XCV. CALCIFICATION OF HYPERTROPHIC CARTILAGE *IN VITRO*.

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Introduction.

In a previous paper on the experimental calcification of cartilage *in vitro* [Robison *et al.*, 1930] the conclusion was stated that two distinct mechanisms are concerned in normal calcification of cartilage and bone. The first of these, phosphatase-phosphoric ester, brings about the necessary supersaturation of tissue fluid with bone salt [Robison, 1923; 1932]. The second becomes effective only when supersaturation in sufficient degree has been achieved; it provokes the rapid and orderly deposition of the calcium salt in the ground substance of the tissue [Shipley *et al.*, 1926; Robison, 1926].

The share contributed by each mechanism towards the ultimate deposition of the solid calcium salt may depend on the state of the blood with respect to this salt and on the amount of phosphoric ester available.

The distinction between these two mechanisms is empirical. Their activity becomes apparent under different experimental conditions; but it is not affirmed that the mechanisms are entirely independent.

Previous experiments brought to light the favourable effect of bicarbonate on calcification and the inhibitory effect of other ions, notably Mg^{++}, and of protein, and the inhibition of the second mechanism by potassium cyanide, chloroform or by dehydration of the cartilage by acetone *etc.*

Experiments planned to obtain more information on the nature and properties of the dual calcifying mechanism, and particularly of that component which we have termed the second mechanism, are described in this paper.

Experimental.

The technique was in general the same as that previously employed [Robison *et al.*, 1930]. Longitudinal slices cut from the heads of the long bones of rachitic rats were immersed for periods of 16–20 hours in sterile salt solutions at 37° and p_H 7·4. In these experiments the constant-flow apparatus previously described was not used; but the flasks (20 or 100 ml.) containing the bone slices were slowly and continuously rotated at 37° so that all surfaces of the slices were equally exposed to the calcifying solutions. The slices were subsequently fixed, stained with silver nitrate and cleared in cedar wood oil, so that the amount of new calcification in the zone of hypertrophic cartilage could be more readily judged. This is shown in the tables by a figure (maximum 10) representing the extent of the deposit and bars (maximum 5) representing the density, thus $6\equiv$.

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The composition of the basal solution is given in Table I. It differs slightly from that previously used, approximating still more closely to normal plasma in salt content.

Table I. *Composition of experimental solutions.*

g. per litre basal solution		Ion	Millimols per litre: Experimental solution 8 : 5 : 0	Millimols per litre: Normal plasma
NaCl	6·0	Na	130·0	146·0
$NaHCO_3$	2·2	K	5·6	5·1
KCl	0·3	Ca	2·0	2·5
$MgSO_4, 7H_2O$	0·25	Mg	1·0	1·25
		Cl	108·0	101·0
		HCO_3	26·0	26·7
		HPO_4	1·6	1·0
		SO_4	1·0	2·0

The molar concentrations of the acid and basic radicals in a typical inorganic calcifying solution, 8 : 5 : 0[1], and typical values for normal human plasma [Kramer and Tisdall, 1922] are compared in the same table.

Calcium was added to the basal salt solution as $CaCl_2$ and inorganic phosphate as $NaKHPO_4$.

Comparison of the effects of various phosphoric esters on calcification in vitro.

In view of the rôle ascribed to the phosphatase mechanism experiments were undertaken to compare the effects of different phosphoric esters on calcification *in vitro* with that of the syrupy sodium glycerophosphate (chiefly α-ester) which had been generally used. The effect may be dependent on such factors as the rate of diffusion into, or specific adsorption by, the cartilage and on the rate of hydrolysis by the bone-phosphatase.

In these experiments the period of immersion and the concentration of ester were varied, so that any differences in behaviour might be brought out more clearly. The various esters used and the experimental results are shown in Table II. One rat was used for each experiment.

No very marked differences were observed in the effectiveness of the first five esters in the table. The smaller deposits obtained in 5 hours with hexose-monophosphate may possibly signify that this ester diffuses somewhat more slowly than glycerophosphate into the cartilage, although the calcification produced in 18 hours was equally great with both esters.

In the inorganic solutions, in absence of phosphoric ester, no calcification was obtained in periods up to 6 hours. Diphosphoglycerate, which is one of the phosphoric esters of the red blood corpuscles, was ineffective. This ester was not hydrolysed in separate tests with extracted bone-phosphatase; moreover the calcium salt was so insoluble as to be precipitated in the experimental solutions. It is possible, however, that a monophosphoglycerate is also formed in the red corpuscles, and this being readily hydrolysable would prove a suitable substrate for the bone-enzyme. The question is at present being investigated in this laboratory.

[1] The experimental solutions are referred to in this way throughout the text. Thus solution 8 : 5 : 3 contains 8 mg. Ca, 5 mg. P as inorganic phosphate and 3 mg. P as organic phosphate per 100 ml. basal salt solution.

Table II. *The effect of various phosphoric esters on calcification* in vitro.

