CCXVII. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

IX. CALCIFICATION *IN VITRO*.

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IN the preceding paper [Robison and Soames, 1930] it was shown that, although bone slices from rachitic animals will calcify in solutions of inorganic salts supersaturated with respect to the bone phosphate, at lower concentrations of calcium and inorganic phosphate calcification will only occur in presence of phosphoric ester. At critical concentrations of the inorganic radical as little as 0.5 mg. per 100 cc. of phosphorus in the form of ester is sufficient to determine calcification, this amount being similar to that found in plasma. The solutions used for these experiments contained no protein and no other organic constituent but phosphoric ester. The concentration of calcium ions was, therefore, higher than in plasma even when the total calcium content was the same. The solutions further differed from plasma in containing much less bicarbonate.

The experiments described in the present paper deal with the effect of bicarbonate, sodium, potassium and magnesium on calcification in protein-free solutions at different levels of calcium and inorganic phosphate, in absence and in presence of phosphoric ester. Experiments on the effect of antiseptics, of previous treatment of the bone slices with organic solvents or of desiccation *in vacuo* have also been carried out. The results of these have set in sharp distinction two mechanisms in calcifying hypertrophic cartilage, the phosphatase mechanism which can produce in the matrix a local supersaturation sufficiently great to cause deposition of the bone phosphate, and the second, still obscure, mechanism, which favours, and may determine, deposition of the bone phosphate from its supersaturated solution.

Method of experiment.

The experiments were carried out by comparing the calcification *in vitro* of slices cut under sterile conditions from the bones of rachitic rats (fed three to four weeks on McCollum diet 3143). The distal heads of the femora and the proximal heads of the tibiae and humeri were cut longitudinally by hand, 12-14 slices, about 1 mm. thick, being in this way obtained from each rat.

Solutions containing all the salts except the calcium chloride were sterilised in 100 cc. graduated flasks by heating for 30 minutes in steam. The p_H was adjusted by allowing carbon dioxide to diffuse through the cotton wool plug, or by adding sterile acid or alkali when the solution contained no bicarbonate. The requisite amount of sterile calcium chloride solution was then added, the volume made up to the mark and the plugs replaced by rubber stoppers.

In most of the experiments one or more bone slices were placed in each flask and kept at 37° for the stated period. The inorganic phosphate in the solutions was determined at the beginning and end of each experiment. In absence of phosphoric ester the initial and final values did not differ appreciably except in rare cases when general precipitation of calcium phosphate occurred. In presence of phosphoric ester a rise in the concentration of inorganic phosphate was observed. The increase varied with the duration of the experiment, the size of the bone slices and the concentration of ester, and was due partly to hydrolysis of ester in the external solution by phosphatase diffusing out of the bone, and partly to hydrolysis of ester within the bone and subsequent diffusion into the surrounding solution of the inorganic phosphate which escaped precipitation. This increase was usually between 0.1 mg. and 0.5 mg. P per 100 cc., but sometimes exceeded the latter amount when the concentration of phosphoric ester was high. The lower initial level of inorganic phosphate in these "organic" solutions ensured that in spite of this increase the final concentration never rose above that in the corresponding "inorganic" solutions in the same experiment.

A modification of the large constant-flow apparatus previously described was used for experiments in which it was important to avoid any change in the level of inorganic phosphate. With this modified apparatus the calcifying power of twelve different solutions could be compared simultaneously, 100 cc. of each solution being made to flow over a bone slice during a period of about 16 hours at 37°. Each slice was placed in a small bulb tube connected on one side with the vessel containing the solution and on the other, by a length of rubber tubing, with a glass capillary outlet. The twelve outlets, fixed together, were lowered at a constant rate by clockwork mechanism, a continuous flow of solution over each slice being thus maintained. The p_H was kept between 7.3 and 7.4 by equilibrating the solutions with air containing 5 % carbon dioxide, supplied to each vessel from a large aspirator.

When the bone slices were removed from the calcifying solutions they were placed in 10 % neutral formalin (2 hours), washed and placed in 2 % silver nitrate solution (2 hours). After thorough washing in distilled water they were blackened by exposure to light, dehydrated with acetone, cleared in cedar wood oil and examined with low magnification under a binocular microscope. The approximate extent and density of the new deposit is indicated in the tables by a number and by bars, the numbers (maximum 10) showing the area of the zone of hypertrophic cartilage calcified *in vitro*, and the bars (maximum 5) the density of the new deposit. Thus the maximum mark 10≡ assigned to

any slice would indicate that the new deposit extended throughout the entire area and thickness of the zone of hypertrophic cartilage previously uncalcified. One slice from each bone was fixed at the time of cutting and examined to ascertain the extent of the zone and its freedom from patches of calcification due to healing rickets which, if present, invalidated the experiment. In humeri and femora, after immersion in the calcifying solutions, a cap-like zone of newly calcified matrix was sometimes observed in the deeper layers of the cartilage forming the articular surface of the epiphysis, the appearances in this zone reproducing on a smaller scale those seen in the metaphysis. In control slices of these bones the zone of hypertrophic cartilage present in this region was entirely uncalcified. The new deposit in the epiphysis, as seen in thin section, is illustrated in Plate XI, fig. 1. Calcification in this area was not taken into account in marking the extent of the deposit in the experimental slices.

