XCVI. THE POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION. PART III.

THE ACTION OF THE BONE ENZYME ON THE ORGANIC PHOSPHORUS COMPOUNDS IN BLOOD.

By HERBERT DAVENPORT KAY (*Beit Memorial Research Fellow*) and ROBERT ROBISON.

From the Biochemical Department, Lister Institute.

(*Received May 1st, 1924.*)

The occurrence in bone and ossifying cartilage of an enzyme which hydrolyses hexosephosphoric esters is not sufficient evidence that such esters are concerned in the process of ossification, for it must further be shown that they are present in the blood and are available wherever calcium phosphate is being deposited. The belief that this would prove to be the case was based on the fact that considerable quantities of acid-soluble organic phosphorus compounds are known to be present in blood, chiefly in the corpuscles, though small amounts have also been found in the plasma. The following averages are taken from Bloor's [1918] analyses of human blood (males).

mg. P per 100 cc.

			Acid-soluble	
	Total	Lipin	Inorganic	Other forms
Corpuscles	79	18·0	5·9	55
Plasma	10·2	7·0	2·7	0·5

The chemical composition of these acid-soluble compounds is still unknown, but their hydrolysis on boiling with dilute acids indicates that they are esters of phosphoric acid. The evidence produced by Embden and his collaborators that the lactacidogen of muscle is a hexosediphosphoric ester suggested that this or similar compounds might occur in blood. The diphosphoric ester itself could not serve as the required substrate, as it is hydrolysed by an enzyme present in almost all tissues including muscle and non-ossifying cartilage, but both mono- and di-phosphoric esters may be formed in the metabolism of carbohydrate within the animal body, as they are formed during fermentation of sugar by yeast juice. Thus a hexosemonophosphoric ester, synthesised perhaps in the liver, may not only play a part in ossification but, being also absorbed by the muscle, may be there converted into the lactacidogen or alternatively into glycogen. These speculations carry us, however, beyond our present knowledge and it must be granted that glycerophosphoric ester would serve as well as hexosemonophosphoric ester in the suggested scheme of ossification, since these two compounds are hydrolysed with equal rapidity by the bone enzyme [Robison and Soames, 1924].

The complete answer to this problem requires the isolation and identification of the various phosphorus compounds occurring in the blood and considerable progress has been made in this direction, but the experiments described in this paper prove definitely that the blood contains phosphoric esters which are rapidly hydrolysed when brought into contact with the bone enzyme under suitable conditions.

The presence of the necessary substrate is thus demonstrated and the probability that the enzyme plays a part in the ossification process is thereby increased.

The positive evidence so far obtained refers almost entirely to the corpuscles and the objection may be raised that calcium deposition must be determined ultimately by the concentration of calcium and phosphate ions in the plasma, in which the amount of phosphoric ester must in any case be very small. If the concentrations of the ester in plasma and corpuscles are in equilibrium the corpuscles would act as reservoirs. Moreover a very small amount of ester in plasma saturated with calcium phosphate would be sufficient to bring about deposition of the solid phase by increasing the concentration of phosphate ions when hydrolysis occurred. Some experiments here described furnish evidence of the correctness of this view but the difficulty remains for the present a real one.

The combined phosphoric acid which is readily hydrolysed by the bone enzyme represents 14 % to 36 % of the total organic phosphorus in the acid-soluble fraction. The remainder, though very resistant to the action of the enzyme, is hydrolysed by boiling with dilute acids. At least two different acid-soluble phosphoric esters are therefore present and a method by which the respective amounts of each may be estimated with a fair degree of accuracy has been worked out. This method is being applied to the investigation of phosphorus compounds in the blood in certain conditions of health and disease in the hope that the further information thus obtained may throw some light on the problems of phosphorus and of carbohydrate metabolism.

The phospholipins, although derivatives of glycerophosphoric ester, are not acted on by the bone enzyme.

Action of the bone enzyme on the acid-soluble phosphorus compounds of blood.