mg. per 100 ml. Ca	mg. per 100 ml. P inorg.	Phosphoric ester	Conc. of ester mg. P per 100 ml.	Exp. 1 Time (hours) 18	Exp. 2 Time (hours) 5	Exp. 3 Time (hours) 6	Exp. 4 Time (hours) 4	Exp. 5 Time (hours) 6	Exp. 6 Time (hours) 6	Exp. 7 Time (hours) 18	Exp. 8 Time (hours) 18
8	2	0	0	.	.	.	.	.	.	0	.
8	3	0	0	.	.	.	.	.	.	3=	.
8	4	0	0	8≡	0	0	0	0	.	.	6≡
8	4	α-Glycerophosphate	2	.	.	.	0	.	.	.	.
			3	9≡	.	.	.	0	4=	7≡	.
			4	.	.	8=	.	.	.	.	.
			6	9≡	7= 5=	8≡	5=	.	.	.	.
			10	.	.	.	.	5≡	.	.	.
8	4	β-Glycerophosphate	3	9≡	.	.	.	.	.	.	.
			6	9≡	7≡ 7≡	8≡	.	.	.	.	.
8	4	Hexosemonophosphate (mixed ester of fermentation)	3	8≡	.	.	.	.	.	.	.
			6	9≡	1= 2=	.	.	.	.	.	.
8	4	Fructosediphosphate	4	.	.	6−	.	.	.	.	.
			6	.	.	7≡	.	.	.	.	.
8	4	Trehalosemonophosphate	2	.	.	.	0	.	.	.	.
			6	.	.	.	4−	.	.	.	
8	4	Diphosphoglycerate	3	.	.	.	.	0 *p*	.	.	.
			4	.	.	0	.	.	.	.	.
			10	.	.	.	.	0 *p*	.	.	.
8	4	Lecithin	7	.	.	.	.	.	0	.	.
			15	.	.	.	.	.	0	.	.
			30	.	.	.	.	.	0	.	.
8	2	Sphingomyelin	3	.	.	.	.	.	.	0	.
			8	.	.	.	.	.	.	2=	.
8	0	Pyrophosphate	3	.	.	.	.	.	.	.	0 *p*
			4	.	.	.	.	.	.	.	0 *p*
8	2	Pyrophosphate	1	.	.	.	.	.	.	.	0 *p*

p signifies precipitation in the solution during the experimental period.

Lecithin, which is not hydrolysed by the bone-phosphatase, had no favourable effect on calcification; but on the other hand sphingomyelin[1], which is hydrolysed to a slight extent, had some beneficial effect.

Substitution of pyrophosphate for glycerophosphate yielded negative results, since precipitation of the very insoluble calcium pyrophosphate occurred in solutions containing no more than 1 mg. P per 100 ml. in this form.

Experiments with other esters which might function as substrates for the bone-enzyme are in progress.

Deposition of salts other than those of calcium in the hypertrophic cartilage in vitro.

The object of a further series of experiments was to discover whether the second mechanism of hypertrophic cartilage is specific for the bone salt, the complex carbonato-phosphate of calcium, or whether it can also function for other salts of low solubility. The range of possible salts was restricted by the necessity of obtaining solutions sufficiently supersaturated, yet with total ion concentration not unduly removed from that of the tissue fluids.

[1] We are much indebted to Dr O. Rosenheim, who very kindly provided us with a specimen of sphingomyelin.

Attempts to substitute arsenate for phosphate were unsuccessful owing to the high solubility of calcium arsenate. A solution containing 0·025 *M* calcium and as much as 3 *M* arsenate failed to give any precipitate in 24 hours at p_H 7·4.

The replacement of calcium by other alkaline earth metals was more practicable although the phosphates of these metals are more soluble than those of calcium.

In order to find for these experiments the conditions most comparable with those leading to deposition of calcium salt in the cartilage, preliminary tests were undertaken to establish the lowest concentrations of metal and phosphate at which general precipitation occurred in the salt solutions under the experimental conditions. Concentrations slightly lower than this were chosen for the experiments.

For the above tests and subsequent experiments in this group potassium, magnesium and sulphate were omitted from the basal solution and the bicarbonate content was varied as shown in Table III[1].

Table III. *Deposition of Ca, Ba, Sr and Mg salts in the hypertrophic cartilage* in vitro.

17–20 hours; 37°; p_H 7·4.

Metal	Conc.	g. per 100 ml. $NaHCO_3$	mg. per 100 ml. P inorganic	mg. per 100 ml. P organic	Deposition in cartilage: From inorganic solution	Deposition in cartilage: From solution + phosphoric ester
Ca	0·0025 *M*	0·22	3	0	3 =	.
	,,	0·22	2	1	.	4 ≡
Ba	0·01 *M*	0·6	6	0	0	.
	0·005 *M*	0·6	10	0	0	.
	,,	0·6	14	0	0 *p*	.
	,,	1·0	18	0	0	.
	,,	0·05	0	30	.	6 ≡
	,,	0·05	5	10	.	0
	,,	0·05	5	30	.	7 ≡
Sr	0·01 *M*	0·22	10	0	0 *p*	.
	,,	0·6	4	0	0	.
	,,	0·6	8	0	3 ≡	.
	,,	0·6	12	0	8 ≣	.
	0·005 *M*	0·05	12	0	0	.
	,,	0·6	16	0	0	.
	,,	0·6	18	0	3 ≡	.
	0·0025 *M*	0·6	50	0	4 ≡	.
	0·01 *M*	0·05	0	30	.	5 =
	0·005 *M*	0·05	8	4	.	0
	,,	0·05	8	10	.	7 ≡
	0·0025 *M*	0·05	32	2	.	0
	,,	0·05	32	4	.	4 =
Mg	0·1 *M*	0·05	14	0	0	.
	,,	0·05	16	0	0 *p*	.
	,,	0·05	10	4	.	0
	,,	0·05	10	10	.	4 =
	,,	0·05	10	30	.	10 ≣

p signifies precipitation in the solution during the experimental period.

[1] In the case of barium and strontium it was found that as the bicarbonate concentration was raised, progressively higher concentrations of inorganic phosphate could be reached before general precipitation in the solution occurred.

Deposition of barium, strontium and magnesium salts in the hypertrophic cartilage could not at first be realised by agency of the second mechanism alone, that is by immersing the bone slices in inorganic solutions containing these metals in place of calcium. Suitable conditions were at length established in the case of strontium; but deposits of barium and magnesium salts have not yet been obtained in absence of phosphoric ester, even with the highest degree of supersaturation which can be realised short of general precipitation in the solution. On the other hand, deposits of the salts of barium, strontium and magnesium were readily obtained in the hypertrophic cartilage *in vitro* by agency of the bone-phosphatase in presence of phosphoric ester. Typical results are set out in Table III, which shows the relative concentrations required for deposition.

The appearance of the deposits, stained with silver nitrate[1] can be seen in Plate I, Figs. 1–4. Fig. 1 shows a deposit of magnesium salt obtained in solution 0·1 *M* Mg : 10 : 60. The deposit was formed in the matrix of the cells, entirely filling the zone of uncalcified hypertrophic cartilage between the epiphysis and the diaphysis. In appearance it closely resembled that obtained with calcium salts.

