CCI. THE ACTION OF BONE PHOSPHATASE ON THE ESTERS OF PHOSPHORIC ACID OF THE BLOOD.

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THE presence in bone of an enzyme capable of hydrolysing organic esters of phosphoric acid to yield inorganic phosphate was demonstrated by Robison [1923]. A considerable body of evidence has since been brought forward by Robison and his co-workers in support of the suggestion that the bone enzyme is intimately concerned in the process of ossification in bone formation. It was suggested that the deposition of bone salts resulted from the hydrolysis by the enzyme (phosphatase) of organic compounds of phosphorus to yield free phosphate, any increase of which would, in the presence of inorganic calcium, lead to precipitation of calcium phosphate. The presence, in the fluid bathing the bone, of a suitable substrate for the phosphatase was, of course, a necessary part of this hypothesis, and Kay and Robison [1924] were able to demonstrate a partial hydrolysis in the presence of the enzyme of the "acid-soluble organic phosphorus" of the blood. Their experiments were carried out on neutralised trichloroacetic acid filtrates of whole blood, on which the bone phosphatase was allowed to act at 37° and at its optimum p_H of about 9. Under these conditions only a fraction (usually about 25 %) of the acid-soluble organic phosphorus compounds present in the blood was hydrolysed by the enzyme. Since dilute solutions of primary esters of phosphoric acid are readily and almost completely hydrolysed by phosphatase in a few hours, it was supposed that in blood there were at least two phosphoric esters, one of which was rapidly hydrolysed by bone phosphatase, while the other was very resistant to the action of the enzyme. In contrast to the limited hydrolysis by enzymic action, almost complete hydrolysis of the acid-soluble phosphorus of blood resulted from boiling with dilute mineral acid.

In some experiments in this laboratory on the combined action of phosphatase and lipase on lecithin, an unexpectedly large increase of inorganic phosphorus was observed in control flasks which contained phosphatase, lipase and buffer, but no lecithin. The source of this phosphorus was traced to the lipase preparation, an extract of pig's pancreas, which was found to contain large amounts of acid-soluble phosphorus. It seemed worth while to investi-

gate the effect of phosphatase on these phosphorus compounds contained in the pancreas, and the action of the enzyme on neutralised acid extracts of the pancreas was accordingly studied. At p_H 9, the optimum hydrogen ion concentration for phosphatase, about a quarter of the total acid-soluble phosphorus was liberated. At or near p_H 7, however, a somewhat larger proportion, corresponding to about 50 % of the organic phosphorus present, was hydrolysed under the influence of the enzyme during 24 hours.

With acid extracts of liver, kidney and muscle the p_H effect was not so marked, maximum hydrolysis being effected under the action of the enzyme at a p_H intermediate between 7 and 9. In the case of blood, only relatively small proportions of the acid-soluble phosphorus were hydrolysed at the optimum p_H of the enzyme, as was found by Kay and Robison. The hydrolysis proceeded, however, to a much greater extent, if the reaction of the mixture was maintained near neutrality. In some instances over 90 % of the phosphoric esters contained in the acid-soluble fraction of whole blood were hydrolysed by the bone phosphatase at or near p_H 7. Not much more than this amount of hydrolysis was obtainable even under the action of boiling $N/2$ sulphuric acid.

EXPERIMENTAL.

In the enzymic hydrolysis of lecithin the method used [King, 1931] for estimating the "lecithinase" activity of a tissue extract consisted in measuring the increase of inorganic phosphate, during 48 hours at 37° , in mixtures of buffered lecithin and tissue extract. Since it has been demonstrated that lipase acts on lecithin as well as on neutral fat to liberate fatty acids [Contardi and Latzer, 1927; Paal, 1929], it seemed possible that the combined action of lipase and lecithinase might lead to a greater increase of free phosphate than the action of lecithinase alone. The first lipase preparations used were crude extracts of pig's pancreas made according to Cole [1928] by treating finely minced pancreas with aqueous alcohol. This extract shows a high lipolytic activity for olive oil and other neutral fats, particularly in the presence of bile salts. When 1 cc. of Cole's lipase solution was added together with 2 cc. of a preparation of lecithinase (rabbit's intestine) to 10 cc. of a buffered 0.25 % solution of lecithin there was an increase in inorganic phosphate (F.P.) of 0.127 mg. P in 48 hours, while 0.111 mg. F.P. were liberated by the action of the lecithinase alone. If a preparation of bone phosphatase was substituted for the rabbit's intestinal extract there was practically no increase of F.P. due to the phosphatase alone, whereas the addition of 1 cc. of lipase solution led to an increase of 0.139 mg. in the case of a 10 % rabbit's bone extract and of 0.118 mg. in the case of an extract of rachitic chicken bones. The increase of total acid-soluble phosphorus was, however, very small in all cases, there being a large amount present in the initial trichloroacetic acid filtrates of mixtures containing lipase. The increase of F.P. was mainly due, therefore, to hydrolysis of this organic phosphorus (O.P.) of the lipase solution rather than to