The first experiments were carried out with sheep's blood (100–200 cc.), corpuscles and plasma being separately examined. Proteins were precipitated with trichloroacetic acid and the filtrates were repeatedly extracted with ether to remove the excess of this acid and also any phosphorus compounds of lipin nature. The neutralised protein-free filtrate from the laked corpuscles was treated with bone extract and kept at 37° for 24 hours. At the end of this period a decided increase was observed in the amount of inorganic phos-

phate (estimated by the modified Neumann method), showing that hydrolysis had occurred. Similar treatment of the plasma filtrate yielded inconclusive results.

The extraction of the filtrate with ether was very troublesome and was soon given up as it was found that sodium trichloroacetate did not interfere with the activity of the enzyme and that the ethereal extracts never contained more than a trace of phosphorus.

In all our later experiments the inorganic phosphate was estimated colorimetrically by the Briggs modification of the Bell-Doisy method, thus enabling us to use much smaller quantities of blood. The method of experiment was modified from time to time as experience suggested but only the procedure finally adopted will be described in detail.

Method of experiment. For the investigation of whole blood 10–20 g. was the amount usually taken, although satisfactory experiments have been carried out with as little as 5 g. Mixed venous and arterial blood was obtained by severing the carotid and jugular after anaesthetising the animal[1]. In other cases the blood was taken from a vein or from the carotid with the aid of a canula. The blood was received in a weighed vessel containing 1–2 cc. of a 1 % solution of potassium oxalate. It was immediately weighed and was then transferred to a stoppered cylinder and laked by adding three to four volumes of distilled water. Proteins were precipitated by the addition of a 25 % solution of trichloroacetic acid in amount equal to the original volume of the blood. Water was added to make the total volume equal to seven times the weight of the blood, frothing being reduced by the aid of the minimum quantity of capryl alcohol. The liquid was vigorously shaken and allowed to stand for ten minutes, after which it was filtered through a No. 30 Whatman paper into a second dry stoppered cylinder. The volume (v) was noted and the filtrate then neutralised by the cautious addition of a 40 % solution of sodium hydroxide, phenolphthalein being used as indicator. By the addition of water the new volume was made equal to $7{\cdot}5/7\ v$. Up to this point the operations were carried out with the utmost expedition owing to the possibility of hydrolysis of phosphorus compounds and consequent increase in the inorganic phosphate. In the neutralised filtrate no appreciable hydrolysis occurs at room temperature while even at 37° such hydrolysis, if it takes place at all, is extremely slow.

The bone extract was prepared by thorough maceration of the bones of young rats or other animals with twenty times their weight of water saturated with chloroform. After standing overnight in the cold room these extracts were filtered and were then kept at 0° in presence of sufficient chloroform. The same extract served for a series of experiments extending over a month or longer and showed no loss of activity.

[1] It is possible that the effects of anaesthesia and the method by which the blood was subsequently laked may have given rise to changes in the amounts of phosphorus compounds. *v.* Martland and Robison [1924].

For the enzyme experiments flasks were prepared containing the neutralised blood filtrate, bone extract, etc. in the following proportions. (The actual quantities varied, but were usually less than those shown.)

	FB Filtrate + enzyme cc.	FO (control 1) Filtrate + water cc.	OB (control 2) Water + enzyme cc.
Blood filtrate (dilution 1 g. blood in 7·5 cc.)	100	100	—
5 % bone extract	10	—	10
Water	—	10	100
Chloroform	1	1	1
Additions for adjustment of p_H (indicator, alkali, etc.) ...	1	1	1

