CCXCI. THE DEVELOPMENT OF THE CALCIFYING MECHANISM IN THE LONG BONES OF THE RABBIT.

BY JANET SIMPSON FERGUSON NIVEN¹ AND ROBERT ROBISON.

From the Strangeways Research Laboratory, Cambridge, the Pathological Department of the University and Western Infirmary, Glasgow, and the Lister Institute, London.

(Received November 1st, 1934.)

MUCH of the information which has been gained regarding the nature of the calcifying mechanism has been derived from experiments on the calcification *in vitro* of hypertrophic cartilage from the long bones of young rats or rabbits rendered rachitic by deficient diet. Since this material was abnormal in that the tissue was taken from unhealthy animals it was desirable to carry out similar experiments with healthy normal cartilage. Mammalian hypertrophic cartilage rapidly becomes calcified during normal development and cannot then be used for such experiments; but suitable material was found in the rudimentary long bones of rabbit embryos. In embryos removed from the doe 12–13 days after mating, the rudimentary femora and tibiae consist of early cartilage in which no differentiation into epiphyses and diaphysis has taken place. Between the 18th and 19th days, sections of the long bones in general show differentiation of the cartilage into a central area of hypertrophic cells merging on each side into a zone of flattened cells which are arranged in transverse rows. These in turn are succeeded by the small, round-celled chondroblasts of the future epiphyses. Round the hypertrophic cartilage the mesoderm has differentiated to form a 2-layered periosteum consisting of an inner layer of osteoblasts and an outer layer of fibroblasts. Between the former and the surface of the cartilage a layer of osteoid tissue may be found. At this stage of development the deposition of calcium salts begins in the matrix both of the osteoid tissue and of the hypertrophic cartilage. In the series examined calcification appeared, with one exception, to begin in the osteoid layer. Calcification of this layer occurs at first in a localised area and, adjacent to this, deposition begins in the cartilage and extends inwards in wedge-shaped fashion. Soon, however, the calcified periosteal bone forms a ring round the centre of the shaft, and calcification extends completely across the cartilage. In the latter it extends further towards the limits of the hypertrophic area at the periphery than in the centre of the shaft, and the zone of calcification in cartilage is thus concave at each end, giving the appearance of a cup. The histological development is accompanied by a marked rise in the phosphatase activity of the entire rudiment, as may be seen from the values of A/W given in Table I. These were determined in 1930 by the method of Fell and Robison [1930], α -glycerophosphate being used as the substrate.

¹ This work was done during the tenure of a Carnegie Research Fellowship.

Table I. *Phosphatase activity of bone rudiments.*

A = inorganic phosphate (mg. P) liberated from 0.1 *M* Na α -glycerophosphate in 24 hours at p_H 8.6 and 37°. *W* = weight of dried tissue (mg.).

Period after mating (days)	No. of embryos	Tissues	<i>W</i>	<i>A/W</i>
12 $\frac{1}{4}$	2	Hind limb buds	1.10	0.01
13 $\frac{3}{4}$	3	Hind limb buds	3.43	0.01
15 $\frac{1}{2}$	4	Tibiae and femora	1.40	0.02
19	1	Tibiae and femora	2.54	0.13

The degree of histological differentiation and of calcification varies in different bones of the same embryo, the femur and humerus always showing a more advanced stage than the tibia, fibula, radius and ulna. Moreover, the bones of embryos from the same uterus vary considerably in degrees of development; thus, the femur from one embryo showed only a thin layer of uncalcified osteoid tissue, while that from another embryo showed both calcified cartilage and periosteal bone. This variability would have rendered the material useless for the purposes of our experiments but for the fact that corresponding bones of the same embryo were found to exhibit a remarkably similar picture both morphologically and with respect to calcification.

These entire rudiments could be freed from the surrounding tissue without injury; and by preserving one as a control the other could be used to demonstrate the activity of the calcifying mechanism.

The material proved less satisfactory for the comparison of the effects of different factors on calcification since it was impossible to obtain a series of rudiments at exactly the same stage of development.

It is to be noted that no difference in the degree of development or of calcification was observed between the rudimentary bones of embryos taken from rabbits which had been deprived of direct sunlight throughout the period of gestation and those which had been kept in outside pens and thus exposed to full daylight during the summer months.

