CCXVI. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

VIII. CALCIFICATION *IN VITRO*.

By ROBERT ROBISON and KATHARINE MARJORIE SOAMES.

From the Biochemical Department, The Lister Institute, London.

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In the first two papers of this series [Robison, 1923; Robison and Soames, 1924] it was shown that when bones of rachitic rats were immersed in solutions of calcium hexosemonophosphate or glycerophosphate, a dense deposit of calcium phosphate was formed in the matrix of the hypertrophic cartilage, where calcification would have occurred *in vivo* if the rats had been fed on a normal diet. The location of the bone phosphatase and its power to effect calcification were thus demonstrated, since only by the agency of this enzyme could the insoluble calcium phosphate have been produced from the phosphoric ester. It was further suggested that a second mechanism might also play a part in normal calcification by bringing about a slight increase in the p_H of the matrix fluid, thereby increasing the concentration of PO_4 and CO_3 ions, and also the activity of the enzyme.

Shipley [1924] found that slices of cartilage and bone of a rachitic rat became calcified when placed in the serum of normal rats and kept at 37° for a number of hours, and later it was shown by Shipley, Kramer and Howland [1926] that calcification could be effected by immersing such bone slices in solutions of inorganic salts, provided that "the concentrations of the bone-forming constituents and the reactions are nearly those of normal serum." In a reply [Robison, 1926] to the criticism of the enzyme hypothesis by these authors it was pointed out that the apparent similarity of the concentrations of calcium and inorganic phosphate in their calcifying solutions with those in normal serum was misleading, owing to the absence from these solutions of proteins and other organic constituents, which greatly depress the ionisation of calcium in plasma. These solutions were, indeed, highly supersaturated with respect to the bone salt, but the fact that calcification, normal in character, occurs when such solutions are allowed to diffuse into the cartilage is none the less of great significance.

Our own experiments[1] confirm the results of Shipley, Kramer and Howland

[1] The results described in this paper were communicated to the Biochemical Society in Feb 1928 [1928].

in nearly all respects. These authors found that calcification was obtained with salt solutions for which the value of [Ca] × [P] was 35, while in the experiments described in the present paper calcification did not occur with values of [Ca] × [P] below 40 and only rarely at this level. This apparent divergence in our results is probably accounted for by the great difference in the concentrations of sodium chloride in the solutions used. According to a footnote in a later paper by Shipley and Holt [1927] the concentration of sodium chloride in the solutions used by Shipley, Kramer and Howland was only 0·013 *M*, and not 0·1 *M* as stated in the original description of their experiments. The later work of Shipley and of Kramer and their collaborators and the experiments described in Part IX of this series [Robison, Macleod and Rosenheim, 1930] show that the level of the product [Ca] × [P] required for calcification depends to a considerable extent on the concentrations of sodium chloride and of the other salts present in the solution.

The experiments described in the present paper deal with the effect of phosphoric ester on calcification *in vitro* in solutions containing calcium and inorganic phosphate in low concentrations. They prove that while calcification does undoubtedly take place in absence of phosphoric ester, provided the value of [Ca] × [P] is sufficiently high, a much lower level of this product is required when phosphoric ester is present even in very small amounts.

Method of experiment.

For these experiments we used bones of young rats and of rabbits which had been fed for three to four weeks on a rickets-producing diet (McCollum 4026, 3143 or Steenbock 2965). The animals were killed by a blow on the neck and the bones were removed with precautions to avoid bacterial contamination. The distal heads of the femora and the proximal heads of the tibiae were used, the latter being preferred on account of the broad regular zone of epiphysial cartilage. The head of the bone was either cut in half longitudinally (as in our former experiments), one half being placed in each solution, or into a number of thinner longitudinal slices. For this purpose a sterile safety razor blade was found convenient. One half-head or slice was stained with silver nitrate and examined for calcification due to healing. Experiments in which such healing was observed in the controls are not included in the table.

The basal solution used in most of these experiments had the following composition:

	g. per 100 cc.	*mM*
NaCl	0·6	103
$NaHCO_3$	0·03	3·6
KCl	0·04	5·4
$MgSO_4$, $7H_2O$	0·025	1·0
Phenol red	0·0003	—

In the experiment shown in Table I the solution contained 0·9 % NaCl and in Exp. 1 and 2 of Table II the solutions contained 0·7 % NaCl, but were otherwise similar to the above.

