XCVI. THE EFFECT OF SERUM-PROTEINS ON CALCIFICATION *IN VITRO*.

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THE inhibitory effect of egg-albumin on the calcification of bone slices in organic salt solutions was noted by Shipley *et al.* [1926]. The concentrations of calcium and inorganic phosphate in the solutions used by these workers were approximately those found in normal serum; but as was pointed out by Robison [1926], this apparent similarity is misleading owing to the absence of the other constituents of plasma, particularly the proteins, which are present in a concentration of 7 % and greatly reduce the ionisation of calcium. In Shipley's solutions as little as 2 % egg-albumin was sufficient to inhibit calcification. To bring about deposition of the bone salt from plasma, therefore, some mechanism for increasing the concentration of calcium or phosphate ions would be required, and such a mechanism is furnished by the bone-phosphatase.

The experiments described in this paper were planned to study the effect of serum-proteins on the calcification of hypertrophic cartilage *in vitro*, both in inorganic solutions and in those containing phosphoric ester.

EXPERIMENTAL.

The method of experiment was similar to that described by Robison *et al.* [1930]. Slices from the heads of the long bones of rachitic rats were immersed for periods of 16 to 20 hours in the sterile experimental solutions at 37° and p_H 7.4. They were then fixed, stained with silver nitrate and cleared in cedar-wood oil. The amount of new calcification in the zone of hypertrophic cartilage was judged by examination under low magnification and is shown in the tables by a figure (maximum 10) representing the extent of the deposit, and bars (maximum 5) indicating the density, thus 6≡. Except where otherwise stated each experiment was carried out with bone slices from the same rachitic rat.

Only those modifications in the technique necessitated by addition of protein will be described in greater detail.

Serum-proteins were obtained from normal horse serum by saturation with ammonium sulphate, followed by solution of the precipitate and reprecipitation. The ammonium sulphate precipitate was dialysed in cellophane sacs against hot tap water, distilled water and finally 0.8 % NaCl, and the stock protein solution thus obtained was filtered through Seitz filters and stored at 0° in a sterile bottle fitted with a syphon for withdrawal of samples. The protein content of

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the solution was estimated by Kjeldahl nitrogen determinations and also by refractometer¹.

The stock protein solution used for most of the experiments quoted contained 0.76 g. N per 100 ml. ($\equiv$ 11 % protein), and further, 0.8 g. NaCl, 9.55 mg. Ca, 0.53 mg. Mg and 0.1 mg. P per 100 ml. This was diluted as required with a basal salt solution of such concentration that the final salt contents of the calcifying solutions containing protein and of the protein-free solutions should be the same.

Protein-salt solutions containing the requisite amounts of calcium and inorganic and organic phosphate, and made up in slightly more concentrated form than finally required, were sterilised by filtration through a Seitz filter into a pyrex flask².

From the sterile protein-salt solutions the final solutions were prepared in 20 ml. flasks by addition of water, glucose or other constituents as required. The p_H of these solutions was adjusted to 7.35–7.4. Measurements were made with a capillator with correction for protein error and were checked by occasional electrometric measurement.

The sterility of the solution in each flask was tested at the end of the experimental period by incubating a drop on an agar slope. Experiments in which there was serious contamination with consequent fall in p_H were rejected.

*The inhibitory effect on calcification in vitro of protein
in various concentrations.*

In the experiment quoted in Table I, bone slices from the same rat were immersed in solutions containing 0, 1.5 and 3 % serum-proteins at various

Table I. *Effect of serum-proteins on calcification in vitro.*

mg. per 100 ml.			Protein g. per 100 ml.		
Ca	P	P	0	1.5	3
	inorg.	org.	Calcification <i>in vitro</i>		
10	4	0	6 $\equiv$	2 $\equiv$	0
10	6	0	.	6 $\equiv$	7 $\equiv$
10	4	3	8 $\equiv$	8 $\equiv$	.
10	4	10	.	8 $\equiv$	7 $\equiv$

All the bone slices were from the same rachitic rat.

levels of calcium, inorganic and organic phosphate. In absence of protein there was good calcification in the inorganic solution; 10:4:0³ but calcification in

¹ For assistance in preparing these solutions, and for the refractometer measurements I am indebted to Dr W. T. J. Morgan.

