Collagen Synthesis and Carbohydrate Metabolism of Rachitic Bone

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1. This paper reports studies on the metabolism of bone from normal chicks and from chicks with vitamin D-deficiency rickets. Both *in vitro* and *in vivo* there was an increased incorporation of [^{14}C]proline into collagen hydroxyproline by rachitic bone. The proportion of the collagen that was soluble in cold salt solutions was greater with the rachitic bone. These results show that in rickets there is an increased synthesis of bone collagen, but they do not provide any evidence of a defect in the maturation of collagen. 2. Rachitic bone incubated aerobically *in vitro* consumed more glucose and released more lactate than normal bone. Bone from rachitic chicks treated with vitamin D 48 hr. previously had rates of glycolysis that were nearly normal. Though we were unable to show any direct action of vitamin D *in vitro*, we consider that vitamin D probably has a direct action on bone, possibly related to matrix biosynthesis.

In vitamin D-deficiency rickets in children the rate of mineralization of bone measured with ^{32}P or ^{45}Ca is low (Bauer, Carlsson & Lindquist, 1956; Harris, Hoffenburg & Black, 1965*a,b*), yet the urinary excretion of hydroxyproline may be increased (Klein & Curtiss, 1964), which suggests that there is an increased turnover of bone collagen.

Rohr (1965) gave [^3H]glycine to normal and vitamin D-deficient rats. He found that osteoblasts in the rachitic bone were labelled for a shorter time than those in the control bone, and suggested that the rate of collagen synthesis was increased in rickets.

We have studied the synthesis of bone collagen in normal and rachitic chicks with three independent techniques: the amount of proline incorporated into collagen hydroxyproline *in vivo*, and *in vitro*, and the proportion of the collagen that was soluble in cold neutral salt solutions. All three techniques showed an increased rate of synthesis of bone collagen in rickets.

MATERIALS AND METHODS

Chicks. Male Rhode Island $\times$ Sussex chicks were obtained 1-day-old and fed on Olsson's rachitogenic diet (Jones & Elliot, 1943). Control birds received weekly intramuscular injections of 40 i.u. of vitamin D₃. After 4 weeks the untreated birds had severe rickets: they failed to gain weight normally, they had muscular weakness and radiological examination showed that the epiphyses were widened and the bones were generally less dense. The

alkaline phosphatase activity of the plasma was raised [range 530–1700 KA units (Kind & King, 1954)/100 ml. in six rachitic birds compared with 140–410 KA units/100 ml. in eight control birds].

Incubation of bone. Chicks were decapitated and within 1 min. their calvaria were incubated in a Krebs bicarbonate medium (Umbreit, Burris & Stauffer, 1964) containing glucose (2 mg./ml.) and [$\text{U-}^{14}\text{C}$]proline (10 $\mu\text{g.}/\text{ml.}$, specific activity 0.05–0.3 $\mu\text{C}/\mu\text{mole}$).

After 4 hr. incubation the calvaria were washed twice with deionized water and blotted. The washings were added to the incubation fluid and the proteins in it were precipitated by the addition of 2 ml. of 5% (w/v) ZnSO_4 and 2 ml. of 0.3 N-NaOH. The volume was made up to 10 ml. with water. The concentrations of glucose and lactate were determined on the filtrate.

The calvarium was cut in two halves: one was for the determination of DNA, and the other was hydrolysed for the determination of the specific activity of proline and hydroxyproline.

Soluble collagen. The procedure was based on that of Jackson & Bentley (1960), who showed that neutral salt solutions of increasing ionic strength extracted collagen of increasing maturity. Bone or soft tissue was crushed in a stainless-steel mortar cooled with liquid N_2 . The soluble collagen was extracted by shaking at 4° either for 4 days with 2 M-NaCl or with four salt solutions of increasing ionic strength each for 4 days. The four solutions were (a) 10% (w/v) disodium EDTA adjusted to pH 7.5 with NaOH; (b) 0.14 M-NaCl; (c) 0.45 M-NaCl; (d) 2 M-NaCl. Preliminary experiments showed that a single extraction for 4 days yielded almost as much soluble collagen as four successive daily extractions. In a second 4-day period only a further 10% was extracted. At the end of each extraction the solution was centrifuged, filtered and dialysed twice against a large volume of deionized water at 4°. The amounts of collagen in the non-diffusible material were expressed as the amount of hydroxyproline after hydrolysis.

