CLXXXVI. BLOOD-PHOSPHATASES.

By JEAN ROCHE.

From the Biochemical Department, Lister Institute, London.

(*Received August 28th, 1931.*)

Previous workers [Martland, Hansman and Robison, 1924; Demuth, 1925; Martland and Robison, 1926; Kay, 1929, 1930, 1 and 2] have shown that the red corpuscles and plasma of mammalian blood each contain a phosphatase, but that these enzymes differ in certain characteristics notably in their optimum p_H. Kay [1930, 1] also noted the occurrence of a phosphatase in the leucocytes but made no further study of its properties.

In the present work these phosphatases of the red and white cells and of plasma have been studied and compared with those of other tissues, especially of bone and kidney. It is probable that the phosphatase of the red cells participates in the mechanism of glycolysis in blood [Roche and Roche, 1929] and it was therefore considered that further knowledge of the hydrolytic and synthetic powers of this enzyme was desirable and might help towards understanding the glycolytic process.

Experimental.

The cells were separated from the serum in defibrinated blood or from the plasma in oxalated blood by centrifugation and were washed with a 0·9 % solution of sodium chloride. The upper layer, consisting largely of leucocytes, was removed by pipetting, suspended in isotonic saline and again centrifuged at slow speed. By repeating this process three or four times, the leucocytes were obtained free from red cells. Similarly, by pipetting the lower layer of cells and repeating the centrifugation, the red cells were obtained practically free from leucocytes.

For the enzyme tests, the red cells were laked by adding an equal volume of distilled water and allowed to remain at room temperature for 8 to 10 hours, so that the major portion of the blood-phosphoric esters became hydrolysed. The white cells were lysed with a volume of water equal to the original volume of the blood. One cc. of the lysed cells or of the cell-free serum or plasma was added to 1 cc. of a 0·2 *M* solution of sodium glycerophosphate together with a small drop of chloroform and, after adjustment of the p_H, kept at 37° for 1 hour or longer, the increase in inorganic phosphate being estimated by the Briggs method. The phosphatase content of laked red cells was not appreciably diminished by keeping the solution for periods of 6–10 days at 0° in presence of chloroform. A loss of activity amounting to about 20 % was observed in

one case after 3 weeks at 0°. Blood of rabbits, guinea-pigs and horses was used in these experiments. The red cells of horse blood had a lower phosphatase activity than those of the other animals, but this may possibly have been due to age or condition of the animal.

Attempts to purify the phosphatase of the red cells.

The separation of the phosphatase from the haemoglobin of the laked corpuscles was attempted (*a*) by precipitating the haemoglobin, leaving the enzyme in solution, (*b*) by adsorbing the enzyme on various substances. Precipitation of the haemoglobin with ammonium sulphate yielded a colourless solution, which was, however, completely inactive, both before and after dialysis. Removal of haemoglobin by precipitation as cathaemoglobin with chloroform was more successful. The laked cells were shaken with one-tenth to one-fifth their volume of chloroform and left for some hours at room temperature or 2–3 days at 0°. A considerable proportion of the pigment was thus precipitated, and by repeating this operation three or four times with fresh quantities of chloroform it was possible to remove about 95 % of the haemoglobin with a loss of only 20–30 % of the phosphatase activity. The further removal of the pigment by this method resulted, however, in rapid inactivation of the solution.

Adsorption of the phosphatase was attempted with aluminium hydroxide, kaolin, kieselguhr, barium carbonate and barium sulphate. The first three adsorbents proved satisfactory in so far that they removed a large proportion of the enzyme from the haemolysed cells, but its subsequent elution proved neither easy nor quantitative. This elution was attempted, using water, sodium phosphate solution ($M/15$) and sodium glycerophosphate solution ($M/10$) at various p_H. The best results were obtained with glycerophosphate, but even with this solution less than 20 % of the adsorbed phosphatase was recovered.

These results were not considered sufficiently good to allow the enzyme to be studied in purified condition and the experiments described below were, therefore, carried out using the crude solution obtained by laking the cells.