A fourth flask containing filtrate + boiled enzyme was added to the above when the quantity of blood was sufficient, and the activity of the enzyme was tested in a fifth flask (MB) in which the blood filtrate was replaced by the same volume of an $M/1000$ solution of sodium hexosemonophosphate or sodium glycerophosphate. For the adjustment of the p_H either phenolphthalein or thymolphthalein was used. These indicators being colourless in acid solutions did not interfere with the later colorimetric estimations. By using phenolphthalein and adding alkali until a faint pink colour was visible, a p_H near the lower limit of the optimum range was obtained, but unless the reaction was readjusted frequently, the rate of hydrolysis was apt to be somewhat irregular. With thymolphthalein a p_H of 9·0–9·3 was obtained. This was well within the optimum range but carried the disadvantage of a somewhat greater tendency towards hydrolysis in the controls. The flasks were placed in a thermostat at 37·5° and at intervals the inorganic phosphate was estimated in measured volumes of their contents. For the first estimation portions were removed immediately after mixing the contents of the flasks and before the adjustment of the p_H, the necessary volume correction (1 %) being applied to the amount of phosphate found. 5 cc. of the liquid were pipetted into a test-tube containing 1 cc. of N sulphuric acid[1] to precipitate the proteins of the bone extract. (For "OB" 1 cc. of a 15 % solution of trichloroacetic acid was used.) The tubes were shaken and allowed to stand during ten minutes, after which the contents were filtered and 5 cc. of the final filtrates taken for phosphate estimation. This quantity corresponded with $\frac{5}{6} \times 5 \times \frac{100}{111} \times \frac{1}{7{\cdot}5} = 0{\cdot}50$ g. blood.

The total acid-soluble phosphorus was also estimated in the final filtrates by ignition with nitric and sulphuric acid. Hydrolysis was usually complete in about six hours but the experiment was continued for several hours longer, the p_H being readjusted when necessary. If the duration was extended to 24 hours the results were apt to be complicated by slight irregular increases in the inorganic phosphate, due partly to chemical hydrolysis, but possibly

[1] Sufficient sodium trichloroacetate was already present in the blood filtrates. The addition of further trichloroacetic acid at this stage leads to errors in the colorimetric measurements [Martland and Robison, 1924].

also to the very slow enzymic hydrolysis of the second ester. The results of a typical experiment are given in detail below.

No. 33. *Whole blood of an adult rabbit taken from carotid and jugular. Hydrolysis by* 5 % *bone extract at* 37·5°. p_H 9·3.

Inorganic phosphate (mg. P) in 5 cc. final filtrate—equivalent to 0·5 g. blood.

	Time in hours							Increase after hydrolysis	Total P, mg.
	0	½	1	2	3	5	10		
FB. Blood filtrate + bone extract	·0475	·055	·062	·0685	·074	·080	·080	·0325	—
FO. Blood filtrate + water	·024	—	—	—	—	·025	·024	0	·155
OB. Water + bone extract	·023	—	—	—	—	·023	—	0	·023
MB. *M*/1000 sodium hexosemonophosphate + bone extract	·025	·053	·071	·092	·103	·115	·130	·105	·133

	mg. P per 100 g. blood	
Total acid-soluble phosphorus	= 31·0	
Inorganic phosphate	= 4·8	
Phosphoric esters	= 26·2	
of which (*a*) esters hydrolysed by bone enzyme	= 6·5	= 24·8 % of organic P
(*b*) esters not hydrolysed by bone enzyme	= 19·7	= 75·2 % ,,