As the size of the different bones and the area of the uncalcified zone varied considerably, endeavour was made to use slices from the same bone for the most critical comparisons in any experiment. Where more than one rat was used the results obtained with bone slices from the different animals are given in separate columns.

Effect of bicarbonate.

The results of six experiments are set out in Table I, showing the effect of bicarbonate in different concentrations on calcification *in vitro*. Four basal solutions, *A*, *B*, *C* and *D* were used, their composition being given at the head of the Table. They were made up in concentrated form and diluted after the addition of phosphoric ester, inorganic phosphate and calcium in the amounts stated, these constituents being added as solutions of sodium glycerophosphate, potassium dihydrogen phosphate neutralised with sodium hydroxide, and calcium chloride prepared from Iceland spar. Solution *A* contained bicarbonate in concentration similar to that in plasma, solution *B* contained the much smaller amount used in previous experiments, while *C* and *D* contained none. As this omission involved a reduction in the sodium content, extra sodium chloride was added to solution *D* to bring the sodium concentration up to that in solution *A*.

The results of these experiments, and of seventeen others not quoted in the table, show that in absence of phosphoric ester calcification regularly takes place at lower concentrations of calcium and inorganic phosphate (that is, at lower levels of the product $[Ca] \times [P]$), when bicarbonate is present than when it is absent. In solutions containing no bicarbonate calcification occurred at 8 : 6 : 0¹ (Exp. 183) but only in one slice out of twelve at 8 : 5 : 0 and never at levels lower than this. In bones from the same animal calcification in presence of 0.03 % bicarbonate was more extensive at the above levels and also occurred at 8 : 4.5 : 0 (Exp. 147). In presence of 0.2 % bicarbonate the extent and density of the new deposit were still greater, while calcification occurred at

¹ The concentrations of calcium, inorganic P and organic P respectively in mg. per 100 cc. are thus indicated throughout the text.

levels as low as 6:5:0 and 8:4:0. Calcification failed at 6:4:0 but was observed at 10:2.5:0 in one experiment not shown in the table.

Table I.

					Solution A	Solution B	Solution C	Solution D			
NaHCO ₃ g. per 100 cc.					0.2	0.03	.	.			
NaCl "					0.5	0.5	0.5	0.65			
KCl "					0.03	0.03	0.03	0.03			
MgSO ₄ , 7H ₂ O "					0.02	0.02	0.02	0.02			
mg. per 100 cc.											
Exp.	Time hrs.	P									
		Ca	P inorg.	P org.							
99*	17	6	4	0	0	.	.	0			
		6	5	0	1=	.	.	0			
		6	6	0	2=	.	.	0			
		6	4	3	2=	.	.	0			
		6	5	3	4=	.	.	0			
114	17	10	4	0	3=	.	.	0			
		10	2.5	3	3=	.	.	0			
		10	2.5	10	8=	.	.	6=			
134	17	8	5	0	3=	.	0	0			
		8	4.5	3	7=	.	4=	4=			
					Rat a Rat b		Rat a Rat b		Rat a Rat b		
146	10	8	4.5	0	1=	3=	.	.	0	0	.
		8	5	0	2=	7=	.	.	0	0	.
		8	4.5	1	1=	7=	.	.	0	0	.
		8	4.5	3	5=	9=	.	.	1=	4=	.
		8	4.5	10	8=	7=	.	.	7=	7=	.
		8	4.5	30	8=	9=	.	.	8=	9=	.
147	23	8	4	0	6=	4=	0	0	0	0	.
		8	4.5	0	4=	8=	2=	3=	0	0	.
		8	5	0	6=	5=	4=	4=	2=	0	.
183	16	8	5	0	5=	6=	3=	0	0	0	.
		8	6	0	7=	6=	5=	5=	2=	2=	.

* An asterisk after the number of an experiment signifies that the constant-flow apparatus was used.

These results suggest that a less soluble calcium salt is formed in presence of bicarbonate than in bicarbonate-free solutions, and thus provide some evidence that the deposit normally formed in calcifying cartilage is a complex carbonato-phosphate (carbonato-apatite), rather than a mixture of hydroxy-apatite and calcium carbonate.