METHOD OF EXPERIMENT.

Material. The rabbit was killed by a blow on the neck, and the uterine horns were removed with aseptic precautions and transferred to sterile Petri dishes. Generally 6–7 embryos were obtained but the number varied from 2 to 10. The embryos were removed from the uterine horns and the rudimentary bones were carefully dissected out. One of each pair of rudiments was placed in neutral 10 % formalin while the other was rinsed in the experimental calcifying solution and then placed in a small stoppered tube containing 3 ml. of this solution at p_H 7.3–7.4. The tubes were kept at 37° during periods up to 22 hours, after which the p_H and concentration of inorganic phosphate were again checked. The rudiments were removed from the solution, fixed in neutral formalin for 2 hours, dehydrated in progressive concentrations of alcohol, cleared in cedar wood oil, embedded in paraffin and cut into serial sections. These were stained with silver nitrate or sodium alizarinsulphonate. The controls were treated similarly and each experimental slide and its control were stained under identical conditions.

In all, more than 120 pairs of bone rudiments from rabbit embryos were used.

Calcifying solutions. The calcifying solutions used for these experiments were similar to those described by Robison and Rosenheim [1934]. They are referred

to in this paper by figures representing the concentrations of calcium, inorganic and organic phosphate in mg. per 100 ml.; thus, solution 8:4:3 contains 8 mg. Ca, 4 mg. P as inorganic phosphate and 3 mg. P as organic phosphate per 100 ml. basal salt solution, while solution 8:4:0 contains no organic phosphate. The organic phosphate was glycerophosphoric ester.

RESULTS.

Owing to the diversity of the experimental material the results cannot be stated in tabular form. They are summarised below.

(a) *Rudiments in which the controls showed no calcification but contained clearly defined hypertrophic cartilage surrounded by a thin osteoid layer.* In these rudiments, calcification occurred *in vitro* in varying degree in both hypertrophic cartilage and osteoid tissue in solutions 8:4:0 and 8:5:0 (Plate XIII, figs. 1 and 2) and in the ester solution 8:3:3. In one rudiment calcification was observed in the osteoid tissue but not in the cartilage after immersion in solution 8:3:0. With the same solution, calcification occurred *in vitro* in both cartilage and osteoid tissue in a rudiment whose control showed calcification in osteoid tissue only.

(b) *Rudiments in which the controls showed some calcification both in the hypertrophic cartilage and osteoid layer.* In these rudiments, calcification was invariably increased in both osteoid tissue and hypertrophic cartilage after immersion in solutions ranging from 8:2:0 to 8:6:0 and from 8:3:3 to 8:4:10 (Plate XIII, figs. 3 and 4). The amount of new calcification was greatest in the inorganic solutions of high concentration and in those containing phosphoric ester. Particularly with the latter, the uncalcified "cup" of hypertrophic cartilage was often fully calcified *in vitro* to the limit of the sheath of periosteal bone.

Calcification in solutions containing protein and glucose.

Experiments were also carried out with solutions containing 7 % horse serum-proteins and 0.1 % glucose, for the preparation of which we are indebted to Dr A. H. Rosenheim. Calcification occurred in both osteoid tissue and hypertrophic cartilage at Ca $\times$ P levels of 12 $\times$ 6 and over in absence of phosphoric ester; in two rudiments there appeared to be very slight deposition at the level 12 $\times$ 4. In presence of ester, calcification at the lower level was definite and extensive (solutions 12:4:3, 12:4:10). The Ca $\times$ P levels at which calcification of these bone rudiments was observed in absence of phosphoric ester are notably lower than those found by Rosenheim [1934, 1] to effect calcification, with similar protein-glucose solutions, in the hypertrophic cartilage of rachitic rats.

The effect of acetone on the calcifying mechanism.