A stock solution was made up of twice this salt concentration and diluted after addition of calcium chloride, potassium phosphate and glycerophosphoric ester as required to give the concentrations of calcium, inorganic P and organic P stated in the tables. The methods of preparation and sterilisation of these solutions and the adjustment of p_H are described in the next paper [Robison, Macleod and Rosenheim, 1930]. The half-heads or bone slices were immersed in the solutions, previously warmed to 38°, for the stated periods and were then removed, fixed in 10 % neutral formalin, washed, and stained by von Kossa's silver nitrate method. The extent of new deposit, if present, was judged by examining the slice under low magnification and is shown in the Tables by a numeral, the maximum value, 10, indicating that the deposit extended over the entire area of hypertrophic cartilage. In the later experiments the slices were dehydrated and cleared before examination.

Results.

Table I shows the results of an experiment in which slices from the femora and tibiae of the same rachitic rat were immersed in solutions containing constant concentrations of calcium and of inorganic phosphate but different concentrations of phosphoric ester. Each slice was placed in a small stoppered flask, containing 40 cc. of the sterile solution at p_H 7·35, and kept at 38° during 16 hours. The extent to which calcification occurred *in vitro* is approximately indicated by the number in the last column, and is shown in the photomicrographs reproduced in Figs. 1–6 (Plate X).

Table I.

Solution	mg. per 100 cc. Ca	Inorg. P	Org. P	Relative extent of new calcification (max. 10)
A	10	3·5	0	0
B	10	3·5	0·5	1
C	10	3·5	1	4
D	10	3·5	2	6
E	10	3·5	4	8

No calcification occurred in any of the slices immersed in solution *A*, which contained no phosphoric ester. The addition of glycerophosphate equivalent to only 0·5 mg. organic P per 100 cc. caused some calcification to occur, while the extent of the deposit was increased with each further increase in the amount of ester.

Apart from the store of hydrolysable phosphoric esters in the red corpuscles such esters have been shown to occur in plasma in amounts usually between 0·2 and 0·5 mg. P per 100 cc. but sometimes exceeding this limit [Martland and Robison, 1926].

In drawing conclusions from the results of this and similar experiments, objection might be raised to one point. During the immersion of the bone slices in the solution containing phosphoric ester, a slight rise occurred in the concentration of inorganic phosphate in the external solution, due partly to

diffusion of enzyme from the bone and partly to diffusion of some of the phosphate set free by enzyme hydrolysis within the cartilage. The rise did not usually exceed 0·5 mg. P per 100 cc. and occurred chiefly towards the end of the experiment. It was, moreover, due directly to the phosphatase activity. It was desirable, nevertheless, to avoid confusing the argument by these variations in the composition of the surrounding fluid. We therefore carried out a number of experiments in which the sterile solutions were caused to pass in a slow continuous stream over the bones in the apparatus shown in Fig. 1.

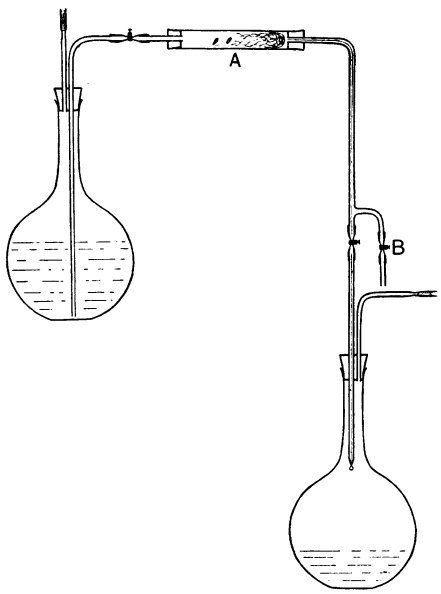

Fig. 1. *A*, tube containing bone slices and glass wool plug; *B*, outlet for withdrawal of samples of the solution.