In presence of phosphoric ester in low concentration calcification occurred at lower levels of inorganic phosphate in all solutions, but the favourable effect of bicarbonate was still noticeable. The effect was masked when the concentration was raised to 30 mg. P per 100 cc., the calcification being then nearly maximal in both solutions (Exp. 146).

Solution A was used as the basal solution for all further experiments described in this paper, the necessary modifications being made when the effect of magnesium, potassium or sodium salts was being investigated.

The concentrations of the basic and acidic radicals calculated for the

solution 10 : 4 : 0 are given below. Those of sodium, magnesium and chloride are somewhat below the average values for normal human plasma.

	g. per litre	Millimols per litre
Na	2.53	105.8
K	0.20	5.3
Mg	0.02	0.8
Ca	0.10	2.5
Cl	3.35	87.0
HCO ₃	1.45	24.0
SO ₄	0.08	0.8
PO ₄	0.126	1.3

Effect of sodium chloride.

Shipley and Holt [1927, 1 and 2], using solutions containing inorganic salts alone, found that sodium chloride in concentrations even lower than occur in blood has an inhibitory effect on calcification. Our own experiments confirm this. The results given in Table II show that calcification occurred at the low level 8 : 2.5 : 0 in absence of sodium chloride but was inhibited by this salt in 0.2 % concentration. At 8 : 3 : 0 calcification was obtained with solutions containing 0.2 % sodium chloride but was inhibited by 0.5 %. At higher levels of inorganic phosphate (8 : 4 : 0 and 8 : 4.5 : 0) calcification occurred in all solutions but to a greater extent in those containing 0.2 % sodium chloride than in those containing 0.5 %. In the solutions containing no sodium chloride general precipitation took place at these levels. In presence of phosphoric ester the inhibitory effect of sodium chloride was still discernible but was less marked owing to the much greater calcification obtained in all solutions. This experiment, indeed, shows very clearly the pronounced effect of phosphoric ester on calcification.

Table II.

Basal solution: solution A without sodium chloride.

Exp.	Time hrs.	mg. per 100 cc.			NaCl g. per 10 cc.		
		Ca	P inorg.	P org.	0	0.2	0.5
174	16	8	2.5	0	5=	0	0
		8	3.0	0	2=	2=	0
		8	2.5	3	7≡	7≡	6=
		8	2.5	10	9≡	8≡	8=

Effect of potassium and magnesium salts.

Shipley and Holt [1927, 1] found that the effect of potassium chloride on calcification was similar to, though somewhat less than, that of sodium chloride, but no inhibition was observed with such low concentrations of potassium as are present in plasma. They found, however, that magnesium in very low concentrations (less than 1 *mM*) inhibited calcification, a conclusion also reached by Kramer, Shelling and Orent [1927]. Our own experiments with potassium (Table III) were confined to concentrations not exceeding 10 *mM* (twice the concentration in plasma) and no definite inhibitory effect was observed, either

in inorganic solutions or in presence of phosphoric ester. On the other hand, 2 mg. magnesium per 100 cc. (0.84 mM) had an adverse effect on calcification and with twice this concentration inhibition was pronounced in experiments of 5 hours' and 19 hours' duration. It was apparent also in presence of small amounts of phosphoric ester. With higher concentrations of the ester the effect of magnesium was masked.

Attempts to obtain deposits of magnesium phosphate in bone slices by immersion in solutions free from calcium but containing magnesium in high concentration (up to 48 mg. per 100 cc.) were unsuccessful.

Table III.

Basal solution: solution A without potassium and magnesium salts.

Exp.	Time hrs.	mg. per 100 cc.											
		Ca	P inorg.	P org.	K Mg	5 0	5 4	20 0	20 2	20 4	0 .	} mg. per 100 cc.	
76	5	10	4	0	3≡	0	0	3≡	2≡	1≡	.		
		10	3.5	10	7≡	2≡	8≡	7≡	5≡	.	.		
					K 0 Mg 0	0 2.4	0 4.8	19.5 0	19.5 2.4	19.5 4.8	.	} mg. per 100 cc.	
150	19	8	4	0	5≡	2≡	0	6≡	4≡	0	.		
		8	5	0	6≡	6≡	2≡	6≡	4≡	0	.		
151	19	8	3.5	3	3≡	1≡	1≡	8≡	3≡	1≡	.		
		8	3.5	30	9≡	7≡	6≡	6≡	9≡	7≡	.		
152	19				K 0 Mg 0	19.5 0	39 0	0 2	39 2	.	.	} mg. per 100 cc.	
		10	3	0	3≡	7≡	4≡	.	.	.	.		
		5	6	0	4≡	4≡	4≡	.	.	.	.		
		5	8	0	4≡	4≡	5≡	.	.	.	.		
158	18	10	2.5	3	.	.	.	8≡	3≡	.	.		
		5	5	3	.	.	.	9≡	5≡	.	.		
		2.5	10	3	.	.	.	0	0	.	.		
		2.5	10	30	.	.	.	4—	5≡	.	.		