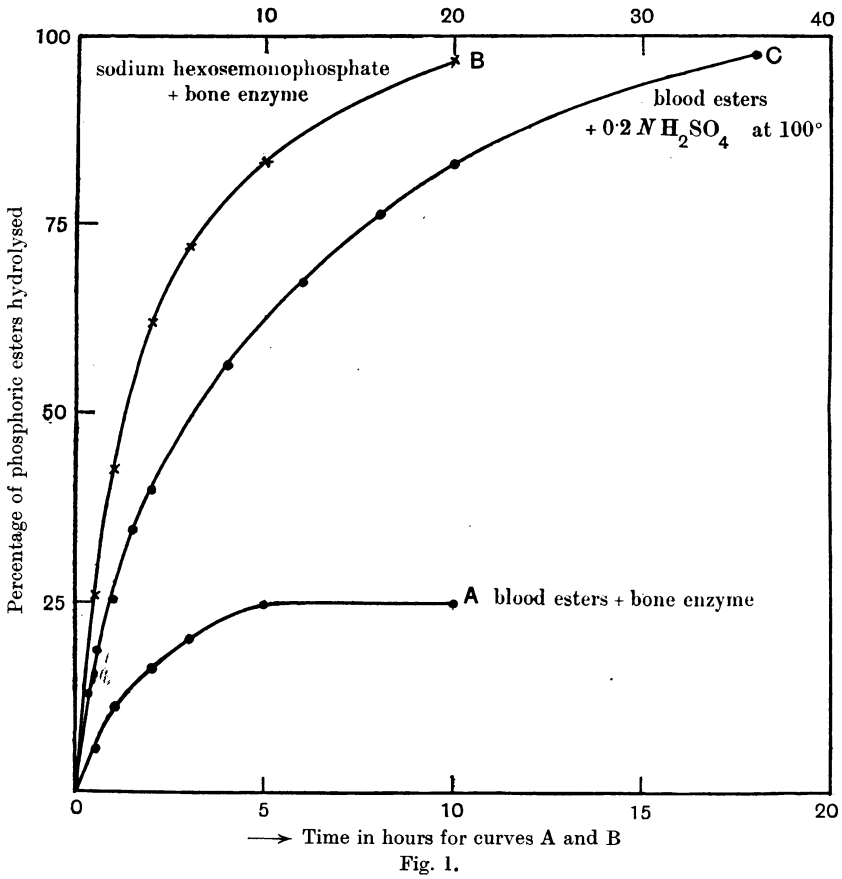

Fig. 1.

The results are shown graphically in Fig. 1 in which curve A expresses the amount of hydrolysis of the blood esters calculated as a percentage of the total organic acid-soluble phosphorus, and curve B the amount of hydrolysis of hexosemonophosphoric ester also as a percentage of the total ester. Curve C shows the hydrolysis of the blood esters by dilute acids and will be referred to later.

Table I. *Distribution of phosphorus in the acid-soluble compounds of blood.*

No. of exp.	Animal	Age	Type of blood	mg. P per 100 g. blood: Total acid soluble	Inorganic	Organic	Hydrolysed by bone enzyme	Not hydrolysed by bone enzyme	% of organic P hydrolysed by bone enzyme
9	Cockerel	6 months	Mixed	30·1	7·1	23·0	3·2	19·8	14
10	Rats	24 days	,,	38·5	10·9	27·6	9·5	18·1	34
8	,,	3 months	,,	28·7	4·5	24·2	7·4	16·8	31
11	,,	3 ,,	,,	27·8	5·0	22·8	7·4	15·4	32
12	,,	2½ ,,	,,	26·0	7·4	18·6	4·7	13·9	25
	,,	9 ,,	,,	29·1	5·4	23·7	5·4	18·3	23
15	,,	3 ,,	,,	33·6	5·4	28·2	6·0	22·2	21
	,,	9 ,,	,,	38·5	6·8	31·7	4·9	26·8	15
6	Rabbit	Adult	,,	28·4	5·4	23·0	7·0	16·0	30
17	,,	3½ months	,,	44·1	8·5	35·6	9·9	25·7	28
	,,	18 ,,	,,	37·1	7·8	29·3	6·5	22·8	22
33	,,	Adult	,,	31·0	4·8	26·2	6·5	19·7	25
42–49	,,	Young adults	Average of 7	38·4	6·2	32·2	10·5	21·7	32
27	,,	Adult	Venous (ear)	32·0	5·1	26·9	8·5	18·4	32
			Arterial (carotid)	32·3	7·8	24·5	8·7	15·8	35
16	Child (O. H.) ♀	6 years	Venous (arm)	21·9	4·3	17·6	6·4	11·2	36
28	Child ♀	10½ ,,	,,	22·8	4·1	18·7	5·7	13·0	30
	Child ♂	11 ,,							
26	Man (R. R.)	Adult	,,	25·2	2·6	22·6	6·0	16·6	27
16	,,	,,	,,	26·6	3·3	23·3	7·0	16·3	30
	Man (S. S. Z.)	,,	,,	21·9	3·2	18·7	6·1	12·6	33