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Studies of collagen synthesis in vivo. Five control chicks and four rachitic chicks had intraperitoneal injections of $5\mu\text{C}$ of [^{14}C]proline (specific activity $270\text{mC}/\mu\text{mole}$) in 0.9% NaCl. Then 22 hr. later the animals were decapitated and specimens of bone and skin taken. The bones were the calvarium and the middle third of the tibia; the skin specimens were from the axilla.

Hydroxyproline. Tissue or fractions of soluble collagen were hydrolysed in 6N-HCl for 6 hr. at 120° . The hydrolysates were cleared of 'humin' with a resin-charcoal mixture (Prockop & Udenfriend, 1960), and a sample of each was evaporated to dryness over solid KOH *in vacuo*. The dried material was redissolved in water and the hydroxyproline content measured by the method of Grant (1964).

Hydroxyproline and proline specific activity. The amino acids in the hydrolysate were separated by descending paper chromatography with butan-1-ol-acetic acid as solvent (Smith, 1960). The approximate position of the imino acids on the paper was found by staining with isatin a parallel strip from a standard solution. A 20 cm. length of the paper containing both imino acids was then cut into sections 2 cm. long and the ^{14}C content of each section was determined with an automatic liquid-scintillation counter with xylene-based scintillator (NE213; Nuclear Enterprises Ltd., Edinburgh). The efficiency was such that a 1nc standard on paper gave 1218 counts/min. (overall efficiency 55%). There was no quenching as estimated by the channels-ratio method (Bush, 1963). The sections with radioactivity were washed with xylene, dried and eluted with water. Hydroxyproline was determined on the eluates by the method of Grant (1964) and proline by the method of

Wren & Wiggall (1965) adapted for the Technicon Auto-Analyser (Paterson & Metcalfe, 1967). The counts from proline and from hydroxyproline were each found in two of the sections of the original chromatogram and, when the specific activity of each section was determined, there was good agreement between each pair of determinations. The coefficient of variation of the duplicate measurements of specific activity was 8.5% for proline and 7.4% for hydroxyproline.

Glucose. Glucose was estimated by the method of Huggett & Nixon (1957) adapted as follows for the Technicon Auto-Analyser. Fig. 1 shows the manifold. The sampler 2 was run at 20 samples/hr. with a sample/wash ratio 1:2. The range of glucose concentrations was 0.1–0.5 $\mu\text{g}/\text{ml}$. The mixed enzyme-oxygen-acceptor reagent is prepared as follows: 0.5 ml. of Fermcozyme 653 (glucose oxidase) [Hughes and Hughes (Enzymes) Ltd., London, W. 1] and 0.5 ml. of 1% *o*-dianisidine (in 95% ethanol) are taken, made up to 100 ml. with 0.5M- NaH_2PO_4 (adjusted to pH 7.0 with NaOH) and filtered.

Lactate. Lactate was measured with lactate dehydrogenase and NAD (Olson, 1962). The enzyme and NAD for this procedure were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

DNA. Pooled half-calvaria were extracted with N-HClO₄ at 4° for 18 hr. to remove RNA. DNA was then extracted with 0.5N-HClO₄ for two 20 min. periods at 70° (Ogur & Rosen, 1950). The E_{260} of the extracts was determined, and the DNA content was determined by the diphenylamine reaction (Davidson & Waymouth, 1944).

Plasma alkaline phosphatase. Because of the very high alkaline phosphatase activities 0.01 ml. of plasma was incubated for 5 min. with phenyl phosphate (Kind & King, 1954).