*Calcification at different levels of calcium, inorganic
phosphate and phosphoric ester.*

Table IV shows the calcification obtained with concentrations of calcium between 2.5 and 50 mg. per 100 cc. and of inorganic phosphate between 0 and 20 mg. per 100 cc. in absence of phosphoric ester. It will be seen that the lowest levels for calcification in solutions containing phosphate were 2.5 : 14, 5 : 7, 10 : 3, 20 : 1, giving products of 35, 35, 30, 20 respectively. These results would appear to show that calcification occurs at lower levels of the product $[Ca] \times [P]$ when the concentration of calcium is high and that of inorganic phosphate is low than when the conditions are reversed. This is not inherently improbable since, for example, if the deposited salt were $Ca_3(PO_4)_2$, supersaturation of the solution would depend on the product $[Ca^{++}]^3 \times [PO_4^{--}]^2$. It will be seen that when the concentration of calcium was 20 or more mg. per 100 cc., small deposits were sometimes obtained in solutions containing no phosphate. Presumably these deposits consisted of calcium carbonate unless

phosphate was taken from the bone slice itself, either from the cells or from the calcified portions. The latter possibility, which might be of some interest in connection with resorption and re-deposition of the inorganic material during bone growth, is being further investigated.

Table IV.

Basal solution A. 16 hrs.

P mg. per 100 cc.	Ca mg. per 100 cc.													
	2.5		5		10		20			30		40		50
0	.	.	.	.	.	0	0	1—	0	0	1=	1—	0	2=
1	.	.	.	.	.	.	2—	0	1=	1=	3=	3=	.	2=
1.25	.	.	.	.	.	.	2—	.	.	.	.	.	.	.
1.5	.	.	.	.	.	.	.	+	3≡	.	3≡	+	.	.
2	.	.	.	.	.	.	.	5=	4≡	+	.	.	.	.
2.5	.	.	.	.	.	0	.	8≡	+	.	.	.	.	.
3	.	.	.	.	2—	2=	.	.	.	.	.	.	.	.
3.5	.	.	.	.	3=	4≡	.	.	.	.	.	.	.	.
4	.	.	.	0	3=	4≡	.	.	.	.	.	.	.	.
5	.	.	.	0	.	4≡	.	.	.	.	.	.	.	.
6	.	.	0	0	.	.	.	.	.	.	.	.	.	.
7	.	.	4—	.	.	.	.	.	.	.	.	.	.	.
8	.	0	3=	5=	.	.	.	.	.	.	.	.	.	.
10	.	0	.	7≡	.	.	.	.	.	.	.	.	.	.
12	0	0	.	.	.	.	.	.	.	.	.	.	.	.
14	1—	.	.	.	.	.	.	.	.	.	.	.	.	.
16	4—	2—	.	.	.	.	.	.	.	.	.	.	.	.
20	.	2=	.	.	.	.	.	.	.	.	.	.	.	.
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>c</i>	<i>a</i>	<i>d</i>	<i>e</i>	<i>d</i>	<i>e</i>	<i>d</i>	<i>e</i>	<i>e</i>

Five rats, *a*, *b*, *c*, *d* and *e*, were used in these experiments, the results in any columns being obtained with bone slices from the rat specified at the foot of that column.

+ signifies that general precipitation occurred in the solutions.

Table V.

Basal solution A.

Exp.	Time	mg. per 100 cc.		Glycerophosphoric ester mg. per 100 cc.			
		Ca	Inorg. P	0	0.5	1	3
88*	16	10	3	2≡	4≡	7≡	5≡
97*	16	10	3	1=	.	.	4≡
		8	4	1=	.	.	5≡
		6	4	0	.	.	1=
		6	3	0	.	.	1≡

Table V shows the effect of phosphoric ester in low concentrations on calcification at different levels of calcium and inorganic phosphate. Both the experiments here quoted were carried out in the constant-flow apparatus so that the concentration of inorganic phosphate in the solutions surrounding the bone slices did not appreciably alter. At the level 10×3 , calcification occurred in absence of ester but was definitely increased in presence of glycerophosphate equivalent to only 0.5 mg. P per 100 cc. With 3 mg. organic P the extent and density of the deposit were still greater, while definite calcification was obtained at a $[\text{Ca}] \times [\text{P}]$ level as low as 6×3 .