Deposits of barium and strontium were usually similar in appearance though sometimes they were less sharply outlined, and occasionally the barium salt was deposited in isolated clusters of relatively large crystals, as seen in Plate I, Figs. 5 and 6.

Attempts were made to obtain deposits of lithium and beryllium salts in the cartilage, but the relatively high solubility of lithium phosphate and the extreme insolubility of beryllium phosphate, carbonate and hydroxide offered considerable difficulties, and no definite evidence of deposition was obtained.

We conclude from these experiments that the second mechanism is not strictly specific for calcium, but that it undoubtedly functions better for the calcium salt than for the others which have been tested. It may be that calcium alone forms a highly complex carbonato-phosphate of solubility so much lower than that of the simple phosphates and carbonates that solutions not supersaturated with the latter may be greatly supersaturated with respect to the complex salt which is ultimately deposited.

Effect of potassium cyanide, of fat solvents and of desiccation on the calcifying mechanisms.

In our previous paper [Robison *et al.*, 1930] it was stated that potassium cyanide strongly inhibited calcification in inorganic solutions but had no appreciable effect in solutions containing phosphoric ester. Further experiments have made it clear that the inhibition of the second mechanism by potassium cyanide is only partial and that calcification may still occur in the more highly concentrated inorganic solutions, particularly if the original activity of the second mechanism in the bone is high. This is illustrated by the results in Table IV. The calcification of the control bone slices in solution 8 : 5 : 0 shows that the second mechanism activity was low in rats *a* and *b* and much higher in rats *c* and *d*. In presence of 0·001 *M* potassium cyanide, calcification was still obtained in this solution in bones from the latter rats but not in those from rats *a* and *b*. The effect of 0·01 *M* cyanide was not markedly greater than that

[1] The deposits of barium, strontium and magnesium salts in the hypertrophic cartilage were also found to stain almost as well as those of calcium with alizarin S. In gelatin models, deposits of the phosphates of calcium, barium, strontium, magnesium, beryllium and lithium stained deep red with this dye, as was shown for calcium and strontium by Cameron [1930].

Table IV. *The effect of potassium cyanide on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.																
			Control				Potassium cyanide									
							0·0001 *M*		0·001 *M*				0·01 *M*			
Ca	P inorg.	P org.	*a*	*b*	*c*	*d*	*c*	*d*	*a*	*b*	*c*	*d*	*c*	*d*		
8	5	0	1≡	1=	5≡	9≡	2=	6≡	0	0	1–	4≡	2=	2=		
8	6	0	.	.	5≡	.	6≡	.	.	.	2=	.	3=	.		
8	3	10	.	.	7≡	.	6≡	.	.	.	7≡	.	.	.		

of 0·001 *M*. No inhibitory effect was observed in solution 8 : 3 : 10, containing phosphoric ester.

The previous conclusion regarding inactivation of the second mechanism by treatment of the bone slices with acetone, alcohol or chloroform, or by desiccation, probably requires similar modification. It would seem that the second mechanism, though greatly weakened, is not entirely destroyed by such treatment.

Effect of various organic substances on the calcifying mechanisms.

Calcification both in presence and absence of ester was strongly inhibited by 0·5 % phenol and by 0·3 % thymol; but 0·1 % phenol had little apparent effect. Neither ethylurethane in 1 % solution nor guanidine in 0·1 % solution appeared to influence the calcifying mechanisms.

Inactivation of the calcifying mechanisms by heat[1].

Experiments were undertaken to determine the effect on the two calcifying mechanisms of heating the bone slices at different temperatures. The slices were placed in bicarbonate-free solution 8 : 2 : 0 and maintained at temperatures between 45° and 60° for periods of 10, 15 and 20 minutes. The effect on the two mechanisms was judged by the subsequent calcification of the slices in solutions 8 : 5 : 0 and 8 : 3 : 10, the deposits being compared with those in unheated control slices. No effect on either mechanism was observed after 20 minutes at 45°; but some reduction in the activity of the second mechanism and probably also in that of the phosphatase was apparent after 15 minutes at 48°. The loss of activity was greater at 50° and 55°; but the results were too irregular to justify any conclusion as to the relative rates of destruction. Both mechanisms, however, appeared to be inactivated in 10 minutes at 60°.

Inactivation of the calcifying mechanisms by acid and alkali.

Unsuccessful attempts were made to inactivate the two mechanisms differentially by soaking bone slices for one hour in isotonic salt solutions at different p_H levels. Little effect on either mechanism was noted between p_H 5·4 and 8·9 at 37°; but both were inactivated, at least partially, at p_H 5.

In 1 % ammonia both mechanisms were destroyed in one hour at room temperature. It may be added that preparations of bone-phosphatase are very rapidly inactivated at p_H values below 5·2 or above 10. It is not known to what extent the tissue may be able to maintain an internal p_H differing from that in the surrounding medium.

[1] Other experiments bearing on this question and those discussed in the following two sections have been carried out by Niven and Robison using embryonic rabbit femora. The results are in course of publication.

Survival of the calcifying mechanisms in excised bone slices under various conditions.

The question of the survival of the calcifying mechanisms in cartilage after excision from the body and immersion in various solutions was investigated on account of its theoretical interest and its bearing on the interpretation of experimental results. Some typical experiments are summarised in Table V. The control (0 hour) bone slices from each rat were placed directly in the calcifying solutions 8 : 5 : 0 and 8 : 3 : 10 at 37° and left there for 18 hours. The other slices were first immersed for varying periods in salt solutions, or distilled water, at 0°, 20° or 37° as specified in Table V. They were then transferred to the calcifying solutions for an 18-hour period at 37°.

