A Model for Studying the Initiation of Normal Calcification *in vivo*

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1. Rat costal cartilage was found to begin to calcify normally when the rats weigh 35–45 g. 2. The cartilage is suggested as a model for the study *in vivo* of mechanisms concerned with normal calcification. 3. The model was tested by studying the incorporation of fluoride into the mineral deposited in the tissue. 4. The percentage of inorganic material in cartilage rose from approx. 3% of the dry weight in the uncalcified tissue to 62% in the tissue from rats weighing 300 g. 5. Mineral deposited had a calcium/phosphorus molar ratio of 1.65. 6. After the oral administration of sodium fluoride to rats, fluoride was incorporated into cartilage mineral. 7. The concentration of fluoride in cartilage ash increased rapidly with calcification and the mineral became more highly fluoridated than the corresponding rib bone. 8. Fluoridated mineral showed a marked decrease in citrate concentration.

Rachitic cartilage has frequently been used as a tissue to study various aspects of the process of calcification. As a result of studies with this abnormal tissue in systems *in vitro*, numerous hypotheses about the mechanism of calcification have been put forward (Robison, 1923; Strates, Neuman & Levinskas, 1957; Glimcher & Krane, 1964). A critical review of the many theories postulated (Urist, 1966) emphasized the uncertainty of the exact mechanism for the initiation of crystal formation during hydroxyapatite deposition in bone and other calcified deposits. In the present paper, the results of investigations with rat costal cartilage as a calcified tissue are presented. Some of these have been presented in preliminary form (Alcock, Shils & Reid, 1968). The tissue is suggested as a model for studying the process of calcification. The model has been tested by studying the incorporation of fluoride into the mineral deposited.

MATERIALS AND METHODS

Animals and diets. All rats were of the Wistar strain.

Expt. 1. Litters of animals given a stock diet (Drug Houses of Australia, Rural Division, Sydney, Australia) were used. The young were weaned when they weighed 45 g., and random selection was made from animals weighing 15–300 g. Rib bone and cartilage from these animals were used to determine the ash content of the two tissues. Calcium, magnesium and inorganic phosphate were determined in selected samples of cartilage.

Expt. 2. The mothers of three litters of 1-day-old rats

were given *ad lib.* a diet based on that of MacIntyre & Davidsson (1960) with the following modifications; the diet given to the first two mothers (I and II) was supplemented with 25 mg. of fluoride (as NaF)/100 g. of diet; no fluoride was added to the diet given to the third mother (III), whose litter served as control animals. All rats were weaned when they weighed 45 g. The control animals, after weaning, were given the same diet as their mother had received. The weanlings from mothers I and II were given the following diet: on the first day after weaning 10 mg. of fluoride (as NaF)/100 g. of diet was added, and the fluoride content was increased daily until it was 25 mg./100 g. on the fourth day and thereafter. This modification was desirable because previous observations with weanling rats had shown that weight loss resulted if a diet with a high fluoride content was given initially, but the animals adapted quickly to increasing doses.

Rats were killed at intervals when they weighed 15–120 g. Costal cartilage and rib bone were used to determine the ash content, the citrate concentration and, where sufficient material was available, the fluoride concentration of the tissue.

Preparation of tissues. Rib cartilage and bone were removed and freed of muscle. A portion of the cartilage (about 1 mm. long) adjacent to the chondrocostal junction was discarded to avoid contamination from the adjoining rib bone. The cartilage and bone samples were dried separately for 16 hr. at 105° and either used whole or ground to a fine powder with an agate mortar and pestle. Bone was defatted by extraction with ethanol–light petroleum (b.p. 40–60°) (1:1, v/v), but cartilage was not defatted.

Analytical methods

Ash content. Tissues were ashed at 500°. The addition of 1 drop of conc. HCl to the residue after 24 hr. accelerated the complete destruction of organic matter, as indicated by the attainment of constant weight.

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Calcium, magnesium, inorganic phosphate and citrate. Cartilage or bone powder was extracted overnight in 5% (w/v) trichloroacetic acid containing 10% (w/v) acetic acid (5 mg. dry wt. of cartilage/ml. of acid) and then shaken for 3 min. on a Vortex mixer. Modification of the acid mixture described by Sparrow & Johnstone (1964) resulted in the complete extraction of calcium, magnesium, inorganic phosphate and citrate, which could then be determined on a single sample, whereas the more concentrated acid used by Sparrow & Johnstone (1964) resulted in turbidity during the colorimetric determination of phosphate. Inorganic phosphate was determined on a portion of the acid extract by the method of Sumner (1944) and citrate on another portion by the method of McArdle (1955). A further portion was diluted appropriately (1:10 to 1:50, depending on the calcium concentration in the tissue) with 1 M-HCl containing 100 mM-KCl, and was used for the determination of calcium by atomic-absorption spectrometry with an N_2O -acetylene flame. The presence of potassium in the diluting fluid prevented ionization of calcium in the N_2O -acetylene flame. No interference from phosphate was observed. Magnesium was determined on the same solution by atomic-absorption spectrometry.

Fluoride. Fluoride was determined on a sample of bone and cartilage by the method of Alcock (1968).