hydrolysis of the lecithin. Controls consisting of the same proportion of rabbit's or chicken's bone phosphatase, lipase, and buffer (with no lecithin solution) showed increases of 0.114 mg. and of 0.099 mg. F.P. during the same time at 37°, which is almost as much as that obtained when lecithin was present.

This increase of free phosphate at the expense of the acid-soluble phosphorus of pig's pancreas appeared to demonstrate the presence of phosphoric esters capable of acting as a substrate for phosphatase. Primary esters of phosphoric acid are optimally hydrolysed at p_H 9 [Robison, 1923], secondary esters at p_H 7.4 to 7.6 [Asakawa, 1929] and pyrophosphate at p_H 7.2 to 7.8 [Kay, 1928]. A study of the effect of hydrogen ion concentration on the hydrolysis of the phosphoric esters of pancreas by bone phosphatase indicated that maximum hydrolysis occurred between p_H 7.0 and 7.5. Cole's lipase extract of pig's pancreas (90 cc.) was treated with 10 cc. of 25 % trichloroacetic acid, and the precipitate filtered off. 40 % sodium hydroxide was added drop by drop to the filtrate until pink to phenolphthalein and the solution filtered from a further precipitate. For the hydrolysis, test solutions of 10 cc. of the neutralised trichloroacetic acid filtrate, 10 cc. of buffer and 5 cc. of a dilute phosphatase solution were kept at 37° and 6 cc. portions withdrawn at intervals for the determination of free phosphate by the Martland and Robison colorimetric procedure [1926]. The results are illustrated in Table I.

Table I. *Phosphatase hydrolysis of the acid-soluble phosphoric esters of pig's pancreas.*

p_H	F.P. liberated		
	4 hours mg.	8 hours mg.	24 hours mg.
5.7	0.011	0.015	0.046
6.6	0.018	0.036	0.111
7.0	0.059	0.095	0.172
7.5	0.080	0.125	0.196
8.7	0.054	0.083	0.137
9.2	0.044	0.068	0.115

O.P. = 0.420 at zero time; maximum hydrolysis (24 hours) = 47 %.

Fresh dog's liver (64 g.) was removed from an anaesthetised (sodium amytal) animal and ground immediately in 250 cc. of ice-cold 5 % trichloroacetic acid. The filtrate was neutralised with 6 cc. of 40 % sodium hydroxide and a further precipitate removed. Test solutions consisted of 5 cc. of the extract, 5 cc. veronal buffer [Michaelis, 1930], 2.5 cc. of 10 % rat's bone extract, acid to give the desired p_H and water to 25 cc. Each 5 cc. of this mixture represented 1 cc. of liver extract and 0.5 cc. of bone phosphatase solution. Portions of 5 cc. were removed at intervals for estimations of free phosphate. For the acid hydrolysis at 100° a mixture of equal parts of liver extract and *N* sulphuric acid was kept under a reflux condenser in a boiling water-bath. The kidneys and portions of muscle (sartorius) were removed from the same

dog and treated as in the case of the liver. The results of these experiments are illustrated in Table II.

Table II.

Hydrolysis of the phosphoric esters of dog's liver.

Initial O.P. (in 1 cc. of trichloroacetic extract)=0.152 mg.

	8 hours	24 hours	48 hours	72 hours
	Percentage hydrolysis			
p_{H}				
7.0	12	14	18	30
7.1	11	18	21	29
7.5	14	22	25	36
8.3	18	28	34	49
9.1	9	18	29	45
9.7	7	10	16	24
$N/2 \text{ H}_2\text{SO}_4$	27	52	76	84

Hydrolysis of the phosphoric esters of dog's kidney.

Initial O.P. (in 1 cc. of trichloroacetic extract)=0.067 mg.