RESULTS

Collagen synthesis in vitro. The specific activity of hydroxyproline was significantly higher in the calvaria from rachitic birds than in those from the control birds after they had been incubated with [^{14}C]proline (Table 1a). This difference could have been caused by (a) an increased rate of collagen synthesis by rachitic bone, (b) a decrease in the amount of hydroxyproline originally present, or (c) a higher specific activity of proline in the intracellular space of the rachitic chick than in the normal chicks. If it is assumed that the specific activity of the proline used in collagen synthesis was the same as the specific activity of the proline

Fig. 1. Manifold for the determination of glucose with the Technicon Auto-Analyser. D_0 and H_3 are standard Technicon glass connexions.

Table 1. *Collagen synthesis in normal and rachitic chicks*

Results are expressed as means $\pm$ S.E.M. with numbers of observations in parentheses. The significance of the difference between the normal and rachitic animals was determined with Student's *t* test.

	Normal bone	Rachitic bone	<i>P</i>
(a) Hydroxyproline specific activity in calvarium after incubation (counts/min./ μg .)	7.42 ± 0.62 (8)	9.61 ± 0.49 (6)	0.0125
(b) Proline incorporation into collagen hydroxyproline ($\mu\text{mole/g.}/\text{hr.}$)	0.105 ± 0.007 (8)	0.133 ± 0.010 (6)	0.025
(c) Soluble collagen in rib (as % of total collagen)	0.75 ± 0.10 (4)	0.94 ± 0.11 (5)	0.15

Fig. 2. Specific activity of hydroxyproline in serial fractions from pooled calvaria incubated with [^{14}C]proline *in vitro*. Solid columns: calvaria from normal chicks. Open columns: calvaria from rachitic chicks. The composition of the four solvents used for the collagen extracts designated (a)–(d) is given in the text.

in the incubation medium, the rate of collagen synthesis may be calculated as μmoles of proline hydroxylated/unit time. Table 1(b) shows that this was significantly higher in the rachitic calvaria.

In a further experiment, the calvaria were pooled after incubation with [^{14}C]proline and serial fractions of collagen were obtained with salt solutions of increasing ionic strength. Fig. 2 shows that the specific activity of hydroxyproline was much higher in the soluble collagen than in the insoluble collagen. Every fraction from rachitic bone had a higher specific activity than the corresponding fraction from normal bone.

It is likely that the insoluble material remaining at the end of this fractionation was almost entirely collagen. In this fraction the ratio:

$$\frac{\text{Specific activity of proline}}{\text{Specific activity of hydroxyproline}}$$

was 1.25 for both rachitic and control calvaria.

Fig. 3. Specific activity of hydroxyproline in the bone and skin from normal (▲) and rachitic (■) chicks that had received intraperitoneal injections of $5\mu\text{C}$ of [^{14}C]proline 22 hr. earlier.

We sought evidence of a direct action of vitamin D on bone collagen synthesis *in vitro*, but vitamin D₃ hydrosol (Wander) added to the incubation medium at concentrations of 2–2000 units/ml. had no effect.

Soluble collagen. Table 1(c) shows the proportion of the bone collagen that could be extracted with cold 2M-sodium chloride. This soluble fraction was 1.25-fold greater in the rachitic than in the control chicks and was thus increased by the same factor as the rate of proline incorporation into hydroxyproline (Table 1b).

Collagen synthesis in vivo. Fig. 3 shows the specific activity of hydroxyproline in the calvarium and in the skin of rachitic and control animals 22 hr. after the injection of [^{14}C]proline. There was a very clear difference between bone from the rachitic and the control animals. This difference is not explained by differences in the specific activity of the proline in the circulation because there was little difference between the specific activities of hydroxyproline in the skin.