The optimum p_H *of the blood-phosphatases.*

The optimum p_H of these enzymes was first determined for the hydrolysis of glycerophosphoric ester. The p_H was measured colorimetrically by means of a capillator, a minute drop of the solution being diluted with 10 times its volume of boiled, distilled water in order to reduce the protein error. In judging the p_H of the solutions containing haemoglobin a compensating tube containing the same proportion of pigment but without indicator was used in conjunction with the standards. The results of the first experiments appeared to indicate two definite optima for the phosphatase of the red cells, one near p_H 6 and the second, less pronounced, near p_H 9. The latter was, however, found to be due to the presence of leucocytes mixed with the red cells and was no longer apparent when the cells had been more completely separated.

Table I shows the amount of sodium glycerophosphate hydrolysed by the phosphatases of red and white cells and of serum, at different p_H. In these tests the syrupy 50 % sodium glycerophosphate, which consists chiefly of the α-isomeride, was used.

The figures are calculated for 100 cc. of the lysed cells or of serum.

Table I. *Hydrolysis of glycerophosphoric ester at 37°.*

Red cells		White cells		Serum	
p_H	Hydrolysis in 5 hours mg. P	p_H	Hydrolysis in 8 hours mg. P	p_H	Hydrolysis in 10 hours mg. P
4·0	20·0	4·5	0	4·5	1·2
4·6	25·9	5·3	0	5·5	2·3
5·3	32·2	6·0	1·3	6·0	5·3
5·8	36·7	6·5	6·0	6·5	7·2
6·2	**39·0**	7·3	6·7	6·9	20·3
6·6	34·8	7·9	30·4	7·5	27·2
6·8	23·2	**8·6**	**52·0**	8·3	38·5
7·4	10·5	8·9	48·3	**8·8**	**55·9**
8·3	4·9	9·3	5·1	**9·1**	**55·8**
9·1	0·7	9·6	0·7	9·8	20·0
10·0	0·8	10·2	0	—	—

These results show that the optimum p_H for the phosphatase of the red cells is near 6·2, in agreement with the findings of Martland, Hansman and Robison [1924]; for the phosphatase of serum the optimum p_H is 8·8–9·2, which is the same as the value found by Martland and Robison [1926] and by Kay [1929, 1930, 1], but much higher than that given by Demuth [1925]. The phosphatase of the white cells has an optimum p_H 8·6–8·8, very close to that of the serum. The similarity between the optimum p_H for the phosphatases of serum and leucocytes with that of the bone phosphatase [Robison and Soames, 1924; Martland and Robison, 1927] suggests that these enzymes are probably identical. It is indeed possible that the serum phosphatase may be derived from the bones but, on the other hand, it may partly arise from the leucocytes, some of which are always broken down in blood *in vitro*.

The hydrolytic activity of the blood-phosphatases was next investigated on other substrates, namely, β-glycerophosphate, glucosemonophosphate[1], fructosemonophosphate[2], fructosediphosphate, monophenylphosphate, adenylate and guanylate[3], diphosphoglycerate[4], and the mixed acid-soluble phosphoric esters of the red blood corpuscles. The method of experiment was the same as described above, except that the amounts of the various esters added to 1 cc. of the enzyme solution were equivalent to 1·2–1·7 mg. P, instead of 6 mg. as in previous experiments. The p_H-activity curves for the three phosphatases on these different substrates are shown in Figs. 1–4, while the p_H optima are shown in Table II.

[1] Prepared by Dr R. Robison.

[2] Prepared from hexosediphosphate by Neuberg's method [1918].

[3] Prepared by hydrolysis of yeast nucleic acid and isolated in crystalline condition.

[4] Prepared from horse blood by Greenwald's method [1925].