Two similar sets of apparatus were used, each containing 2 litres of solution which was allowed to flow over the bones during 20 to 48 hours, the temperature being maintained at 38°. Tests carried out on samples of the solution which had passed over the bones showed that the concentration of inorganic phosphate had not risen appreciably but that phosphatase continued to diffuse out of the bones at an increasing rate throughout the whole period. The total phosphatase activity of the solution in the lower flask was so great as to suggest that the living cartilage cells were continuing to produce and secrete the enzyme, but further experiments did not confirm this. The continuous loss of enzyme must involve a severe handicap to the phosphatase mechanism. Further, the concentration of inorganic phosphate was always made higher in the inorganic solution than in the solution containing phosphoric ester. Nevertheless, the results given in Table II show that calcification is regularly obtained in presence of ester at levels of calcium and inorganic phosphate which produce no calcification when ester is absent.

Table II.

The concentrations of Ca, inorganic P and organic P are given in mg. per 100 cc. The inorganic solution contained no phosphoric ester. The letters *h* and *s* in column 3 indicate half-heads of bones, and slices, respectively. Where more than one value is shown for calcification these refer to the separate bone slices immersed in the same solution.

					Inorganic solution			Organic solution			
Exp.	Animal		p_H	Duration hrs.	Ca	Inorg. P	Calcification (max. 10)	Ca	Inorg. P	Org. P	Calcification (max. 10)
1	Rat	*h*	7·2	22	10	4	0	10	3·8	3	5
2	„	*h*	7·4	18	10	4	0	10	3·5	6	10
38	„	*s*	7·35	28	10	4	0, 0	10	3·5	3	5, 5
41	„	*s*	7·3	24	10	3·6	0, 0	10	3·2	3	8, 9
		h	7·3	24	10	3·6	0	10	3·2	3	8
46	„	*s*	7·3	24	—	—	—	10	2·8	3	3
								10	2·8	9	9
24	Rabbit	*s*	7·3	22	10	5·5	10, 10, 10	10	5	4	10, 10, 10
7	„	*s*	7·4	18	10	4	2, 2, 1, 0	10	3·5	3	4, 3, 3, 3
3	„	*s*	7·35	18	10	4	0, 0, 0	10	3·5	3	10, 3, 2
13	„	*s*	7·3	24	10	3·75	0, 0, 0, 0	10	3·5	3	10, 10, 10, 10
4	„	*s*	7·3	18	10	3·5	0, 0, 0	10	3	3	4, 0
26	„	*s*	7·4	18	7·5	4·5	0, 0, 0, 0	7·5	4	9	6, 5, 3, 2
8	„	*s*	7·5	27	7·5	4	0, 0, 0	7·5	3·5	3	4, 0, 0
14	„	*s*	7·3	48	7·5	3·75	0, 0, 0	7·5	3·5	3	7, 3, 1
15	„	*h*	7·3	48	7·5	3·75	0	7·5	3·5	1	2
21	„	*s*	7·4	24	5	5	0, 0	5	4·5	3	2, ½
18	„	*h*	7·2	24	5	5	0	5	4	3	1

Summary.

It is shown that while calcification *in vitro* may occur in solutions of inorganic salts supersaturated with respect to the bone salt, calcification will take place with lower concentrations of calcium and inorganic phosphate if phosphoric ester is also present, even in very small amount.

References.

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DESCRIPTION OF FIGURES IN PLATE X.

Figs. 1–6. Slices cut from the distal heads of the femora and proximal heads of the tibiae of a rachitic rat. The slices were immersed 16 hours at 37° in solutions all containing 10 mg. Ca and 3·5 mg. P as inorganic phosphate per 100 cc., but containing different concentrations of glycerophosphoric ester. Slices stained by von Kossa's silver nitrate method and photographed, without clearing, by reflected light. Mag. × 11.
X = new deposit of calcium salts in the zone of hypertrophic cartilage (see Table I).