Earlier experiments [Shipley *et al.*, 1926; Robison *et al.*, 1930] appeared to show that the mechanism responsible for the calcification of cartilage in super-saturated inorganic solutions (the "second mechanism") was destroyed when the cartilage was treated with acetone or other organic solvents, or was desiccated. Later work [Robison and Rosenheim, 1934] suggested that this conclusion was not entirely correct, that the mechanism, though weakened, was not inactivated by such treatment and that calcification could still be brought about in the cartilage provided that the Ca $\times$ P level of the solution was sufficiently raised. The present series of experiments has provided definite confirmation of this view. Bone rudiments were immersed in acetone for 22 hours and subsequently

transferred to calcifying solutions in which they remained for a further 22 hours at 37°. Greatly increased calcification was observed in both hypertrophic cartilage and osteoid tissue after immersion in 8 : 6 : 0 (Plate XIII, figs. 5 and 6). As previously observed, calcification occurred regularly at lower levels of the product $\text{Ca} \times \text{P}$ when phosphoric ester was also present (solutions 8 : 3 : 3, 8 : 4 : 3). The phosphatase mechanism is not injured by the acetone treatment.

The effect of desiccation on the calcifying mechanism.

Calcification of osteoid tissue was observed in two bone rudiments which had been desiccated *in vacuo* and subsequently immersed in solutions 8 : 5 : 0 and 8 : 6 : 0. Both of the control rudiments were free from calcification. The second calcifying mechanism is thus not entirely destroyed by desiccation.

The effect of heat on the calcifying mechanism.

A number of experiments were carried out to investigate the effect of heat on the phosphatase and second calcifying mechanisms. The bone rudiments after removal from the embryos were placed in tubes containing 2 ml. of Ringer's solution which were heated in a water-bath at different temperatures for periods of 30 minutes. They were subsequently immersed in the calcifying solutions for 22 hours at 37°. After heating at 45° and 50° calcification still occurred *in vitro* in both inorganic and ester solutions (8 : 5 : 0, 8 : 3 : 10). After heating at 55° the phosphatase mechanism appeared to be inactivated, no increased calcification being observed in solution 8 : 3 : 10. In several rudiments which had been heated at 55° and 60°, evidence of increased calcification was noted after immersion in the inorganic solution 8 : 5 : 0, indicating apparently that the inorganic mechanism still survived. This last result is not in agreement with those of similar experiments carried out with bone slices from rachitic rats, from which it appeared that both the phosphatase and second mechanisms are inactivated by heating for 10 minutes at 60° [Robison and Rosenheim, 1934]. The discrepancy cannot at present be explained.

DISCUSSION.

In the rudimentary long bones of the rabbit both hypertrophic cartilage and osteoid tissue normally become calcified soon after their first appearance. The evidence of the experiments described in this paper goes to show that both these tissues possess the calcifying mechanism before a visible deposit can be demonstrated, although the mechanism may not have acquired its full activity. The rapid development of the calcifying mechanism in the hypertrophic cartilage is in marked contrast to the delayed appearance of this mechanism in the corresponding tissue of the long bones of the fowl [Fell and Robison, 1934].

Deposition of calcium salts may be brought about in the as yet uncalcified hypertrophic cartilage and osteoid tissue of rabbit long bone rudiments by immersion in solutions supersaturated with the bone salt, and such deposition is increased in the presence of phosphoric ester. With suitable solutions deposition may extend almost to the limit of the hypertrophied cartilage and osteoid tissue.

It is probable that the activity of the calcifying mechanism in this healthy tissue is greater than in the hypertrophic cartilage of rats which have been kept for some weeks on a rachitogenic diet, since calcification occurs in inorganic solutions at lower levels of the product $\text{Ca} \times \text{P}$ (8×2) than are usually effective

with the latter material [Rosenheim, 1934, 2]. Calcification was also observed at relatively low $\text{Ca} \times \text{P}$ levels in solutions containing protein and glucose.

The calcifying mechanism has been empirically divided into two components, *viz.* the phosphatase and the second, or inorganic, mechanism, although the likelihood of their intimate relationship is recognised. Our experiments confirm the conclusion [Robison and Rosenheim, 1934] that the inorganic mechanism as well as the phosphatase can still function after the tissues have been soaked for 22 hours in acetone or desiccated.