In another experiment, two chicks, one rachitic and one normal, received $7.5\mu\text{C}$ of [^{14}C]proline by intraperitoneal injection. They were killed 24 hr. later and serial extracts were made of bone and skin from each. Fig. 4 shows the specific activity of hydroxyproline in each fraction. All fractions, including the insoluble collagen from rachitic bone, had a higher specific activity than the corresponding fractions of normal bone. This difference cannot be

Fig. 4. Specific activity of hydroxyproline in serial fractions from bone and skin from normal chicks (solid columns) and rachitic chicks (open columns). The animals had received $7.5 \mu\text{C}$ of $[^{14}\text{C}]$ proline 24 hr. earlier. The composition of the four solvents used for the extracts designated (a)–(d) is given in the text.

attributed to differences in the specific activity of circulating proline as there was no difference between the specific activities in fractions from skin in the two animals.

Glucose consumption and lactate production in vitro. Table 2 shows the DNA content, the glucose consumption and the lactate production by the fragments of calvarium from control chicks and from rachitic chicks. It is seen that, whether these measurements were related to the wet weight of bone (used by Borle, Nicols & Nicols, 1960a,b) or to the DNA content of the bone, the rachitic bone consumed more glucose and produced more lactate than the normal bone. Bone from control and rachitic animals had a high rate of lactate production despite the aerobic conditions. About 70–80% of the glucose consumed could be accounted for as lactate; this proportion was almost the same in the bones from control and rachitic animals.

Calvaria from six rachitic chicks given vitamin D_3 24 hr. before death had rates of glycolysis intermediate between those of the control and deficient chicks. If the vitamin D was given 48 hr. before death the rate of glycolysis was nearly normal (Table 2).

DISCUSSION

In soft tissues very nearly all of the hydroxyproline of collagen is derived from proline (Udenfriend, 1966). When a collagen-forming tissue is incubated with labelled proline, the proline and hydroxyproline in the newly formed collagen should have the same specific activity, and this is true of formation of soft-tissue collagen *in vivo* (Stanley, Dawson, Field & Glicksman, 1966). We found that the ratio of specific activities was close to 1.0; the higher specific activity of proline was at least partly due to contamination of the collagen with some non-collagenous proteins containing proline. As collagen hydroxyproline is formed from proline the formation of labelled hydroxyproline

Table 2. *Glucose consumption and lactate production by bone surviving in vitro*

		Results are expressed as means $\pm$ s.e.m.			
Source of bone		Control chicks	Vitamin D-deficient chicks	Deficient chicks given vitamin D 24 hr. before	Deficient chicks given vitamin D 48 hr. before
No. of animals		8	6	6	6
Glucose consumption (μ moles/hr./g. wet wt.)		5.04 $\pm$ 0.35	8.15 $\pm$ 1.00	6.38 $\pm$ 0.60	5.68 $\pm$ 0.44
Lactate production (μ moles/hr./g. wet wt.)		7.74 $\pm$ 0.51	11.13 $\pm$ 0.82	9.04 $\pm$ 0.68	8.92 $\pm$ 0.11
Proportion of glucose recovered as lactate (%)		77.1	71.4	72.0	79.3
DNA content (mg./g. wet wt.)		3.16	3.67	3.55	3.89

from proline provides a measure of bone collagen synthesis *in vivo* or *in vitro*.

Both *in vivo* and *in vitro* the specific activity of the newly formed collagen depends on the specific activity of the proline in the intracellular pool used in collagen synthesis. Green & Lowther (1959) suggest that there may be an intracellular pool of free proline that exchanges only slowly with that of the incubation medium or of the pool from which the collagen is synthesized. Accordingly the differences in specific activity of collagen hydroxyproline could have been caused by differences between the intracellular transport mechanisms for proline between rachitic and normal bones.

However, there is a clear difference between the bones (but not the skins) of the two groups of animals, and the studies of soluble collagen favour the view that there is an increased rate of collagen synthesis in rickets.