RESULTS

Ash content (Expt. 1). The changes in ash content of rib bone and costal cartilage with increasing rat weight are shown in Table 1. Bone from the 15g. rat contained 47% of ash, and the value increased slowly with rat weight to 68% in the 305g. rat. The ash content of costal cartilage in the 15–40g. rats was 3–5% of the dry weight, but it increased suddenly in rats weighing more than about 50g. The ash content increased sharply with rat weight, and was 42% in the 120g. rat. The rate of increase of ash content then diminished with increasing rat weight, and cartilage from the 300g. rat contained 62% of ash.

Calcium, magnesium and inorganic phosphate (Expt. 1). Calcium, magnesium and inorganic phosphate were determined on samples of costal cartilage from animals weighing 20–120g. (Table 2). The variation in cartilage calcium concentration with rat weight followed the same pattern as that described above for the ash content. The concentration of calcium in the cartilage from 20–40g. animals was about 0.3%, and cartilage from the 60g. rats contained 4% of calcium. Inorganic phosphate and magnesium concentrations increased with the calcium concentration. Although the Ca/P molar ratios in the tissues from animals weighing less than 50g. were variable, the ratio was constant at about 1.65 in tissues from older animals (Table 2).

Citrate and fluoride (Expt. 2). The ash contents of the tissues taken from animals in Expt. 2 were similar to those found in Expt. 1. Changes in

Table 1. *Comparison of the percentages of ash in samples of rat costal cartilage and rib bone in rats of different weights*

The values for cartilage are expressed per dry weight, and those for rib bone per defatted dry weight. For experimental details, see the text. The values for rats weighing 15–40g. are the means for 15 rats; other values are for single rats.

Weight of rat (g.)	Percentage of ash	
	Costal cartilage	Rib bone
15–40	3.5	50
42	4.0	50.5
44	5.0	50.0
48	6.8	51.0
54	11.1	51.0
60	13.5	56.0
72	20.4	58.0
84	24.5	58.9
91	29.5	58.6
100	34.0	60.0
110	39.9	61.0
116	42.5	59.8
120	42.0	58.6
174	58.7	66.8
279	60.4	63.8
305	62.6	68.1

Table 2. *Calcium, magnesium and inorganic phosphate concentrations in costal cartilage from rats of different weights*

Ca/P molar ratios are also shown. For experimental details, see the text.

Weight of rat (g.)	Calcium content (mg./g. dry wt. of tissue)	Magnesium content (mg./g. dry wt. of tissue)	Inorganic phosphate content (mg./g. dry wt. of tissue)	Ca/P molar ratio
20	2.8	0.79	3.63	0.60
25	2.8	0.79	3.63	0.60
32	1.6	0.70	2.22	1.08
34	4.0	0.72	3.76	0.82
38	5.0	0.73	3.78	1.03
46	17.2	0.86	9.12	1.46
48	29.0	1.27	13.7	1.64
55	18.4	0.91	9.63	1.48
61	38.8	1.10	19.1	1.58
72	64.4	1.49	30.0	1.65
80	69.2	1.63	33.7	1.58
91	85.0	2.00	39.6	1.66
120	106.1	2.45	49.5	1.66

citrate and fluoride concentrations with increasing rat weight in cartilage from the fluoride-treated and control rats are shown in Figs. 1 and 2 respectively. No fluoride was detected in bone or cartilage in the samples from the control animals. Fluoride was incorporated at a rapid rate into cartilage from the treated animals, its concentration in the ash from

Fig. 1. Changes in citrate concentration in rat costal cartilage of growing control (Δ) and fluoride-treated ($\blacktriangle$) rats. For experimental details, see the text.

Fig. 2. Changes in fluoride concentration of costal-cartilage ash from growing control ($\circ$) and fluoride-treated ($\bullet$) rats. For experimental details, see the text.

rats weighing more than 100g. being higher than that in the corresponding rib bone. Citrate concentration was very low in the cartilage of rats weighing less than 40g., but in the control animals it rose rapidly with increasing rat weight. In the cartilage from the fluoride-treated animals weighing more than 40g., citrate concentration was markedly less than in the control tissues. Bone citrate concentration in fluoride-treated animals was also less than that of the controls.

DISCUSSION

Calcification of costal cartilage was not evident in the rat until it weighed about 40g. About this

time deposition of mineral commenced. Analysis of the mineral from older rats indicated that it has a calcium/phosphorus ratio the same as that found in bone mineral. The low calcium/phosphorus ratios observed in the tissue extracts from young rats (15–45g.) were probably due to the presence in the tissue of organic phosphates extracted and hydrolysed by the procedure used. Incorporation of fluoride into newly deposited mineral in cartilage as well as into the older mineral seems evident from the increasing concentration of fluoride found as the ash content increased while the animals were receiving a constant intake of fluoride in the diet. The difference between citrate concentration in cartilage from fluoride-treated rats and the controls is consistent with observations in bone mineral reported by Zipkin, Schraer, Schraer & Lee (1963). In the present work the content of citrate in rib bone was also decreased in the presence of fluoride. Since citrate is considered to be located on the surface of the mineral crystals, the marked difference in citrate concentration between fluoride-containing cartilage and control cartilage supports the X-ray diffraction data of Posner, Eanes, Harper & Zipkin (1963), in which fluoroapatite crystals were found to be larger than those of hydroxyapatite.

Costal cartilage can be studied with ease in the precalcified state and also at the initiation of calcification, which begins predictably in the rat when it weighs about 40g. The results presented here indicate that the tissue can serve as a good model for investigations of the mechanisms involved in the initiation of normal calcification *in vivo*. It should also provide a model for the study of mineral composition.

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