Table V. *Gradual inactivation of the calcifying mechanisms in bone slices immersed in various solutions at different temperatures.*

Preliminary immersion				Subsequent calcifying solution	Duration of preliminary immersion (hours)						
Rat	Solution	$NaHCO_3$ %	Temp. ° C.		0	½	1	2	4	24	48
					Subsequent calcification in 18 hours; 37°; p_H 7·4						
a	A	0·22	0	8 : 5 : 0	7≡	.	.	.	.	2≡	0
				8 : 3 : 10	7≡	.	.	.	.	3≡	0
b	B_1	0·03	,,	8 : 5 : 0	7≡	.	.	.	.	2=	0
				8 : 3 : 10	7≡	.	.	.	.	3≡	0
c	A	0·22	20	8 : 5 : 0	8≡	7≡	.	6≡	5≡	.	.
				8 : 3 : 10	8≡	.	.	.	9≡	.	.
	,,	,,	37	8 : 5 : 0	8≡	6≡	.	6≡	4≡	.	.
				8 : 3 : 10	8≡	.	.	.	6≡	.	.
d	,,	,,	,,	8 : 5 : 0	5≡	.	.	.	3≡	0	.
				8 : 3 : 10	5≡	.	.	.	5≡	3≡	.
e	B	0·22	37	8 : 5 : 0	6≡	.	6≡	5≡	.	.	.
	B_1	0·03	,,	8 : 5 : 0	6≡	.	6≡	3≡	.	.	.
	B_2	0	,,	8 : 5 : 0	6≡	.	6≡	0	.	.	.
	B	0·22	37	8 : 5 : 0	2=	.	2=	.	.	.	.
				8 : 3 : 10	8≡	.	7≡	.	.	.	.
	B_2	0	,,	8 : 5 : 0	2=	.	0	.	.	.	.
				8 : 3 : 10	8≡	.	7≡	.	.	.	.
g	B_1	0·03	37	8 : 5 : 0	.	5≡	5≡	2=	.	.	.
				8 : 3 : 10	.	.	8≡	.	.	.	.
Distilled water		0	,,	8 : 5 : 0	.	2≡	0	0	.	.	.
				8 : 3 : 10	.	.	5≡	.	.	.	.

Solutions used for the preliminary immersion:

A, 8 : 2 : 0, similar in composition to the calcifying solution but of lower P content. (This solution can itself effect calcification in bones with very exceptionally high second mechanism activity.)

B, basal solution (Table I) containing neither Ca nor P.

B_1 and B_2, similar to B in all respects except bicarbonate content.

In the last experiment (rat *g*) the bone slices in solution B_1 served as controls for those in distilled water.

The results show that when the bone slices were kept at 37° in solution 8:2:0 or in the basal salt solution there was a gradual decrease in the activity of the second mechanism, which became pronounced after 24 hours' immersion. This loss of activity occurred also, though more slowly, at lower temperatures. In salt solutions containing no bicarbonate and in distilled water the inactivation of the second mechanism was more rapid.

These adverse conditions would appear to have had little effect on the phosphatase mechanism over short periods; but calcification in solution 8:3:10 was reduced after prolonged immersion.

These results indicate that in experiments on calcification *in vitro* undue prolongation of the period in the calcifying solutions at 37° can be of little advantage owing to the gradual decrease in the activity of the calcifying mechanisms. Practical experience has, indeed, shown that the maximum calcification obtainable in any given solution at 37° occurs, as a rule, within 18 hours. At room temperature, on the other hand, in solutions 8 : 10 : 0 and 8 : 4 : 10 calcification has been obtained in 43 hours though absent after 19 hours.

Effect of various vitamin and hormone preparations on calcification in vitro.

Experiments were performed to discover whether vitamin D or hormones, which are known to influence the growth and calcification of the skeleton *in vivo*, would have any demonstrable action on the calcification or decalcification of bone slices *in vitro*. The results of these experiments were all negative and will therefore only be summarised briefly.

Vitamin D. (1) Bone slices from a rachitic rat were moistened with 8 : 2 : 0 solution and irradiated in quartz tubes for periods of 15, 30 and 60 minutes before immersion in 8 : 3 : 0, 8 : 5 : 0 and 8 : 3 :10 solutions for 18 hours.

(2) Bone slices from a rachitic rat were immersed for one hour in an olive oil solution of irradiated ergosterol (radiostol) before immersion in the calcifying solutions.

(3) Bone slices were immersed in 20 ml. of various calcifying solutions (with and without ester) in which 0·1, 0·2 or 0·3 ml. of radiostol was emulsified by shaking. A control solution containing 0·2 ml. of olive oil in emulsion was used in addition to the usual controls.

(4) In other experiments calciferol was used instead of radiostol. It was added to the calcifying solutions in amounts equivalent to 400–40,000 antirachitic units per 100 ml. In all the above experiments, untreated slices from the same rat were immersed in similar calcifying solutions (containing no vitamin preparation) to serve as controls. There was no indication of any effect, beneficial or otherwise, of irradiation or of radiostol or calciferol on calcification *in vitro* either in presence or absence of phosphoric ester.

Parathyroid hormone. (1) Bone slices were immersed for 19 hours at 37° in calcifying solutions with and without ester, and containing in addition 10, 20 and 40 units of Eli Lilly parathormone per 100 ml. No increase or inhibition of calcification was apparent; and it was almost impossible to determine by inspection of the stained slices whether there had been any resorption of the previously calcified shaft trabeculae during the experimental period.

(2) Whole bones, heads of bones or half bones of normal young rats were each immersed in 5 ml. of sterile basal salt solution without calcium or phosphate, half the solutions containing 60 units of parathormone per 100 ml. The vessels containing the bones were rotated for 19 hours at 37°. The very slight increase in the inorganic and organic phosphate and the calcium content of the solutions, which occurred during the experiments, was no greater when parathyroid extract was present than when it was not. There was thus no evidence that calcium or phosphate was brought into solution in any form.

Pituitary extract. In two experiments no definite effect on calcification was observed as a result of adding standard posterior lobe extract (equivalent to

0·1 to 15 I.U.) to calcifying solutions with and without phosphoric ester. Short periods of immersion (3 to 6 hours) were chosen in order that any increase in permeability due to the action of the hormone might be more apparent.