The potentialities of the phosphatase mechanism as a factor in calcification have been demonstrated in yet another way, namely, by comparing the minimum time required for calcification, when this mechanism is enabled to function by addition of phosphoric ester to the solution, with that required at higher $[Ca] \times [P]$ levels but in absence of ester. The results of experiments with bone slices from four rats are shown in Table VI. Five slices from one rat were placed in a flask containing 40 cc. of the 8 : 5 : 0 solution at p_H 7.35, and five other slices from the same animal were placed in a similar quantity of solution 8 : 4 : 30. The solutions were changed hourly and the inorganic phosphate estimated, no significant increase being found. Single slices were removed at intervals, fixed and examined. The 23 hours' test was carried out separately, one slice being placed in 100 cc. of each solution which was not changed, so that at the end of 23 hours the concentration of inorganic phosphate was very nearly the same (5 mg. per 100 cc.) in both solutions. It will be seen that in presence of ester calcification was obtained in the first two hours. The deposit increased rapidly both in extent and density and was almost maximal in all slices after 10 hours. In the inorganic solutions calcification was first observed in a slice removed after 8 hours and was far from maximal even after 23 hours.

Table VI.

Basal solution A.

mg. per 100 cc.

Ca P inorg. P org.								
	8				8			
	5				4			
	0				30			
Time hrs.	Rat a	Rat b	Rat c	Rat d	Rat a	Rat b	Rat c	Rat d
2	0	.	0	.	3—	.	6—	.
3	0	0	0	.	4=	4=	8=	.
4	0	.	0	0	6=	.	8=	9=
5	.	0	.	.	.	5—	.	.
6	0	.	0	0	6=	.	7=	9=
7	.	0	.	.	.	6=	.	.
8	.	0	.	3—	.	8=	.	9=
10	4=	0	6—	2=	8=	8=	9=	9=
23	3=	0	4=	5=	9=	7=	10=	9=

The results in Table VI illustrate one of the difficulties encountered in these comparative experiments, namely, the variations in the calcification obtained under similar conditions in the bones from different rats. Rats *a* and *b* were from one litter and *c* and *d* from another. All four animals were kept for 24 days on McCollum diet 3143, and all weighed about 60 g. when killed. The exceptional failure of bone slices from rat *b* to calcify in the 8 : 5 : 0 solution is the more interesting since other slices from this rat calcified well in presence of phosphoric ester, showing that the phosphatase mechanism was unimpaired. Small variations in the response of bones from different rats were not infrequent at the levels 30–35 of the product $[Ca] \times [P]$, while, at the other extreme, a single case of calcification at the exceptionally low level, 10 : 2.5 : 0, has been

noted. The variations were much less apparent when calcification took place in presence of phosphoric ester.

Effect of hydrogen ion concentration.

Shipley, Kramer and Howland [1926], using inorganic solutions at the $[Ca] \times [P]$ level 10×3.5 , found that calcification did not occur at a p_H lower than 7.0, and Kramer, Shelling and Orent [1926] obtained the same negative result with the higher level 10×5 . This failure to calcify may, however, have been due to the increased solubility of calcium phosphate in the more acid medium; the solutions, though supersaturated at p_H 7.3, may have been unsaturated at a p_H below 7.0. By using higher $[Ca] \times [P]$ levels (10×6 , 10×7 , 10×8) we have obtained calcification at p_H 6.5, in inorganic solutions, while even better deposits were obtained at lower levels (6×4 , 8×4) in presence of phosphoric ester (Table VII). Between p_H 7.2 and 8.0 we find no marked optimum either in presence or absence of ester, but if the p_H is higher than 7.8 general precipitation often occurs at the $[Ca] \times [P]$ levels used in our experiments. The results of four experiments out of a total of fourteen are given in Table VII. The figures in brackets refer to calcification in bone slices which had been previously soaked in acetone, so that only the phosphatase mechanism was able to function. (See the next Section.)

Table VII.

Basal solution A.					
Exp.	Time hrs.	mg. per 100 cc.			p_H
		Ca	P inorg.	P org.	
121	17	6	6	0	6.5
		6	4	10	7.1
					7.2
148	20	8	5	0	7.35
					7.5
					7.8
149	5	8	4	30	6.5
					7.1
					7.2
185	18	10	6	0	7.35
		10	7	0	7.5
		10	8	0	7.8

In view of the high optimum p_H of bone phosphatase as determined in aqueous extracts, it was at first sight surprising that the p_H of the solutions had so little effect on calcification in presence of ester, especially after the second mechanism had been destroyed by treating the bone slices with acetone (Exp. 149). It is possible that the optimum p_H is not the same for the enzyme acting in the cartilage as for the extract, but a more probable explanation of our results is that, under the conditions of these experiments, the enzyme activity, even at low p_H , was more than sufficient for the hydrolysis of the ester diffusing into the cartilage, and that the rate of diffusion of the ester was the limiting factor governing the amount of calcification obtained.

Effect of chloroform and other organic solvents.