There is good evidence that the neutral-salt-soluble fraction of soft-tissue collagen represents the recently formed collagen (Gross, 1958; Jackson & Bentley, 1960; Nimni, de Guia & Bavetta, 1967). A much smaller proportion of the collagen of bone and dentine is soluble in cold neutral salt solutions (Veis & Schlueter, 1964; Glimcher & Katz, 1965). The difference has been attributed to the rapid formation of non-covalent intra- and inter-molecular bonds in hard tissues. We found that, after incubation of calvaria with labelled proline, labelled hydroxyproline was present at a much higher specific activity in a collagen fraction extracted with 2M-sodium chloride than in the insoluble collagen. Flanagan & Nichols (1964) obtained a similar result with a fraction extracted with dilute alkali. We concluded that, in bone as in soft tissue, much of the extract in neutral salt solutions is recently formed collagen.

In soft-tissue collagen, the proportion of soluble collagen increases with the rate of collagen synthesis (Jackson & Bentley, 1960). We hoped that measurement of the proportion of bone collagen that is soluble in cold neutral salt solutions would provide another simple measure of the rate of collagen synthesis. Table 1 shows that, though less than 1% of the total collagen in chicken rib is soluble in cold neutral salt solutions, the soluble fraction tends to be greater in the rachitic than in the normal chicks. This increase occurred despite a severe fall in the rate of growth of the chicks. Gross (1958) showed that, when growth of an animal was arrested by dietary restriction, the proportion of soluble collagen in the skin fell rapidly from 10% to 2%. On this basis our rachitic animals might have been expected to have a decreased proportion of soluble collagen.

Thus the results of all the experiments described suggest that there is an increase in collagen synthesis in the bone (but not the skin) of rachitic chicks.

With techniques *in vivo* similar to ours, H. E. Firschein (personal communication) found an increased rate of collagen synthesis in rachitic rats. An exact interpretation of this result is not yet possible. It should be emphasized that some of the collagen normally formed in bone does not go through a stage of mineralization before being broken down again (Lapière, Onkelinx & Richelle, 1967).

Several studies suggest that in hyperparathyroidism collagen synthesis is increased. For example, Flanagan & Nichols (1965) found an increased uptake of [^{14}C]proline when bone from patients with 'continuous' hypersecretion of parathyroid hormone was incubated *in vitro*. Laitinen (1967) measured the incorporation of labelled proline into bone collagen *in vivo* and found that after 8 days of treatment with parathyroid hormone there was a significant increase in collagen synthesis measured in this way. The increased rate of collagen synthesis in rickets could then be a consequence of secondary hyperparathyroidism, but this cannot explain all the abnormalities of the bone in rickets. In particular, the work of Sheldon & Robinson (1961) and of Engfelt & Zetterström (1955) suggests that there are abnormalities of the collagen formed in the experimental rickets of rats.

We wished to determine whether the increased collagen synthesis in rachitic bone was accompanied by any defect in the maturation of collagen. The soluble collagen fractions both from bone after incubation *in vitro* with [^{14}C]proline and from bone from chicks injected with [^{14}C]proline 24 hr. earlier were studied. In both the specific activity of hydroxyproline was higher in fractions from rachitic bone than from normal bone. This was true of every fraction including the insoluble collagen. The soluble collagen from soft tissues probably represents the collagen formed in the previous 1–2 days (Gross, 1958). Bone incubated for only 4 hr. with [^{14}C]proline already had some labelled hydroxyproline in the insoluble collagen, and these results suggest that the soluble fraction of bone collagen is probably that formed in the previous 4–8 hr. Our results therefore do not show a defect in collagen maturation, but only that the earliest stages of collagen maturation appear to be normal in rickets.

The rate of collagen synthesis in the bone was increased. The increased activity of alkaline phosphatase in the serum in these animals and the increased rate of glycolysis are probably further indications of the increased activity of the bone cells. The cause of this increase is not yet clear, but we consider that the possibility that vitamin D has a direct action on bone, possibly related to matrix biosynthesis, has not received the attention it deserves (Irving, 1957).

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