Testicular extract. Experiments similar to the above were carried out with testicular extract, for which we are indebted to Drs D. McClean and W. T. J. Morgan. No indication was obtained of more rapid calcification as a result of increased permeability.

Effect on calcification in vitro *of other substances present in the blood.*

The results of two experiments quoted in Table VI show that neither glycine nor urea in concentrations covering the normal physiological range had any significant effect on the activity of the second mechanism. The amounts of new deposit in tibia slices immersed in solutions 8 : 4 : 0 and 8 : 5 : 0 were nearly the same in presence of these substances as in the controls.

Table VI. *The effect of urea and glycine on calcification* in vitro.

17 hours; 37°; p_H 7·4.

	mg. per 100 ml.				mg. per 100 ml.					
					Urea			Glycine		
Rat	Ca	P inorg.	P org.	Control	30	50	60	20	40	50
a	8	4	0	5=	2≡	5≡	.	7≡	2=	.
	8	5	0	6≡	7≡	4≡	.	7≡	7≡	.
b	8	4	0	1=	.	.	<1–	.	.	<1–
	8	5	0	5≡	.	.	6≡	.	.	5≡
	8	5	0	4≡	.	.	5≡	.	.	4≡

Table VII. *Effect of sugars and sugar alcohols on calcification* in vitro.

	mg. per 100 ml.				mg. per 100 ml.					
					Glucose		Fructose	Sucrose	Dulcitol	Erythritol
Rat	Ca	P inorg.	P org.	Control	50	100	100	100	100	100
a	10	3	0	5=		0	.	.	.	.
	10	5	0	7≡		0	.	.	.	.
b	10	3	0	1–		.	.	.	.	.
	10	4	0	4=	0	<1–	.	.	.	.
	10	5	0	.	4≡	0	.	.	.	.
	10	6	0	.	4≡	2=	.	.	.	.
	10	7	0	.	6≡	5≡	.	.	.	.
c	8	4	0	2=	.	0	3–	4=	.	.
	8	5	0	4≡	.	0	2≡	2≡	.	.
d	8	4	0	4≡	.	0	.	.	3≡	3=
	8	5	0	5≡	.	<1–	.	.	5≡	5≡

On the other hand the results set out in Table VII show that glucose in concentrations of 50 or 100 mg. per 100 ml. had a very definite inhibitory effect on calcification in inorganic solutions. Thus in rat *b* calcification was less extensive in 10 : 6 : 0 solution containing 100 mg. glucose per 100 ml. than in 10 : 4 : 0 solution containing none. In another paper [Rosenheim, 1934, 1] it is shown that this inhibition is exerted also in presence of protein and is additive to the effect of the latter.

It was at first thought that this inhibition might be explained by the combination of the glucose with the calcium ions; but as will be seen from Table VII

no such marked effect could be demonstrated with other sugars or sugar alcohols. Glycogen also, in concentration of 150 mg. per 100 ml., did not affect the amount of calcification in solution 8 : 5 : 0.

Effect of fluoride, iodoacetate and arsenate on calcification in vitro.

The results given in Tables VIII and IX show that both sodium fluoride and sodium iodoacetate exert a most pronounced inhibitory effect on the second mechanism. The former in concentration as low as 0·0001 *M* completely prevented calcification even in solutions 8 : 6 : 0 and 8 : 6 : 10, though calcification still occurred in solution 8 : 6 : 30. In still lower concentration (0·00001 *M*)

Table VIII. *Effect of sodium fluoride on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.			Control			Concentration of NaF: 0·00001 *M*	Concentration of NaF: 0·0001 *M*	
Ca	P inorg.	P org.	*a*	*b*	*c*	*a*	*b*	*c*
8	5	0	5≡	4≡	4=	0	0	0
8	6	0	.	.	.	4≡	0	0
8	3	10	8≡	7≡	5≡	.	0	.
8	5	10	.	.	.	7≡	.	0
8	6	10	.	.	.	.	0	.
8	6	30	.	.	.	.	.	8≡

The results given in columns *a*–*c* were obtained with bone slices from three different rats.

Table IX. *Effect of sodium iodoacetate on the calcifying mechanisms.*

18 hours; 37°; p_H 7·4.

mg. per 100 ml.			Control						Concentration of iodoacetate: 0·0001 *M*	0·001 *M*				
Ca	P inorg.	P org.	*a*	*b*	*c*	*d*	*e*	*f*	*b*	*a*	*b*	*c*	*d*	*e*	*f*
8	5	0	6≡	5≡	4≡	5≡	6≡	5≡	<1–	0	0	0	.	3=	3≡
8	6	0	.	.	.	.	.	.	.	.	.	0	0	.	3≡
8	3	10	6≡	8≡	.	6≡	6≡	.	6≡	0	<1–	.	.	6≡	4=
8	5	10	.	.	9≡	.	.	.	.	.	.	0	.	.	6=
8	6	10	.	.	.	.	.	.	.	.	.	0	2=	.	.
8	6	30	.	.	9≡	.	.	.	.	.	.	5–	7≡	.	.

The results given in columns *a*–*f* were obtained with bone slices from six different rats.

sodium fluoride prevented calcification in 8 : 5 : 0 solution although not in solutions 8 : 6 : 0 and 8 : 5 : 10. The inhibitory effect of sodium iodoacetate, although not apparent in such great dilution, was also very marked. In a concentration of 0·001 *M* it prevented or greatly reduced the calcification in the inorganic solutions, while in presence of ester the amount of deposit was generally reduced.

In separate experiments, summarised in Table X, it was shown that neither fluoride nor iodoacetate in concentrations higher than those used in the above experiments affected the rate of hydrolysis of β-glycerophosphate by a preparation of purified bone-phosphatase.

The presence of 0·001 *M* sodium arsenate in the calcifying solutions 8 : 5 : 0 and 8 : 3 : 10 had neither favourable nor inhibitory effect on calcification *in vitro*.