² Estimations of calcium and magnesium were at first made on the non-sterile solutions before filtration, but later it was found that appreciable amounts of Mg and Ca were being extracted from the Seitz asbestos pads. In this way the concentrations of Mg and Ca were being raised by as much as 0.7 mg. Mg and 1 to 1.5 mg. Ca per 100 ml.

Tests showed (a) that silica was not extracted simultaneously, and (b) that all the soluble Mg and Ca could be extracted by first passing through the pads 50 ml. of a solution similar to the basal salt solution but free from Ca and Mg. This modification of the technique described above was adopted in all further experiments, although the certainty of sterilisation by filtration was thereby somewhat lessened. Only a few of the early experiments in which the Ca and Mg concentrations were raised by filtration have been quoted, and these are marked with an asterisk.

³ The experimental solutions are referred to in this way throughout the text. Thus, solution 10:4:3 contains 10 mg. Ca, 4 mg. P as inorganic phosphate and 3 mg. P as phosphoric ester per 100 ml.

this solution was partially inhibited by 3 % protein. In solution 10 : 6 : 0 and in the ester solution 10:4:10 calcification still occurred even in presence of 3 % protein.

Table II. *Effect of serum-proteins on calcification in vitro.*

mg. per 100 ml.			17 hours; 37°; p_H 7.4.										
			Rat	<i>a</i>		<i>b</i>		<i>c</i>		<i>d</i>		<i>e</i>	
Ca	P inorg.	P org.	Protein g./100 ml.	0	7	0	7	0	7	0	7	0	7
10	3	0		.	.	1—	.	.	.	.	.	0	.
10	4	0		6≡	.	2=	0	7≡	0	2≡	.	2=	0
10	5	0		.	.	6≡	0	.	2=	.	0	.	0
10	6	0		.	1=	.	4≡	.	5≡	.	0	.	0
10	7	0		.	2=	.	5≡	.	7≡	.	0	.	.
10	8	0		.	5≡	.	.	.	.	.	2=	.	.

The concentration of protein was raised to 7 % in the experiments quoted in Table II. Considering first the ester-free solutions, it will be seen that the lowest level of $Ca \times P$ at which calcification regularly took place was 10×6 in presence of protein as compared with 10×3 in absence of protein. In one experiment only, out of a large number, was calcification obtained at the lower level 10×5 in protein solution (rat *c*). Where the calcifying power of the bones was low, as in rat *d*, a level as high as 10×8 was necessary in the protein solutions before calcification occurred.

The inhibitory effect of glucose on calcification in protein-salt solutions.

It is shown elsewhere that glucose has an inhibitory effect on calcification *in vitro* in protein-free solutions [Robison and Rosenheim, 1934]. From the results given in Table III it will be seen that this inhibitory effect was equally marked in 7 % protein solution. Inhibition due to 0.01 % glucose was not demonstrable, but in presence of 0.03 % there was total inhibition of calcification in solution 10 : 7 : 0 and partial inhibition in solution 10 : 9 : 0. In presence of 0.05 or 0.1 % glucose calcification was only twice obtained at a level lower than 11×10 in a total of 11 experiments, but no obvious progressive inhibition of calcification was observed with concentrations of glucose greater than 0.1 %.