Similar experiments have been carried out with the blood of many animals of different species and age and the main results are summarised in Table I. Except when otherwise stated mixed venous and arterial blood from one or more animals was used. Of the human subjects both the adults were normal, the child O.H. was suffering from slight vaginitis and the other children were congenital syphilitics but were not suffering from active disease at the time. Except in the case of O.H. the blood was taken between 10 a.m. and 11 a.m. about two hours after breakfast. The figures for human blood show less variation than the rest but this may be due to the fact that it was in every case taken from the arm vein and possibly greater differences would be found in blood before passage through the capillaries. Experiment 27 was planned to obtain information on this point although the ear vein was probably not the most suitable one for this purpose owing to the relatively low metabolic activity of the tissue through which the blood had passed. Blood was first taken from the ear and the rabbit was then anaesthetised and bled from the

carotid by means of a canula. The results appeared to show that during the passage through the capillaries some of the inorganic phosphate had been converted into phosphoric ester of the type not hydrolysed by the bone enzyme. Later experiments by Martland and Robison [1924] indicate however that this conclusion is probably incorrect and that the difference between the two samples of blood was due to the shock of anaesthesia and the operation, which caused an increase of the inorganic phosphate apparently at the expense of the ester.

In several experiments the hydrolysis was carried out in duplicate, twice as much bone extract being used in one flask as in the other. The total hydrolysis was nevertheless nearly the same in each although the rates were different. In another experiment after hydrolysis had ceased, a further amount of bone extract was added but no increase of inorganic phosphate was found after a further 24 hours at 37°. The cessation of hydrolysis is therefore not due to inactivation of the enzyme but to the exhaustion of the substrate.

Hydrolysis of the esters by dilute acids.

Zucker and Gutman [1923, 1] have stated that only a part (about 40–50 %) of the organic acid-soluble phosphorus compounds in blood is hydrolysed by boiling with dilute acids and have carried out [1923, 2] a large number of estimations of the amounts of hydrolysable and non-hydrolysable phosphorus in the blood of normal and rachitic rats. The experiments were conducted by boiling the protein-free filtrates for four hours with dilute acids and estimating the increase in the inorganic phosphate. No evidence was adduced that hydrolysis had ceased so that their conclusions seemed to be unwarranted. We carried out some experiments to test the truth of their statement and found that hydrolysis continued, naturally with decreasing velocity, until nearly the whole of the phosphorus was present as inorganic phosphate. The results of one experiment are given below, and are also shown in curve C in the figure. They give some indication of the presence of more than one compound which are hydrolysed at different rates but it is obvious that no estimation of the respective amounts of these compounds could be made from any single point on the curve.

Experiment. The protein-free filtrate from the whole blood of a rabbit was boiled under a reflux condenser with $N/5$ sulphuric acid. The inorganic phosphate in 10 cc. was estimated at intervals.

Time of boiling in hours	Percentage of organic phosphorus hydrolysed	Time of boiling in hours	Percentage of organic phosphorus hydrolysed
½	12·8	12	67·2
1	18·9	16	76·4
2	25·4	20	83·0
4	39·7	36	97·6
8	56·2		

ACTION OF BONE ENZYME ON PLASMA AND ON UNLAKED BLOOD.

Several attempts were made to demonstrate the hydrolysis by the bone enzyme of the acid-soluble phosphoric esters in plasma. These all yielded inconclusive results owing to the very small amounts of these esters present. We then tried to find out whether the inorganic phosphate of the plasma could be increased by acting on the unlaked whole blood with bone extract. The experiment was for several reasons a difficult one to carry out satisfactorily. When blood is mixed with saline alone and kept at 37° for some hours the inorganic phosphate in the plasma is increased. Further it was necessary to keep the p_H at 7·4 and to avoid the use of chloroform or other antiseptic since if any haemolysis occurred the results would be valueless. The duration of the experiment was thus limited by the danger of bacterial growth. Nevertheless positive results were obtained (Table II).

Exps. 1, 2 and 3. Mixed venous and arterial blood of adult rabbits. Potassium oxalate used as anticoagulant.

3 cc. of blood were measured into a number of small centrifuge tubes containing 3 cc. normal saline or 3 cc. of bone extract (made isotonic with blood) or 3 cc. of the same extract previously heated to 100° (in the tube itself). Tubes containing blood alone were also taken. Certain of these tubes were centrifuged at once and the inorganic phosphate estimated in 1 cc. of the undiluted plasma and in 2 cc. of the diluted plasma in the tubes containing added solutions. The remaining tubes were stoppered and kept at 37° for five hours after which they were centrifuged and the phosphate estimated as before.

No haemolysis occurred in any of these experiments.