Shipley, Kramer and Howland [1926], in their experiments with inorganic solutions, found that calcification did not occur in bone slices which had been previously soaked in chloroform, 95 % alcohol, ether or toluene, and that it was also inhibited by adding a few drops of toluene to the solution. In the earlier experiments carried out in this laboratory [Robison, 1923; Robison and Soames, 1924] chloroform was usually added to the solutions to guard against bacterial growth, but this did not prevent calcification from taking place. In the two experiments quoted in Table VIII calcification in absence of phosphoric ester, at high $[Ca] \times [P]$ levels, was inhibited, though not completely, by saturating the solution with chloroform (about 1 %) and to a less extent by half saturation. In presence of phosphoric ester, however, good calcification was always obtained, though when the concentration of ester was low (3 mg. P per 100 cc.) calcification was slightly less in presence of chloroform than in the control solutions.

Table VIII.

Basal solution A.

Exp.	Time hrs.	mg. per 100 cc.			Chloroform cc. per 100 cc.		
		Ca	P inorg.	P org.	0	0.5	1 (saturated)
110	17	10	5	0	7≡	2≡	.
		10	6	0	9≡	6≡	.
		10	4	3	9≡	7≡	.
		10	4	10	10≡	8≡	.
		10	4	30	10≡	10≡	.
178	21	10	4	0	7≡	.	0
		10	5	0	6≡	.	0
		10	6	0	8≡	.	4≡
		10	4	3	6≡	.	4≡
		10	4	10	7≡	.	8≡
		10	4	30	8≡	.	10≡

In the saturated solutions the bone slices were suspended by silk threads to avoid contact with any chloroform globules at the bottom of the flask.

We next investigated the effect of soaking the bone slices for 20 hours at room temperature in chloroform, acetone or 90 % alcohol, before immersion in the calcifying solutions, untreated slices of the same bones being placed directly in similar solutions as controls. The results, shown in Table IX, confirm the findings of the American workers, in that calcification was usually inhibited in inorganic solutions even at high $[Ca] \times [P]$ levels. Calcification

Table IX.

Basal solution A. 18 hrs. at 37°. p_H 7.4.

mg. per 100 cc.			Control (untreated)				Acetone			90 % alcohol			Chloroform	
Ca	P inorg.	P org.	Rat a	Rat b	Rat c	Rat d	Rat a	Rat c	Rat d	Rat b	Rat c	Rat d	Rat c	Rat d
8	5	0	2—	1—	6≡	6≡	0	0	1=	0	2=	0	0	0
8	6	0	4≡	2=	7≡	7≡	0	0	0	0	0	3=	3=	0
8	4	10	6≡	3≡	.	9≡	8≡	.	.	5≡	.	.	9≡	9≡
8	4	30	7≡	7≡	9≡	9≡	8≡	9≡	9≡	9≡	9≡	9≡	9≡	9≡

did, however, take place in a few bone slices, though the deposits were much smaller than in the corresponding controls.

In presence of ester very different results were obtained, calcification being apparently unaffected by the previous drastic treatment of the bones. Heavy deposits were obtained in all slices, not only in the four experiments quoted, but also in many others of the same type. These deposits when examined in the stained slices, or in sections of these cut with the freezing microtome, without previous decalcification, appeared remarkably similar in character to the deposits in the control slices. Photomicrographs of five sections are shown in Plate XI.

Essentially the same results were obtained with slices dried *in vacuo* over sulphuric acid at room temperature for 24 hours before immersion in the calcifying solutions. The slices, though much shrivelled when first placed in the solutions, regained their former size in about half an hour. As shown in Table X calcification in inorganic solutions was inhibited in all but one slice, while in presence of ester normal deposits were obtained. Direct phosphatase tests on bone slices similarly desiccated showed that the enzyme activity was unimpaired by this treatment.

Table X.

Basal solution A. 17 hrs. at 37°. p_H 7.4.										
mg. per 100 cc.			Control (untreated)			After desiccation				
Ca	P inorg.	P org.	Rat a	Rat b	Rat c	Rat a	Rat b		Rat c	
8	5	0	2—	6≡	6≡	0	0	0	0	0
8	6	0	4=	5≡	6≡	0	3=	0	0	0
8	4	10	.	5≡	6≡	.	3=	.	5≡	5≡
8	4	30	6≡	9≡	9≡	6≡	7≡	7≡	9≡	7≡

Table XI shows results obtained when formalin or potassium cyanide was added to the calcifying solutions. Formalin in 10 % concentration (4 % formaldehyde) inhibited calcification both in presence and absence of ester. Potassium cyanide in 0.001 *M* or 0.01 *M* concentration prevented calcification in inorganic solutions but not in those containing ester. With low concentrations of the latter the amount of deposit was somewhat reduced in presence of cyanide, but with higher concentrations this slight inhibition was no longer apparent. The different results in presence of these two agents can be understood when their effects on the activity of bone phosphatase are compared. The values at the foot of Table XI show the activities of two phosphatase preparations in presence of formalin and potassium cyanide, as measured by the hydrolysis of glycerophosphate and expressed as percentages of the hydrolysis in the control solution.