Table X. *Hydrolysis of β-glycerophosphate by bone-phosphatase in presence of fluoride and iodoacetate.*

		Sodium fluoride		Sodium iodoacetate	
	Control	0·001 *M*	0·01 *M*	0·001 *M*	0·01 *M*
Inorganic phosphate (mg. P) liberated in 1 hour at 37° and p_H 8·6 by 1 mg. phosphatase	0·285	0·275	0·28	0·28	0·275

Each value is the mean of four determinations.

Discussion.

Although the work described in this and the succeeding papers has not yielded the exact knowledge of the second mechanism which we sought, the results have enabled us to extend and to correct our previous information as to the qualities of this mechanism and its relationship to phosphatase, while new ideas have emerged which may usefully form a basis for further experiment. A brief survey may be attempted.

The phosphatase hypothesis in its simplest form assumed that the blood is normally in equilibrium with the bone salt and that a condition of supersaturation, which is the theoretical requirement for precipitation of this salt, is produced locally in hypertrophic cartilage and osteoid by the phosphatase of these tissues acting on phosphoric esters derived from the blood. That calcification could be effected in this way was made clear by experiments *in vitro*; while further evidence of various nature emphasised the intimate connection of phosphatase with bone development.

But it was apparent that this simple scheme is not in itself sufficient. A difficulty arises from the presence of phosphatase in many tissues other than bone. It is true that phosphatases are required for other physiological processes as well as for calcification; it is true also that the phosphatase activity of the bone is high and that it is highest at the period when calcification is most active; but the question remains, if the enzyme in these other tissues sets free inorganic phosphate in a fluid already saturated with the calcium salt, why is this salt not deposited in these tissues as it is in bone? Certain of these tissues are liable to become calcified in abnormal or pathological conditions and the relation of this tendency to the distribution of phosphatase is being investigated [Rosenheim and Robison, 1934; Macfarlane *et al.*, 1934]. We incline to believe that the effect of the enzyme in such cases can, at most, be supplementary to other factors.

Another difficulty is that solutions may be considerably supersaturated with the bone salt and yet remain relatively stable. Unpublished experiments have shown that materials of different types may be immersed in such solutions for several days without any deposit being formed upon them. Clearly, some further qualities, differentiating hypertrophic cartilage and osteoid from other tissues, were required; and the experiments of Shipley *et al.* [1926], fully confirmed in this laboratory, proved that such qualities exist.

In our previous paper [Robison *et al.*, 1930] we showed how the calcifying power attributable to these special qualities of hypertrophic cartilage and that due to the phosphatase mechanism could be separately demonstrated *in vitro* under suitable experimental conditions. An empirical distinction between the two mechanisms was thus established but this did not exclude the possibility of their intimate relationship. For the present it is convenient to discuss them

as separate components of a dual system by which the normal process of calcification is effected in cartilage and bone.

We have evidence that both mechanisms arise in hypertrophic cartilage and osteoid tissue and reach their highest activity with the full histological development of these tissues, thereafter becoming weaker as tissue degeneration proceeds[1] [Fell and Robison, 1929; 1930; Rosenheim 1934, 2]. It has been shown also that the power of the second mechanism varies considerably in the cartilage of different animals of the same species and even of the same litter and that this variation is connected in some way with the condition of the animal; it may possibly be related to its store of vitamin D, although at present the evidence for this is slight and indirect [Rosenheim, 1934, 2].

The second mechanism appears to be more sensitive than the phosphatase to various agents and conditions. It is greatly weakened by potassium cyanide, by treatment of the cartilage with various solvents or by its desiccation. It is not, however, entirely destroyed by these agencies, for even after such treatment the cartilage retains a greater tendency to become calcified in highly supersaturated inorganic solutions than is ever possessed by the aorta or kidney. The activity of the second mechanism also declines more rapidly than that of the phosphatase after the bones are removed from the body and the slices placed in the experimental salt solutions at 37°. In distilled water or in absence of bicarbonate the decline is still more rapid but is retarded in the salt solutions at lower temperatures. These facts suggest that the second mechanism is bound up with some labile structure of the tissue colloids but does not depend for its functioning on the living cell.

Many of the experimental findings could be readily explained by attributing to this mechanism the properties of a trigger, its effect on the supersaturated tissue fluid being similar to that of a crystal of sodium sulphate on the supersaturated solution of this salt. Conceivably, surface forces at fibrillar interfaces might exert some such action, though experiments in this direction have given only negative results.

Another suggestion [Robison and Soames, 1924] that the p_H within the cartilage is raised above that of the surrounding medium, also lacks experimental support.

Some of the results recorded in this paper suggest strongly that the second mechanism is, like the phosphatase, enzymic in nature; while none of the experimental data is inconsistent with this view. In particular the very pronounced inhibitory action of sodium iodoacetate and sodium fluoride recalls the effect of these substances on the enzymes of yeast and muscle, preventing the fermentative breakdown of phosphoric esters in the cycle of changes.

Prior to these experiments with iodoacetate and fluoride Harris [1932] had drawn attention to the parallelism between the presence of phosphatase and the storage of glycogen in cartilage and had put forward the very interesting suggestion that the chemical system in the ossification process might bear some relation to that in yeast and muscle cells. He suggested that the "hypertrophic cartilage cells provide both the phosphatase enzyme and the glycogen; the latter on hydrolysis yields hexosephosphoric esters which, under the action of the phosphatase and the calcium of the circulating body fluids, lead to the deposition of an insoluble phosphate of calcium in the matrix." Fell and Robison [1933] pointed out that phosphatase is also actively secreted by the osteoblasts which, according to Harris, are devoid of glycogen; but they considered that these facts are not necessarily inconsistent with his suggestion.

[1] Other experiments bearing on this point will shortly be published by Fell and Robison.