In Exp. 3 some of the plasma was separated from the corpuscles and was also treated with bone extract, while a control tube containing 3 cc. $M/10$ sodium glycerophosphate solution and 3 cc. bone extract was made up.

Table II.

Inorganic phosphate (mg. P) in 1 cc. plasma or 2 cc. diluted plasma

	Time at 37° (hrs.)	Whole blood (alone)	Blood + saline	Blood + heated bone extract	Blood + active bone extract
Exp. 1	0	·050	·032	—	—
	5	·0595	·048	·128	·143
Exp. 2	0	·067	·054	(·088)	·088
	5	·084	·076	·098	·136
Increase		·017	·022	·010	·048
Exp. 3	0	—	·044	·064	·064
	5	—	·056	·080	·092
Increase		—	·012	·016	·028

		Plasma + bone extract	Sodium glycerophosphate + bone extract		
Exp. 3					
		·062	·020	—	—
		·062	·086	—	—
Increase		—	·066	—	

In every experiment the increase in the inorganic phosphate in the plasma was greatest in the presence of the active bone enzyme which seems to show that the phosphoric esters in the corpuscles were in some way brought into contact with the enzyme and then hydrolysed. The most likely way in which this could take place is by diffusion of the esters from the corpuscles into the plasma, but the experiments do not justify the assertion that this diffusion actually takes place.

Action of the bone enzyme on the lipin phosphorus compounds in blood.

The increases in the amount of inorganic phosphate found in the preceding experiments might possibly have been due to the hydrolysis of phospholipins, although the absence of such increase when plasma alone was treated with the enzyme in Exp. 3 pointed to the opposite conclusion. Robison and Soames [1924] have shown that egg-yolk lecithin is not acted on by the enzyme and the same was also proved for the blood phospholipins by the following experiment.

The blood of two young cockerels was rapidly centrifuged and separated into 43·2 cc. plasma and 38 cc. corpuscles. These were diluted with water and proteins were precipitated with trichloroacetic acid. The precipitates were filtered in Buchner funnels and washed with 100 cc. $2\frac{1}{2}$ % trichloroacetic acid. They were then extracted three times with a mixture of alcohol and ether. After removal of solvent by distillation and re-extraction with ether, the phospholipins were finally shaken up into an emulsion with 50 cc. of water and tested with the bone enzyme with the usual controls. No increase of inorganic phosphate occurred in any of the flasks during 48 hours at 37°, showing that the phospholipins either from corpuscles or plasma were not hydrolysed by the enzyme.

Summary.

The acid-soluble organic phosphorus compounds present in the blood of various animals consist of at least two phosphoric esters, one of which is rapidly hydrolysed by the enzyme of ossifying cartilage, while the other is very resistant to the action of this enzyme.

Both esters are hydrolysed by boiling with 0·2 *N* sulphuric acid.

A method has been worked out by which the respective amounts of these esters (or types of ester) may be determined, and the results of such estimations are given for the blood of human adults and children, and of rats, rabbits and cockerels.

The amount of ester hydrolysed by the bone enzyme varies between 14 % and 36 % of the total acid-soluble phosphorus.

This ester is present almost entirely in the corpuscles, attempts to demonstrate the presence of hydrolysable esters in the plasma leading to inconclusive results.

By the action of bone extracts on unlaked whole blood, the amount of inorganic phosphate in the plasma is increased. This probably indicates that the esters are able to diffuse from the corpuscles to the plasma, and that the former may act as reservoirs.

The phospholipins present in the blood are not hydrolysed by the bone enzyme.

We are indebted to Dr R. Balmer, Dr E. H. Lepper and Dr D. Nabarro, who kindly supplied us with samples of blood and to whom we wish to express our best thanks.

REFERENCES.

Bloor (1918). *J. Biol. Chem.* **36**, 49.
Martland and Robison (1924). *Biochem. J.* **18**, 765.
Robison and Soames (1924). *Biochem. J.* **18**, 740.
Zucker and Gutman (1923, 1). *Proc. Soc. Exp. Biol. Med.* **20**, 133.
—— —— (1923, 2). *Proc. Soc. Exp. Biol. Med.* **20**, 372.