Fig. 1.	Femur	slice in solution	*A*,	Phosphoric ester	≡0	
Fig. 2.	„	„	„	*B*,	„	≡0·5	mg. P per 100 cc.
Fig. 3.	„	„	„	*C*,	„	≡1	„
Fig. 4.	Tibia	„	„	*A*,	„	≡0	
Fig. 5.	„	„	„	*D*,	„	≡2	„
Fig. 6.	„	„	„	*E*,	„	≡4	„

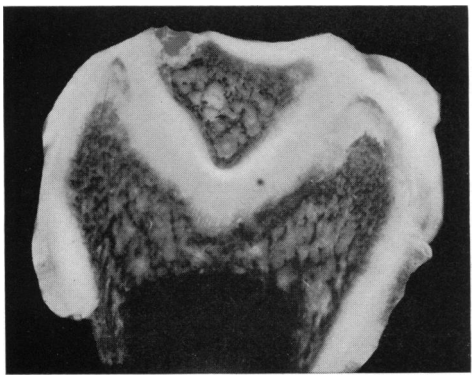


Fig. 1.

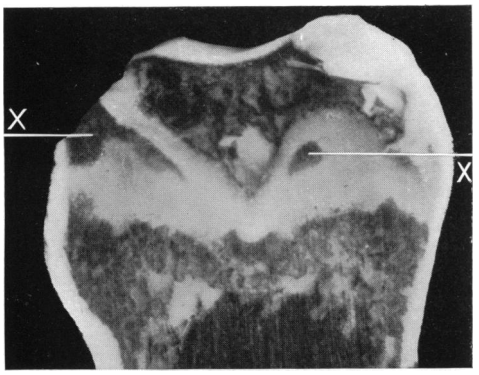
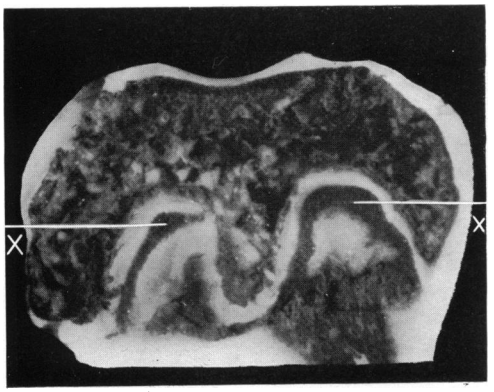


Fig. 2.



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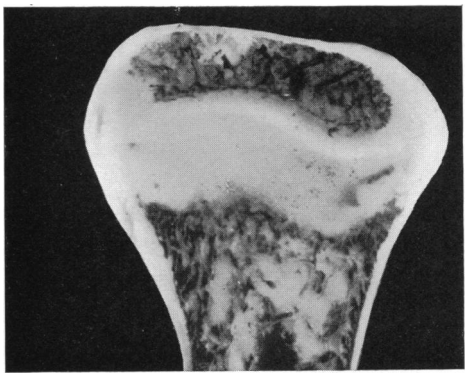

Fig. 4.

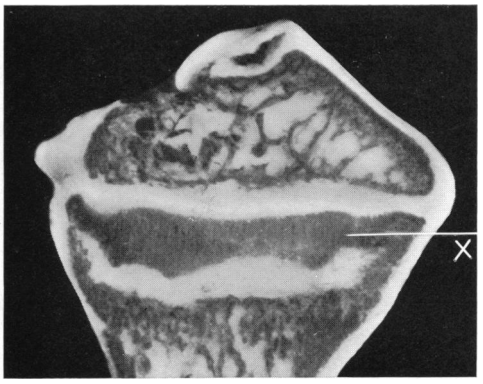

Fig. 5.

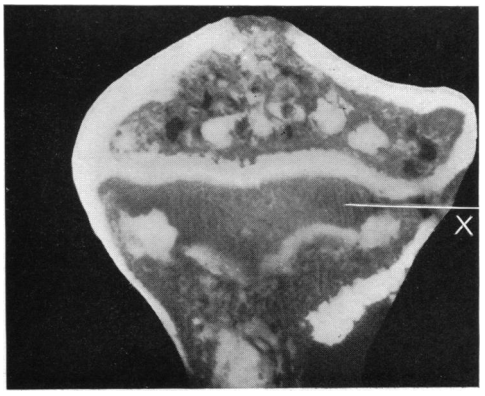

Fig. 6.