Table III. *Effect of glucose on calcification in vitro in presence of protein.*

mg. per 100 ml.				Protein 7 g./100 ml.; 18 hours; 37°; p_H 7.4.						
Exp.	mg. per 100 ml.			Glucose mg. per 100 ml.						
	Ca	P inorg.	P org.	0	10	30	50	75	100	150
34	10	6	0	4≡	.	.	.	.	0	.
	10	7	0	5≡	.	.	.	.	1=	.
30	10	8	0	2≡	.	.	.	.	0	.
	10	10	0	.	.	.	.	.	0	.
17*	10	7	0	4=	4=	0	0	.	0	.
	10	9	0	7≡	7≡	3≡	0	.	0	.
15*	11	9	0	7≡	.	.	0	.	0	.
	11	10	0	9≡	.	.	4=	.	5≡	.
23*	11	10	0	9≡	.	.	5≡	3≡	2=	2=
	10	12	0	9≡	.	.	.	5≡	3=	6≡

*The inhibitory effect of magnesium on calcification
in protein-salt solutions.*

It has been shown [Shipley and Holt, 1927; Kramer *et al.*, 1927; Robison *et al.*, 1930] that magnesium exerts a definitely inhibitory effect on calcification in inorganic solutions, so that to obtain a given amount of deposit progressively higher levels of the Ca $\times$ P product are needed as the magnesium concentration is increased. The two experiments given in Table IV show that the same effect is exerted also in presence of glucose and protein.

Table IV. *Effect of magnesium on calcification in vitro in presence of protein and glucose.*

Protein 7 g./100 ml.; glucose 100 mg./100 ml.; 17 hours; 37°; p_H 7.4.

Rat a					Rat b				
mg. per 100 ml.			mg. Mg per 100 ml.		mg. per 100 ml.			mg. Mg per 100 ml.	
Ca	P inorg.	P org.	1.1	3.2	Ca	P inorg.	P org.	2.6	4.1
11	7	0	2 =	0	10	6	0	0	.
11	8	0	1 =	0	10	7	0	2 =	.
11	9	0	1 =	0	10	8	0	3 =	0
11	10	0	4 =	2 =	10	9	0	6 =	3 =
11	12	0	7 =	6 =	10	10	0	.	5 =
					10	12	0	.	8 =

It will be seen that with a low magnesium concentration of 1.1 mg. per 100 ml. calcification was obtained in bone slices from rat *a* at the Ca $\times$ P level 11 $\times$ 7, in presence of 7 % protein and 0.1 % glucose. When the magnesium concentration was increased to 3.2 mg. per 100 ml. no calcification was obtained at a level lower than 11 $\times$ 10.

In bone slices from rat *b* calcification was obtained in presence of 7 % protein, 0.1 % glucose and 2.6 mg. Mg per 100 ml. at the exceptionally low Ca $\times$ P level of 10 $\times$ 7 and in presence of 4.1 mg. Mg, at the level 10 $\times$ 9. The inhibition due to increased magnesium concentration is here still apparent, although the bones used for this experiment showed a definitely higher calcifying potentiality than usual, thus further illustrating that variability discussed elsewhere [Rosenheim, 1934].

*The beneficial effect of phosphoric ester on calcification in
protein-salt solutions.*

The effect of sodium glycerophosphate on calcification in presence of 7 % protein was next investigated. Results obtained in glucose-free solutions are given in Table V. They show that there was no calcification at levels lower than 12 $\times$ 6 in absence of ester, whereas in presence of glycerophosphate equivalent to 3 mg. P per 100 ml. calcification occurred at the level 12 $\times$ 4. In solution 12 : 4 : 10 calcification was more extensive and denser than in solution 12 : 7 : 0.

The results given in Table VI show that the beneficial effect of phosphoric ester was equally marked in solutions containing 0.1 % glucose.

Table V†. *Effect of phosphoric ester on calcification in vitro in presence of protein.*Protein 7 g./100 ml.; 17 hours; 37°; p_H 7.45.

Exp.	mg. per 100 ml.		Glycerophosphoric ester mg. organic P per 100 ml.		
	Ca	P inorg.	0	3	10
5*	12	4	0	0	6≡
	12	6	2—	.	.
7*	12	4	.	4=	9≡
	12	5	0	5≡	7≡
	12	6	3=	.	.
	12	7	6≡	.	.