It may be that the bone is not entirely dependent on the blood for its supply of phosphoric esters, but that these are also synthesised in the hypertrophic cartilage and osteoid as a stage in the building-up and breaking-down of glycogen. A cycle of synthesis and subsequent hydrolysis in the matrix could obviously serve as a mechanism for raising the level of inorganic phosphate at the site of calcification. Phosphatase might thus prove to be an essential component of the second mechanism, the whole consisting of a complex system analogous to that in yeast and muscle. Part of this system might be inactivated by such agencies as acetone or desiccation, or inhibited by potassium cyanide, iodoacetate or fluoride; while the phosphatase, being unaffected, could still function if phosphoric esters were supplied. To these suppositions a parallel might readily be found in the effect of various agents on the fermentative complex of yeast.

It may be noted that while calcification *in vitro* of hypertrophic cartilage can be effected by agency of the phosphatase mechanism when the second mechanism has been inhibited or destroyed, the converse, calcification by second mechanism activity after destruction of phosphatase, has never been demonstrated. Further, it has been shown that tissues such as kidney may possess a high phosphatase activity without the second mechanism, but no tissue has been found which possesses an active second mechanism without phosphatase.

On the whole we may say that the results of our experiments emphasise the possible enzymic nature of the second mechanism and its close relationship with the phosphatase. They justify certain comparisons with the enzyme systems of yeast and muscle and thus lend support to the stimulating suggestion of Harris. The value of this suggestion may not necessarily depend on the proof that glycogen storage precedes calcification—a contention concerning which there appears to be some doubt.

The analogy between the systems of bone and muscle cannot be pressed too far, since muscle does not contain the bone-phosphatase nor does it become calcified. Nor does the more general conception of the second mechanism as an enzyme system in any way preclude the possibility that factors of a different type, such as surface forces in the colloidal matrix, may also assist in promoting the deposition of calcium salts from the tissue fluids after supersaturation has been achieved by enzyme action.

The experiments on the deposition *in vitro* of barium, strontium and magnesium salts show that the second mechanism is not entirely specific for the calcium salt of bone; but they also indicate that the properties of this calcium salt are very specially appropriate to the function which it fulfils. Although magnesium is a normal constituent of bone and teeth, the very high concentration of this element found necessary for its deposition *in vitro* provides evidence that in the body it is precipitated as part of a much less soluble complex salt in which it replaces an equivalent amount of calcium.

The inhibitory influence of proteins and of magnesium and other ions on calcification may probably be explained by their effects on the ionisation of calcium and phosphate, though the possibility of some direct action on the second mechanism is not excluded. It is not so easy to account for the marked inhibitory action of glucose which has also been investigated by zu Hörste [1932]. The reason may perhaps be bound up with the possible relation of the second mechanism to carbohydrate breakdown.

The beneficial effect of bicarbonate on calcification in inorganic solutions was considered in our previous paper to supply some evidence that the deposited bone salt is a complex carbonato-phosphate. The present experiments suggest

an additional or alternative explanation—that the presence of bicarbonate in the calcifying solutions favours survival of second mechanism activity. Bicarbonate may serve to neutralise lactic or other acids produced by the tissue enzymes, thus preventing a rapid fall in the internal p_H of the cartilage. Before accepting this explanation we must know to what extent the internal p_H is independent of that of the external medium, since the bicarbonate-free solutions which failed to produce calcification did not themselves show any significant fall in p_H, and since immersion of the bone slices in solutions of p_H 5·4 for one hour was not found to affect the calcifying power of the cartilage. The question of the internal p_H of the cartilage and osteoid matrix bears so directly on the problems of calcification that some method by which it could be measured immediately before calcification begins and without injury to the calcifying mechanism would be of the greatest value.

The gradual decline in the calcifying power of the bone slices after excision, and particularly in the activity of the second mechanism, points to a limitation of this experimental method of studying calcification *in vitro*. To counterbalance the effect of this loss of power, somewhat higher concentrations of the essential ions than are adequate for progressive calcification in the body may be required for deposition in the restricted period *in vitro*, so that direct comparison of the limiting concentrations *in vitro* with those *in vivo* may be misleading. The method remains, however, most valuable for the comparative study of factors which may influence either the concentrations of these ions or the activity of the mechanisms.

Summary.

1. The nature and properties of the dual calcifying mechanism of hypertrophic cartilage have been further investigated.

2. It was shown that α- and β-glycerophosphates, hexosemonophosphate, fructosediphosphate and trehalosemonophosphate are satisfactory substrates for the bone-phosphatase in calcification *in vitro*. Diphosphoglycerate and lecithin were not effective.

3. Deposits of the salts of barium, strontium and magnesium were obtained in the hypertrophic cartilage *in vitro* by the phosphatase mechanism. Deposits of strontium salts were also obtained from highly supersaturated inorganic solutions containing no phosphoric ester. The deposits were formed in the matrix of the hypertrophic cartilage and usually resembled those of calcium salts. The second mechanism is, therefore, not entirely specific for the calcium salt of bone.

4. Calcification occurred in presence of 0·001 *M* potassium cyanide in bone slices immersed in highly concentrated inorganic solutions when the original second mechanism activity of the bone was high. The inhibition of the second mechanism by potassium cyanide previously reported is, therefore, not complete.

5. The two components of the calcifying mechanism could not be inactivated differentially by heating the bone slices at different temperatures or by soaking them in isotonic salt solutions at different p_H levels.

6. A gradual decline in the activity of both mechanisms occurred when the excised bone slices were immersed in various salt solutions at 37°. This decline was more rapid for the second mechanism than for the phosphatase. It was more rapid also in absence of bicarbonate or in distilled water, but was retarded at lower temperatures.

7. Vitamin D or hormone preparations known to influence the growth and calcification of the skeleton *in vivo* had no demonstrable action on the calcification or decalcification of bone slices *in vitro*.

8. Glucose in concentrations of 50–100 mg. per 100 ml. had a very definite inhibitory effect on calcification *in vitro* in inorganic solutions, but no such effect could be demonstrated for fructose, sucrose, dulcitol or erythritol. Glycine and urea, also, in concentrations covering the normal physiological range were without significant effect on the activity of the second mechanism.

9. Sodium fluoride in concentrations as low as 0·00001 *M* and sodium iodoacetate in concentration of 0·0001 *M* exerted a pronounced inhibitory effect on the second mechanism, but not on the phosphatase.