	8 hours	24 hours	48 hours	72 hours
	Percentage hydrolysis			
p_{H}				
6.4	16	45	33	36
6.9	21	46	45	48
7.3	28	58	51	54
8.0	31	60	52	55
8.9	25	54	48	48
9.6	—	4	13	27
$N/2 \text{ H}_2\text{SO}_4$	18	52	82	97

Hydrolysis of the phosphoric esters of dog's muscle.

Initial O.P. (in 1 cc. of trichloroacetic extract)=0.081 mg.

	8 hours	24 hours	48 hours
	Percentage hydrolysis		
p_{H}			
6.2	9	—	33
7.0	31	57	81
7.4	47	70	88
8.2	59	80	94
9.0	35	61	65
9.6	5	12	28
$N/2 \text{ H}_2\text{SO}_4$	40	69	84

Mixed whole blood from the carotid artery and femoral vein of this dog was laked with 3 volumes of water and precipitated immediately with 1 volume of 25 % trichloroacetic acid. The mixture was centrifuged, neutralised with strong sodium hydroxide and filtered. The test solutions for enzyme hydrolysis consisted of 7.5 cc. blood-filtrate, 2.5 cc. bone extract, buffer and water to 25 cc. Aliquot portions (5 cc. equivalent to 1.5 cc. blood-filtrate and 0.5 cc. bone extract) were withdrawn at intervals for free phosphate determinations. The results for this dog's blood and for a rabbit's blood are contained in Table III.

Table III. *Hydrolysis of phosphoric esters of blood.*

Dog's blood; initial O.P. (in 1.5 cc. trichloroacetic acid extract)=0.066 mg.

	2 hours	12 hours	21 hours	36 hours	60 hours
	Percentage hydrolysis				
p_H					
3.2	—	2	3	6	8
4.2	—	2	3	5	17
5.7	3	5	23	44	55
6.1	5	20	33	62	77
7.0	5	30	44	68	84
7.5	6	21	33	56	61
8.1	5	11	27	35	41
8.8	3	9	12	21	32
9.2	—	12	21	21	30
$N/2 H_2SO_4$	11	49	55	85	—

Rabbit's blood; initial O.P. (in 1.5 cc. trichloroacetic acid extract)=0.074 mg.

3.2	3	3	4	7	—
4.7	—	3	4	7	—
5.5	4	9	11	15	16
6.3	4	31	34	55	75
6.9	3	49	50	66	84
7.4	19	47	49	62	74
7.9	—	35	45	53	57
8.5	23	34	35	42	43
9.1	16	28	32	32	28
9.8	3	12	16	—	16
$N/2 H_2SO_4$	—	39	51	80	92

Fig. 1. Hydrolysis by acid and by phosphatase of the "acid-soluble phosphorus" of dog's blood.

Fig. 2. Hydrolysis by acid and by phosphatase of the "acid-soluble phosphorus" of rabbit's blood.

The hydrolysis of the acid-soluble phosphorus compounds of the blood of another dog is illustrated in Fig. 1 and of that of a rabbit in Fig. 2.

These results with animal blood appeared to indicate that if the hydrolysis were continued long enough, the greater part of the acid-soluble phosphoric esters could be hydrolysed by the bone enzyme at a reaction in the neighbourhood of neutrality. In order to determine if human blood behaved in like manner, samples were taken from the arm veins of five normal adult males. 10 cc. samples were laked with 30 cc. of water and the proteins precipitated

by the immediate addition of 10 cc. of 25 % trichloroacetic acid. After being shaken thoroughly the samples were centrifuged, the supernatant solutions poured off, neutralised by the careful addition of powdered sodium hydroxide, and filtered. 15 cc. portions of the filtrates were added to 25 cc. volumetric flasks, together with 5 cc. of veronal buffer and 2.5 cc. of 5 % dialysed rat's bone extract. The reaction of each solution was adjusted to p_H 7, and water added to 25 cc. Two drops of a mixture of chloroform and toluene were added and the flasks placed in a water thermostat at 37.5° . At stated intervals 5 cc. portions (equivalent to 3 cc. of trichloroacetic acid filtrate of blood and 0.5 cc. enzyme) were withdrawn and the inorganic phosphate was determined. The results (Table IV) show that the acid-soluble phosphorus of human blood, although attacked relatively slowly by the bone enzyme under these conditions, is, nevertheless, almost completely hydrolysed in a little less than 2 days.

Table IV. *Acid-soluble phosphorus of human whole blood.*

10 cc. blood + 30 cc. water + 10 cc. 25 % trichloroacetic acid. Hydrolysis of 3 cc. neutralised filtrate (equivalent to 0.6 cc. blood) by 0.5 cc. rat's bone extract at p_H 7 and 37.5° .