† It will be noted that the slightly increased Ca and Mg concentrations in the solutions used for the experiments quoted in this and the following table (see footnote 2, p. 700) do not in any way affect the conclusions drawn, as the results in each experiment are on a strictly comparable basis.

Table VI. *Effect of phosphoric ester on calcification in vitro in presence of protein and glucose.*Protein 7 g./100 ml.; glucose 100 mg./100 ml.; 37°; p_H 7.4.

Exp.	Time (hours)	mg. per 100 ml.		Glycerophosphoric ester mg. organic P per 100 ml.				
		Ca	P inorg.	0	0.5	1	3	10
8*	17	12	4	.	.	.	0	3≡
		12	5	0	.	0	0	.
		12	6	0	.	.	.	.
		12	7	2≡	.	.	.	.
9*	17	12	5	.	.	.	0	7≡
		12	6	0	0	0	0	7≡
11*	17	12	6	0	.	.	3≡	5≡
		12	7	0	.	0	2≡	6≡
		12	8	0	.	1=	4≡	.
12*	10	12	8	0	.	3—	4=	8≡
		12	9	4=	4=	7—	7=	8=
14*	17	10	8	0	.	0	5≡	.
		10	9	0	.	0	6≡	.
		10	10	5≡	.	6≡	7≡	.

Calcification in vitro in the serum of various species.

Having obtained the above data with regard to calcification *in vitro* in artificial protein-salt solutions, a further series of experiments was carried out with the sera of normal animals.

It was essential to obtain the blood without the use of anaesthetics, at the same time avoiding any condition involving fright or surgical shock, all of which may lead to a rapid increase in the inorganic phosphate of the serum. It was equally important to avoid haemolysis even in slight degree, since this would liberate appreciable amounts of phosphoric ester from the red blood corpuscles.

Serum was obtained from adult rabbits by bleeding from the ear vein and from young rabbits and rats by heart puncture, precautions being taken to maintain sterility. Some difficulty was encountered in obtaining sufficient sterile serum from young rats for the calcification experiment and for the necessary analyses.

The serum was separated from the corpuscles as rapidly as possible to avoid the alteration in inorganic phosphate content which may occur when whole blood stands at room temperature [Martland and Robison, 1926]. The serum was then measured into small sterile tubes, and when required, inorganic phosphate was added in the most concentrated form possible, the dilution of the serum not usually exceeding 0.5 %. The p_H was adjusted to 7.4 with sterile alveolar air or CO_2 . In all the experiments quoted, control slices from the same animal were immersed for the same length of time in solution 8:4:0 or 8:5:0 and good calcification was invariably obtained even when the volume of solution was reduced to 2 ml. In small volumes of serum the p_H tended to fall slightly during the experimental period.

In Table VII are shown the results of experiments on the calcification of bone slices of rachitic rats in the serum of normal rats. They are arranged in order of increasing $Ca \times P$ product of the serum. Results (1)–(4) in any one experiment were obtained with bone slices from the same animal. The negative result obtained with the serum of one rachitic rat (*R*), in which the $Ca \times P$ product was only 28, is included in this table.

The lowest level of the $Ca \times P$ product at which there was calcification without addition of extra phosphate was 87 (Exp. 15 (1) and (2)). No calcification occurred in 17 hours in a normal serum of $Ca \times P$ level 57 (Exp. 13 (1)), but in samples of the same serum to which inorganic phosphate was added to bring up the levels to 68 and 80 respectively there was slight calcification.