10. The nature of the dual calcifying mechanism is discussed in the light of these and other experimental results.

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DESCRIPTION OF FIGURES IN PLATE I.

Figs. 1 to 4. Deposits of magnesium and strontium salts in the hypertrophic cartilage zone stained with silver nitrate.

Figs. 5 and 6. Crystalline deposit of barium salt unstained.
X = deposit of Mg, Sr or Ba salt formed *in vitro*.

Fig. 1. Microtome section of a tibia slice after immersion for 17 hours in solution 0·1 *M* Mg : 10 : 60 containing high concentrations of magnesium and phosphoric ester. Note the extensive deposit of magnesium salt in the matrix of the previously uncalcified hypertrophic cartilage zone. Magnification ×20.

Fig. 2. Tibia slice after immersion for 19 hours in the inorganic solution 0·01 *M* Sr : 12 : 0. Note the dense deposit of strontium salt formed in the hypertrophic cartilage zone in absence of phosphoric ester. Magnification ×8.

Fig. 3. Microtome section of part of a tibia slice after 19 hours in the solution 0·005 *M* Sr : 8 : 30, containing phosphoric ester. Magnification ×25.

Fig. 4. Deposit of strontium salt formed in 19 hours in solution 0·005 *M* Sr : 0 : 30. The appearance of the deposit in the matrix of the hypertrophic cartilage cells closely resembles that of a normal deposit of calcium salt. Magnification ×160.

Fig. 5. Barium salt deposited in crystalline form in the hypertrophic cartilage zone of the tibia of a rachitic rat immersed for 19 hours in solution 0·005 *M* Ba : 5 : 30. Magnification ×25.

Fig. 6. The same crystals photographed by polarised light between crossed Nicols. Magnification ×50.

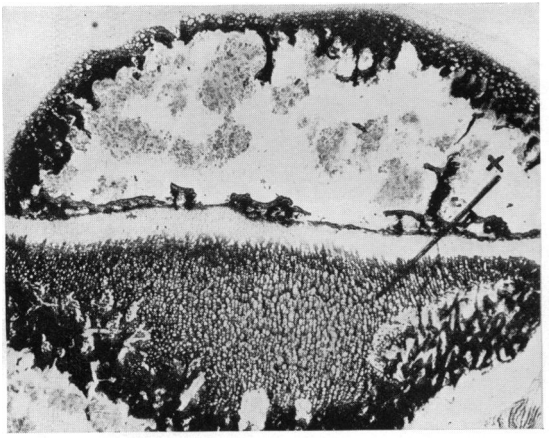
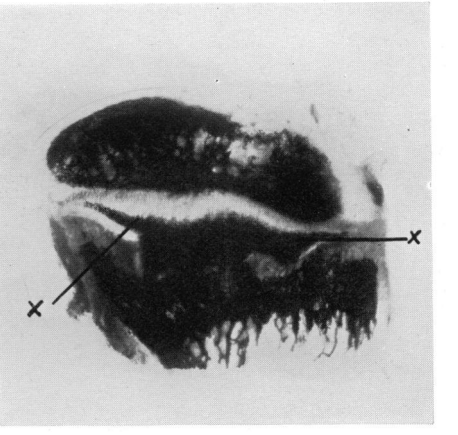


Fig. 1.



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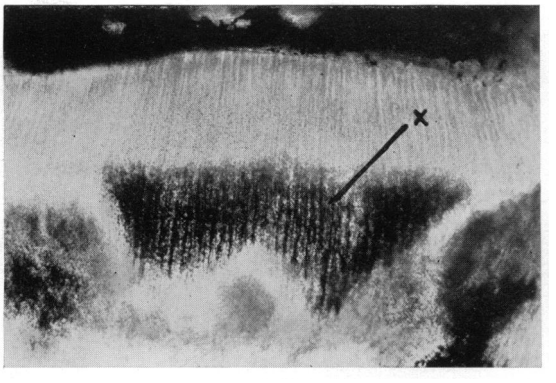

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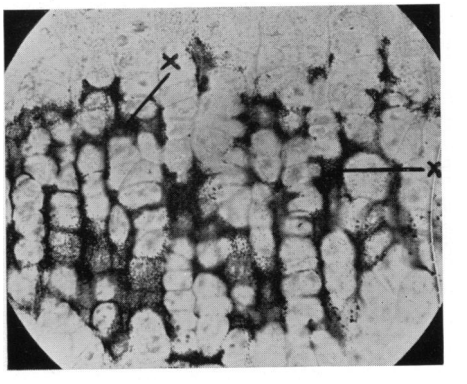

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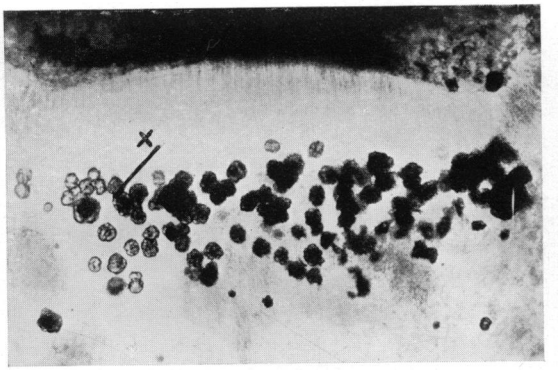

Fig. 5.

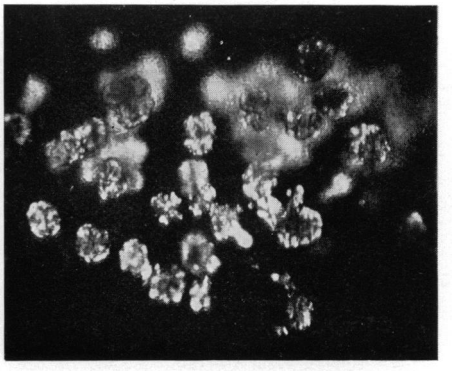

Fig. 6.