)

EXPLANATION OF PLATE I

(A). Tibiae of rachitic (left) and non-rachitic (right) chicks longitudinally split to show the anatomical regions examined chemically

The four regions [epiphysis (Epiph), growth plate (GP), junction (Junct) and diaphysis (Diaph)] are indicated by the dotted lines. The growth plate in the rachitic bone is irregular and distinctly wider than the control. The diaphyseal cortex in the rachitic bone also shows characteristic thickening. Magnification $\times 3.5$.

(B) and (C). Photomicrographs of longitudinal sections of rachitic and non-rachitic bony trabeculae in the diaphysis. In the rachitic bone (B), osteoid seams are very prominent whereas in the non-rachitic bone (C) they are almost undetectable. Haematoxylin and Eosin. Magnification $\times 260$.

Discussion

Osteogenesis of the developing long bone of the chicken, as well as of mammals, involves the replacement of a cartilaginous anlage by bone. Subsequent growth continues to involve change from cartilage to bone in the growth plate and in the establishment of secondary centres of ossification in the epiphysis. At least three different types of histologically recognizable tissues with different extracellular matrices take part in these transformations: cartilage, osteoid and bone. In addition, there may be further modifications within the cartilage matrix during the developmental progression through resting, proliferating, hypertrophic and calcifying zones of the growth plate. Is it possible then to relate changes in chemical composition of the major components of these matrices, e.g. collagen and the glycosaminoglycans, to changes in cell type and tissue morphology and to the mechanism of ossification?

Two types of heterogeneity in the primary and secondary structures of collagen have been reported. The first derives directly from the expression of different structural genes, e.g. the differences between cartilage (Miller & Matukas, 1969; Trelstad *et al.*, 1970; Miller, 1971) and bone or skin collagens (Piez *et al.*, 1963; Miller *et al.*, 1967; Kang *et al.*, 1969). The second results from enzymic modifications of the collagen molecule subsequent to translation at the ribosome, e.g. the variable hydroxylation of proline and lysine (Bornstein, 1967; Butler, 1969; Miller *et al.*, 1969) and glycosylation of hydroxylysine (Spiro, 1969; Morgan *et al.*, 1970; Blumenkrantz & Prockop, 1970; Pinnell *et al.*, 1971), and the different degrees of covalent cross-link formation (Piez *et al.*, 1966; Kang & Gross, 1970).

Rickets is a complex abnormality in which the balance of cartilage deposition and removal and the formation of bone is disturbed. A characteristic

result of this imbalance is enlargement of the cartilaginous growth plate (Park, 1939; Simmons & Kunin, 1970). The extractable lathyritic collagen samples of rachitic and non-rachitic growth plates were found to be similar in α -chain distribution and amino acid composition, being of the type $[\alpha 1(\text{II})]_3$, consistent with histological studies in which the rachitic growth plate had been shown to be cartilaginous (Park, 1939; Sheldon & Robinson, 1961). Whether the observed difference in the glutamate content (Table 1) is due to the possible presence of a contaminant or reflects a true primary structural difference cannot be distinguished at present. The presence of two collagen types, $[\alpha 1(\text{II})]_3$ and $[\alpha 1(\text{I})]_2\alpha 2$, at the site of endochondral ossification may allow investigation of specific control mechanisms for the synthesis and degradation of these two collagen types. If so, the cause of cartilage accumulation in the growth plate could possibly be determined.

A second major feature of rickets is the accumulation of osteoid at the endochondral, periosteal and endosteal sites of ossification (Park, 1939; Engfeld & Zetterstrom, 1955; Sheldon & Robinson, 1961; Baylink *et al.*, 1970). It has been shown that a process of 'maturation' of osteoid to bone matrix occurs before calcification (Baylink *et al.*, 1970), but it is not known whether 'maturation' involves an alteration in the existing osteoid matrix or its complete replacement by that of bone. The rate of synthesis of collagen is probably not increased in rachitic bone (Canas *et al.*, 1969; Baylink *et al.*, 1970), in which case the accumulation of osteoid would result from a decreased rate of 'maturation' (Baylink *et al.*, 1970). It was expected that comparison of the extractable collagens of rachitic animals in which lathyrisms was induced at the same time as osteoid was accumulating, with simply lathyritic animals, where the transformation of osteoid to bone matrix is very

Table 3. Proportions of hydroxylated lysine in α -chains from soluble collagen samples from rachitic and non-rachitic bone

Tissue	Expt. no.	Extract no.	Hydroxylation of lysine (%)			
			Rachitic		Non-rachitic	
			$\alpha 1$	$\alpha 2$	$\alpha 1$	$\alpha 2$
Diaphysis	1	1	25.6	28.5	19.6	24.5
		2	23.0	34.1	18.5	—
	2	1	27.8	33.0	—	—
		2	28.7	40.6	—	—
	3	1	29.4	—	18.6	—
Junction Diaphysis	2	4	25.9	31.9	18.1	23.6
		1	30.2	25.6	—	—
		—	—	—	15.9*	25.0*

* Miller *et al.* (1967).

rapid and the accumulation of osteoid is small (see Plate 1, B and C), would reveal any differences between osteoid and bone-matrix collagens. The present study suggests that osteoid collagen has the $[\alpha 1(I)]_2\alpha 2$ configuration of mature bone-matrix collagen but contains about 50% more hydroxylysine (Table 3). It is notable that the extent of hydroxylation of lysine moieties of normal bone collagen decreases with increasing age (Miller *et al.*, 1967). This change may be a reflexion of the decreasing proportion of osteoid in normal bone with age (Park, 1939).

The carbohydrate groups of $[\alpha 1(I)]_2\alpha 2$ from skin (Butler & Cunningham, 1966) and bone (Segrest & Cunningham, 1970; Pinnell *et al.*, 1971) have been shown to be covalently linked to hydroxylysine. Examination of the carbohydrate moieties of rachitic and non-rachitic bone collagen samples is in progress in our laboratory and preliminary results suggest that differences in carbohydrate parallel the differences in hydroxylysine observed above.

It has been suggested that lysine and hydroxylysine are in some as yet undefined way associated with calcification (Likins *et al.*, 1960; Solomons & Neuman, 1960; Glimcher & Krane, 1968). It is known that initial seeding of calcium apatite crystals occurs in the 'hole' regions of collagen fibrils and that modification of the ϵ -amino groups of lysine and hydroxylysine groups near these regions results in the inability of collagen fibrils to nucleate apatite crystals (Glimcher & Krane, 1968). Furthermore, Morgan *et al.* (1970) have indicated that the Glu-Gal moiety attached to hydroxylysine probably projects into the 'hole' region. Thus extra glycosylated hydroxylysine groups in the osteoid collagen fibrils may block calcification by steric interference with apatite crystal formation in these regions. The 'maturation' of osteoid to bone matrix might then involve an enzymic modification of these strategically located residues.

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