Table VII. *Calcification in vitro in rat serum.*

Exp.	Vol. of serum ml.	Time (hours)	mg. per 100 ml.			Ca × P	Calcifi- cation
			Ca	Inorganic P			
				Serum	Added		
R16	2.5	17	10.3	2.7	.	28	0
13 (1)	2.0	17	9.1	6.3	.	57	0
(2)	"	"	"	"	1.2	68	<1 –
(3)	"	"	"	"	2.5	80	3 =
15 (1)	"	16	9.9	8.8	.	87	3 =
(2)	"	40	"	"	.	"	3 =
10	2.5	17	9.5	9.6	.	91	8 ≡
9	3.0	16	11.2	8.2	.	92	6 =
7	2.0	18	12.1	8.6	.	104	8 ≡
Y 6	2.0	17	10.9	11.7	.	129	7 ≡

R = Rachitic young rat.

Y = Normal young rat.

Experiments on the calcification of rat bones in rabbit serum are summarised in Table VIII. The serum-calcium of rabbits is higher than that of rats and subject to considerable fluctuations [Culhane, 1927; Kapsinow and Underhill, 1929] but in the samples used the product $Ca \times P$ was generally lower than in rat's serum. A $Ca \times P$ level higher than 77 could not be reached without addition of inorganic phosphate, and calcification was not obtained in adult rabbit's serum without such addition, the lowest level for calcification being 87, artificially produced. The inorganic phosphate in the serum of young rabbits is, however, slightly higher than in that of the full-grown animals, and in one such serum with a $Ca \times P$ product of 109, slight calcification occurred in 41 hours.

Table VIII. *Calcification in vitro in rabbit serum.*

Exp.	Vol. of serum ml.	Time (hours)	mg. per 100 ml.				Calcification
			Ca	Inorganic P		Ca x P	
				Serum	Added		
3 (1)	5	40	15.8	3.0	.	47	0
11 (1)	2	17	17.5	2.84	.	50	0
(2)	"	"	"	"	.	"	0
8 (1)	1	18	14.4	3.8	.	55	0
(2)	2	"	14.0	4.0	.	56	0
2 (1)	5	40	14.0	4.0	.	56	0
(2)	"	"	"	"	1.0	70	0
1 (1)	6	16	15.0	5.12	.	77	0
(2)	"	40	"	"	.	"	0
3 (2)	5	19	15.8	3.0	2.5	87	1—
2 (3)	"	40	14.0	4.0	2.3	88	2=
8 (3)	2	18	14.0	4.0	2.3	"	3=
11 (3)	"	17	17.5	2.8	2.5	93	5≡
(4)	"	"	"	"	3.5	110	5≡
3 (3)	5	19	15.8	3.0	2.5	130	8≡
(4)	"	"	"	"	8.0	174	10≡
Y 4 (1)	2	23	12.5	8.6	.	108	0
(2)	5	"	"	"	.	"	0
Y 5 (1)	"	41	13.0	8.4	.	109	1—
(2)	"	"	"	"	1.8	134	4≡

Y = Young rabbit.

Although samples of rat serum containing 10 mg. Ca and 9 mg. P per 100 ml., and rabbit serum containing 15 mg. Ca and 6 mg. P per 100 ml. have the same Ca × P product of 90 there is no suggestion that the same percentage of calcium is ionised in the serum of these two species or that their calcifying power is the same. The assumption that the calcifying power is directly proportional to the simple product Ca × P is theoretically unjustifiable, so that clearly only a limited significance can be attached to a comparison of solutions and sera on this basis.

The fact that calcification in rat serum is not markedly better than in rabbit serum, and only slightly better than in artificial 7 % horse serum-protein solution, disposes of the objection that in some experiments failure to use homologous serum, or rat serum-proteins, might be affecting the results.

 Table IX. *Calcification in vitro in human serum.*

Exp.	Serum	Vol. of serum ml.	Time (hours)	mg. per 100 ml.			Ca × P	Calcifi- cation
				Ca	Inorganic P			
					Serum	Added		
12	Adult	1	17	9.6	3.9	.	37	0
		2	"	"	"	.	"	0
		"	"	"	"	1.1	48	0
		"	"	"	"	2.3	59	5 =
		"	"	"	"	3.4	69	5 =
		"	"	"	"	4.6	81	5 ≡
14	Child 10 years	2	18	10.8	5.3	.	57	0
		"	"	"	"	0.9	66	1 =
	Child 4 years	"	"	"	"	1.8	76	3 ≡
		"	"	10.1	6.3	.	64	1 =

The results of experiments with the serum of human adults and children are given in Table IX. The levels adequate for calcification were lower than in rat and rabbit serum. Definite calcification occurred when inorganic phosphate was added to raise the $\text{Ca} \times \text{P}$ levels from 37 to 59 and from 57 to 66 respectively.