Bone slices soaked for 24 hours in neutral 10 % formalin also failed to calcify on subsequent immersion in inorganic solutions, but slight calcification was usually obtained in solutions containing ester. This indicated that the bone phosphatase could not have been completely destroyed, a supposition

confirmed by direct enzyme tests on half bones after similar treatment with formalin, which showed that about 15 % of the phosphatase activity was still retained.

Table XI.

mg. per 100 cc.					Formalin		Potassium cyanide		
Ca	P inorg.	P org.	Control		1 %	10 % Rat a	0.001 M		0.01 M
			Rat a	Rat b			Rat a	Rat b	Rat b
8	4	0	0	1—	.	0	0	0	0
8	5	0	1≡	1=	.	0	0	0	.
8	4	3	3≡	4≡	.	0	1=	5≡	1≡
8	4	30	7≡	8≡	.	0	6≡	6≡	8≡
Relative activity of bone phosphatase:									
Preparation A			100		25	0	100		33
" B			100		28	0.4	93		58

The fact that bone slices, after treatment with organic solvents or desiccation *in vacuo*, will subsequently calcify in solutions containing phosphoric ester, but not in inorganic solutions even of high $[Ca] \times [P]$ levels, sharply distinguishes the two mechanisms in calcifying hypertrophic cartilage; (a) the phosphatase mechanism which produces in the matrix fluid, by hydrolysis of phosphoric ester, a condition of supersaturation with respect to the bone phosphate, and (b) the second mechanism which favours the deposition of the bone salt from supersaturated inorganic solutions, whether the condition of supersaturation in the cartilage matrix is produced by enzymic hydrolysis of phosphoric ester, or by allowing an external supersaturated solution to diffuse into it.

The nature of the second factor is as yet unknown; a slight increase in the p_H of the matrix fluid, brought about by some membrane equilibrium, or the specific affinity of certain groups in the matrix colloids for some constituent of the bone salt might perhaps account for its action. In some respects it resembles a trigger mechanism, releasing from solution the excess bone salt present in the supersaturated fluid. We have provisionally termed it the "inorganic" mechanism without reference to its nature but because it is responsible for calcification in inorganic solutions.

It is injured by potassium cyanide, by fat solvents and by desiccation of the cartilage, all of which leave the phosphatase mechanism unimpaired. It is probable that the differences in response noted in bones from different rats were due to variations in the efficiency of the "inorganic" mechanism.

Our experiments show that either of these two mechanisms could of itself account for the calcification of cartilage *in vivo*, provided that the conditions necessary for it to function were present. These conditions are, for the phosphatase mechanism, that the plasma should be saturated with respect to the bone salt and contain a hydrolysable phosphoric ester, and for the "inorganic" mechanism, that the plasma should be supersaturated with respect to the bone salt. No satisfactory evidence has, however, been adduced that the

plasma is thus supersaturated. The apparent similarity between the concentrations of calcium and inorganic phosphate adequate for calcification in experiments with inorganic solutions and those in normal plasma is misleading, owing to the absence from the experimental solutions of the protein and other organic constituents which greatly depress the ionisation of calcium in plasma. On the other hand the plasma is known to contain small amounts of a phosphoric ester hydrolysable by the bone phosphatase [Martland and Robison, 1926]. It is therefore reasonable to suppose that both mechanisms play a part in normal calcification of the skeleton, the phosphatase mechanism producing the necessary degree of supersaturation in the matrix and the second ("inorganic") mechanism assisting in the deposition of bone salt, in some way not as yet understood.

The experiments here recorded deal exclusively with calcification in cartilage, but the presence of phosphatase has also been demonstrated in membrane bone [Martland and Robison, 1924; Fell and Robison, 1930] and we have recently obtained evidence of calcification *in vitro* in membrane bone, using the parietal bone of rachitic rats. These experiments and others on calcification in solutions containing protein will be described in a subsequent paper.

SUMMARY.

1. The effect of various factors on calcification *in vitro* has been investigated, using the experimental material and technique previously described.

2. In inorganic solutions with salt concentrations approximating to those in plasma, calcification was obtained with concentrations of calcium varying from 2.5 to 50 mg. per 100 cc. and of inorganic phosphate varying from 1 to 20 mg. per 100 cc. Calcification was observed at somewhat lower levels of the product $[Ca] \times [P]$ when the concentration of calcium was high and that of phosphate low than under the reverse conditions.