The experiments with rudiments heated to different temperatures have provided some unexpected but interesting results. Previous work on cartilage from rachitic rats had failed to reveal any marked difference between the rates of inactivation of the two mechanisms by such treatment, both being rapidly destroyed at temperatures between 55° and 60° . But, in the embryonic bone rudiments the second mechanism, though not the phosphatase, appeared to be still active after the tissue had been heated at 60° for 30 minutes. We are not disposed to accept this conclusion as necessarily correct, but the experimental result has some significance which remains to be discovered.

SUMMARY.

1. The development of the calcifying mechanism in normal embryonic cartilage and osteoid tissue of rabbit long bones has been investigated.

2. The hypertrophic cartilage and osteoid tissue become calcified *in vivo* very soon after their formation, and the presence of the calcifying mechanism has been demonstrated even before deposition of calcium salts has occurred.

3. The activity of the calcifying mechanism in these normal tissues appears to be greater than in the hypertrophic cartilage of rats which have been fed for several weeks on a rachitogenic diet.

4. The effects of acetone treatment, desiccation and heat on the calcifying mechanism have been investigated. The conclusion that the inorganic mechanism as well as the phosphatase can still function in these tissues after acetone treatment or desiccation has been confirmed.

It is with pleasure that we acknowledge our indebtedness to the Staff of the Serum Department of the Lister Institute, Elstree, for their help in these experiments.

One of us (J. S. F. N.) is indebted to the Medical Research Council for a grant towards the expenses of this work.

REFERENCES.

- Fell and Robison (1930). *Biochem. J.* **24**, 1905.
— — (1934). *Biochem. J.* **28**, 2243.
Robison, Macleod and Rosenheim (1930). *Biochem. J.* **24**, 1927.
— and Rosenheim (1934). *Biochem. J.* **28**, 684.
Rosenheim (1934, 1). *Biochem. J.* **28**, 699.
— (1934, 2). *Biochem. J.* **28**, 708.
Shipley, Kramer and Howland (1926). *Biochem. J.* **20**, 379

DESCRIPTION OF FIGURES IN PLATE XIII.

Longitudinal sections of rudimentary long bones of rabbit embryos removed from the doe 18 days after mating. One of each pair of corresponding bone rudiments was fixed directly as a control, the other being immersed for 22 hours in the calcifying solution at p_H 7.4 and 37°. Magnification $\times 55$. *C.H.C.*, calcified hypertrophic cartilage; *C.O.*, calcified osteoid tissue; *U.H.C.*, uncalcified hypertrophic cartilage; *U.O.*, uncalcified osteoid tissue; *Z.F.C.*, zone of flattened cells.

- Fig. 1. Tibia control for Fig. 2, stained with silver nitrate and carmalum. Note the central region of hypertrophic cartilage as yet uncalcified, bounded on each side by a zone of flattened cells. A very thin sheath of uncalcified osteoid tissue bounds the hypertrophic cartilage on one side only.
- Fig. 2. Tibia from the same embryo as that in Fig. 1, immersed for 22 hours in the inorganic calcifying solution 8 : 4 : 0. The osteoid tissue on one side of the shaft has been calcified, while calcification extends throughout a large part of the hypertrophic cartilage. The deposit is confined to the matrix as in normal development.
- Fig. 3. Tibia control for Fig. 4, stained with silver nitrate and carmalum. Note the central area of hypertrophic cartilage with slight calcification in the matrix surrounded by osteoid tissue which is only partially calcified.
- Fig. 4. Tibia from the same embryo as Fig. 3, immersed for 22 hours in the ester solution 8 : 4 : 3. Calcification is greatly increased in osteoid tissue and hypertrophic cartilage and now extends almost to the limit of the latter zone.
- Fig. 5. Humerus control for Fig. 6, stained with sodium alizarinsulphonate. Note the central area of hypertrophic cartilage entirely free from calcification, surrounded by a thin sheath of osteoid tissue which is calcified to a very slight extent.
- Fig. 6. Humerus from the same embryo as that in Fig. 5, soaked in acetone for 22 hours at room temperature and then rinsed and immersed for a further 22 hours at 37° in the inorganic calcifying solution 8 : 6 : 0. Further calcification has occurred *in vitro* in the periosteal sheath of osteoid tissue while the matrix of the hypertrophic cartilage has been heavily calcified.