	O.P. mg.	F.P.					Increase after hydrolysis mg.	Per- centage hydro- lysis
		0 hours mg.	6 hours mg.	12 hours mg.	24 hours mg.	40 hours mg.		
H. M. D.	0.122	0.034	0.096	0.120	0.135	0.149	0.115	94
D. A. I.	0.138	0.035	0.101	0.128	0.149	0.155	0.120	87
J. E. W.	0.120	0.034	0.094	0.119	0.136	0.146	0.112	93
W. R. F.	0.128	0.032	0.067	0.120	0.140	0.150	0.118	92
E. J. K.	0.132	0.032	0.102	0.120	0.136	0.154	0.122	92

Since part of this liberated phosphate might be due to spontaneous decomposition of the phosphoric esters, or possibly derived from organic phosphorus contained in the enzyme preparation, an experiment was conducted in which controls consisting of buffer and trichloroacetic acid filtrate of blood, and of enzyme and water were incubated under the same conditions as mixtures of the blood-filtrate, buffer and enzyme. The results contained in Table V demonstrate that with dog's and rabbit's bloods there was no appreciable increase of inorganic phosphate in either of the controls, and that at least

Table V. *Hydrolysis of acid-soluble phosphorus of blood at p_H 7.*

Dog's blood: 0.152 mg. O.P. in 3 cc. of trichloroacetic acid filtrate.

Hours	F.P. substrate + enzyme + buffer mg.	F.P. substrate + buffer mg.	F.P. enzyme + water mg.	P hydrolysed mg.	Percentage hydrolysis
0	0.035	0.025	0.009	0.001	—
17	0.102	0.031	0.013	0.058	38
47	0.161	0.029	0.010	0.122	80

Rabbit's blood: 0.165 mg. O.P. in 3 cc. of trichloroacetic acid filtrate.

Hours	F.P. substrate + enzyme + buffer mg.	F.P. substrate + buffer mg.	F.P. enzyme + water mg.	P hydrolysed mg.	Percentage hydrolysis
1	0.034	0.016	0.011	0.007	4
24	0.143	0.016	0.013	0.114	69
48	0.161	0.017	0.011	0.133	81
72	0.167	0.018	0.011	0.138	84

four-fifths of the organic phosphorus contained in the acid extracts of these bloods was hydrolysed within 2 days by the enzyme.

It seemed worth while to test the susceptibility of the residual phosphoric esters, unattacked by the enzyme at p_H 9, to bone phosphatase at a lower p_H . 15 cc. samples of human blood-filtrate were adjusted to p_H 9 and maintained at 37.5° in the presence of 2 cc. of bone extract for 24 hours, when equilibrium had apparently been reached with the hydrolysis of some 35 % of the organic phosphorus. At this point 2 cc. more of enzyme were added to each of the flasks, since that originally present had probably been largely destroyed during the 24 hours at an alkaline reaction. Two flasks were adjusted to p_H 7 and two maintained at p_H 9 and further incubated at 37.5° , aliquot portions being withdrawn at intervals for inorganic phosphate and total phosphate determinations. The differences (organic phosphate) indicated that there was only 5 % of organic phosphate left in the mixtures at p_H 7 after 36 hours, while at p_H 9 there was still nearly 60 % unattacked. The results are contained in Table VI.

Table VI. *Hydrolysis of residual phosphoric esters after hydrolysis at p_H 9.*

20 cc. human blood + 60 cc. water + 20 cc. 25 % trichloroacetic acid: 0.129 mg. O.P. in 3 cc. filtrate. Preliminary incubation with enzyme for 24 hours at p_H 9.

Hours	O.P. at p_H 9		O.P. at p_H 7	
	mg.	Unhydrolysed %	mg.	Unhydrolysed %
0	0.084	65	0.083	64
10	0.083	64	0.054	42
24	0.079	61	0.024	19
36	0.075	58	0.007	5
50	0.073	57	0.006	5
		O.P. hydrolysed 43		O.P. hydrolysed 95

SUMMARY.

The esters of phosphoric acid contained in trichloroacetic acid extracts of blood consist of at least two fractions. One is attacked by bone phosphatase at p_H 9, while the other is hydrolysed preferentially at a p_H near neutrality. Both fractions are almost completely hydrolysed at or near neutrality during 2 days at 37.5° .

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