Calcification occurred without added phosphate in serum from a child of 4 years, the $\text{Ca} \times \text{P}$ product being 64¹.

In general it will be seen that the higher the $\text{Ca} \times \text{P}$ product of the solution bathing the bone slices, the denser and more extensive was the new deposit obtained.

DISCUSSION.

It is known that a considerable proportion of the total calcium of blood is present in non-diffusible form and is probably largely combined with the plasma-proteins. The consequent reduction in the concentration of calcium ions must profoundly affect the calcifying power of the plasma, a conclusion which is fully borne out by the results of the experiments described in this paper. The addition of serum-proteins to the inorganic calcifying solutions rendered very much higher levels of the product $\text{Ca} \times \text{P}$ necessary, in order that calcification of the bone slices should take place *in vitro*.

Since calcification in the body may take place more slowly and under more favourable conditions than in these experiments it may reasonably be expected that somewhat lower levels of the product $\text{Ca} \times \text{P}$ than those here required may suffice for calcification *in vivo*. These experiments, therefore, do not give precise information as to the lowest value of this product which would be effective in the body. They show that under the experimental conditions the sera of very young animals, and sometimes also of older animals, may bring about calcification within 20 hours in the hypertrophic cartilage of rachitic rats, but that with many sera it is necessary to increase the phosphate concentration artificially in order that this result may follow.

Still higher levels of the product $\text{Ca} \times \text{P}$ were required with artificial solutions similar to plasma in their content of inorganic salts, protein and glucose, but here, as in all previous experiments, the necessary $\text{Ca} \times \text{P}$ level was notably reduced if phosphoric ester was added.

The better results obtained with natural as compared with artificial sera may well have been due to the small amount of phosphoric ester present in the former; but other constituents of serum not included in the artificial solutions may also have influenced the results.

SUMMARY.

1. The effect of serum-proteins in different concentrations on the calcification *in vitro* of hypertrophic cartilage has been investigated.

2. In presence of horse serum-proteins in 7 % concentration the lowest $\text{Ca} \times \text{P}$ level at which calcification regularly took place in solutions containing no phosphoric ester was 10×6 as compared with 10×3 in absence of protein.

3. The inhibitory effect of glucose on calcification observed in non-protein solutions was apparent also in presence of 7 % protein and was additional to the effect of the latter, so that in salt solutions containing 0.1 % glucose and 7 % protein calcification was not usually obtained at a level lower than 11×10 .

4. The inhibitory effect of magnesium on calcification previously observed in non-protein solutions was apparent also in presence of protein.

¹ Shipley *et al.* [1926] have stated that they obtained calcification in bone slices immersed in children's serum of $\text{Ca} \times \text{P}$ product 50 to 54.

5. In presence of glycerophosphoric ester calcification in protein-salt solutions took place at lower levels of the $\text{Ca} \times \text{P}$ product than in its absence. Thus in bone slices from the same rat calcification was obtained in solution 12 : 4 : 3, but not in solution 12 : 6 : 0.

6. Calcification *in vitro* was obtained in the serum of adult rats and rabbits at a $\text{Ca} \times \text{P}$ level of 80–90, and in human serum at a level of about 60. In the serum as drawn with precautions to avoid alteration in inorganic phosphate concentration the requisite level of the $\text{Ca} \times \text{P}$ product was rarely reached, so that the addition of inorganic phosphate was necessary in order that calcification should take place under the experimental conditions.

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