3. With concentrations of calcium around 10 mg. per 100 cc. some degree of calcification was usually obtained in 16 hours when the concentration of inorganic phosphate was such that the product $[Ca] \times [P]$ was equal to 30. Variations were, however, observed in the minimum level required with bones from different rats. This variability in response was apparent also in the extent of the deposit obtained at higher $[Ca] \times [P]$ levels.

4. Definitely higher $[Ca] \times [P]$ levels were necessary for calcification when the concentration of sodium bicarbonate was reduced from 0.2 % to 0.03 %, and still higher levels were required when the bicarbonate was entirely omitted. This suggests that the compound normally deposited in calcifying cartilage is a complex carbonato-phosphate (carbonato-apatite) rather than a mixture of basic phosphate and carbonate.

5. As observed by other workers, sodium chloride in concentrations as low as 34 mM (0.2 %) was found to have an inhibitory effect on calcification in inorganic solutions.

6. Potassium chloride in concentrations up to 10 *mM* (twice the concentration in plasma) showed no appreciable inhibitory effect.

7. Magnesium in concentration of 0.08 *mM* (slightly lower than that in plasma) adversely affected calcification, and with higher concentrations the inhibitory effect was pronounced.

8. The inorganic solutions in which calcification occurred were all supersaturated with respect to the calcium salt deposited. In presence of phosphoric ester, however, calcification occurred at $[Ca] \times [P]$ levels much lower than 30, while at higher levels the effect of the ester was seen in the greatly increased extent and density of the deposit.

9. The increased calcification which occurred in presence of phosphoric ester tended to mask the favourable effect of bicarbonate and the inhibitory effects of sodium chloride and magnesium. There was no evidence that any of the inorganic radicals directly influenced the enzyme mechanism.

10. Between p_H 7.2 and 7.8 no marked optimum was found for calcification in presence or absence of ester. Calcification was obtained at p_H 6.5 with very high $[Ca] \times [P]$ levels (60–80) in inorganic solutions, and at the low level 24, in presence of phosphoric ester.

11. Formaldehyde completely inhibited calcification in inorganic solutions and almost completely inactivated the phosphatase mechanism.

12. The presence of chloroform or potassium cyanide strongly inhibited calcification in inorganic solutions, but had no appreciable effect in solutions containing phosphoric ester.

13. Bone slices, after being soaked for 24 hours in chloroform, acetone or alcohol, or desiccated *in vacuo* over sulphuric acid, failed to calcify on subsequent immersion in inorganic solutions of high $[Ca] \times [P]$ levels. In solutions containing phosphoric ester, however, extensive calcification occurred and the deposits were normal in appearance.

14. The results of these experiments set in sharp distinction the two mechanisms in calcifying hypertrophic cartilage.

(i) The phosphatase mechanism which produces in the matrix fluid a condition of supersaturation with respect to the bone phosphate, and

(ii) The "inorganic" mechanism which favours deposition of this salt from supersaturated solutions.

The part played by these two mechanisms in the normal calcification of the skeleton is discussed.

Fig. 1

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

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DESCRIPTION OF FIGURES IN PLATE XI.

- Figs. 1-5. Sections of bone slices after immersion in experimental solutions at 37° and p_H 7.35. The sections were cut without previous decalcification. Thickness—sections 1, 2, 3, 5—30 μ ; section 4—50 μ . Sections 1, 2, 3 and 4 were stained with silver nitrate, section 5 was stained with purpurin. Magnification $\times 11$. X denotes hypertrophic cartilage calcified *in vitro*.
- Fig. 1. Humerus of rachitic rat. 18 hrs. in inorganic solution (8 : 5 : 0). Note the calcification, obtained *in vitro*, in the metaphysis and in the cartilage forming the articular surface of the epiphysis.
- Fig. 2. Tibia of the same rat. 22 hrs. in acetone before immersion for 18 hrs. in inorganic solution (8 : 5 : 0). Note the complete absence of new calcification in the broad zone of uncalcified hypertrophic cartilage characteristic of rickets.
- Fig. 3. Tibia of the same rat. 18 hrs. in solution containing phosphoric ester (8 : 4 : 30). Note the extensive calcification, obtained *in vitro*, in the matrix of the hypertrophic cartilage.
- Fig. 4. Tibia of the same rat. 22 hrs. in acetone before immersion for 18 hrs. in solution containing phosphoric ester (8 : 4 : 30). Note the extensive calcification, obtained *in vitro*, in the matrix of the hypertrophic cartilage, in spite of the drastic treatment of the bone slices. The greater density of the deposit, as compared with that in Fig. 3, is accounted for by the greater thickness of the section.
- Fig. 5. Tibia of a rachitic rat. 22 hrs. in 90 % alcohol before immersion for 18 hrs. in solution containing phosphoric ester (8 : 4 : 30). Note the normal appearance of the new deposit in the cartilage matrix.