Table II. p_H *optima of the blood-phosphatases on various substrates.*

Substrate	Phosphatase of: Red corpuscles	White cells	Serum
Sodium glycerophosphate	6·0–6·2	8·6–9·0	8·6–9·0
Sodium glucosemonophosphate	5·9–6·1	9·0–9·2	8·8–9·2
Sodium fructosemonophosphate	6·2–6·3	—	—
Sodium monophenylphosphate	5·8–6·0	8·4–8·6	8·4–8·6
Sodium adenylate	6·4–6·5	—	—
Sodium guanylate	6·4–6·5	—	—
Sodium diphosphoglycerate	6·6–6·8	8·8–9·2	8·8–9·2
Sodium fructosediphosphate	6·4–6·7	8·8–9·2	8·8–9·2
Blood-phosphoric esters	6·7–6·8	—	—

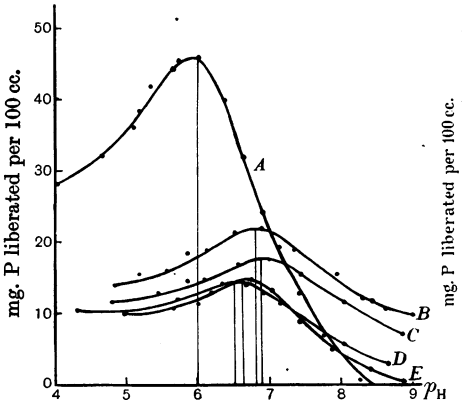


Fig. 1.

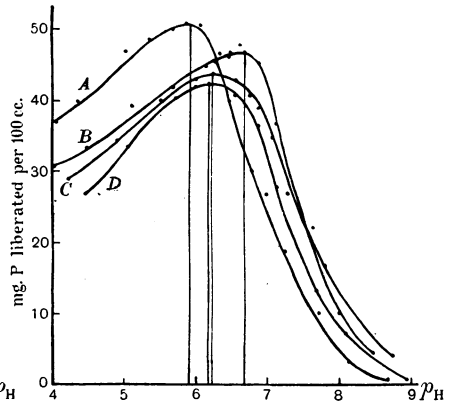
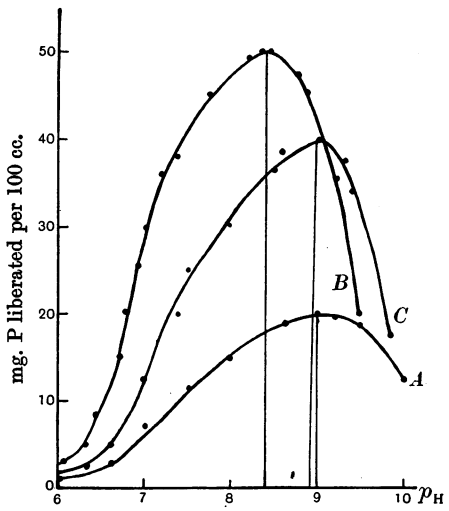


Fig. 2.

Fig. 1. p_H-activity curve for the phosphatase of the red cells. Substrates: *A*, glycerophosphate. *B*, blood-esters. *C*, diphosphoglycerate. *D*, adenylate. *E*, guanylate.

Fig. 2. p_H-activity curve for the phosphatase of the red cells. Substrates: *A*, phenylphosphate. *B*, fructosediphosphate. *C*, fructosemonophosphate. *D*, glucosemonophosphate.



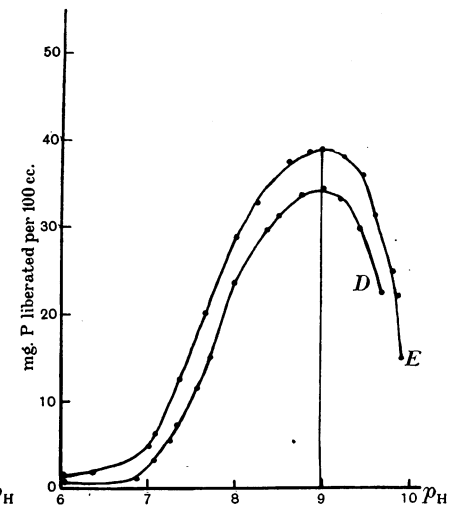


Fig. 3. p_H-activity curves for the phosphatase of the serum. Substrates: *A*, diphosphoglycerate. *B*, phenylphosphate. *C*, glycerophosphate. *D*, glucosemonophosphate. *E*, fructosediphosphate.

It is obvious from these curves that the p_H optimum for each phosphatase varies to some extent with the nature of the substrate. With the enzyme of the red cells it is lower for the monophosphoric ester (5·8–6·4) than for the diphosphoric ester (6·6–6·8). These values are close to that of p_{K_2} (6·72) for

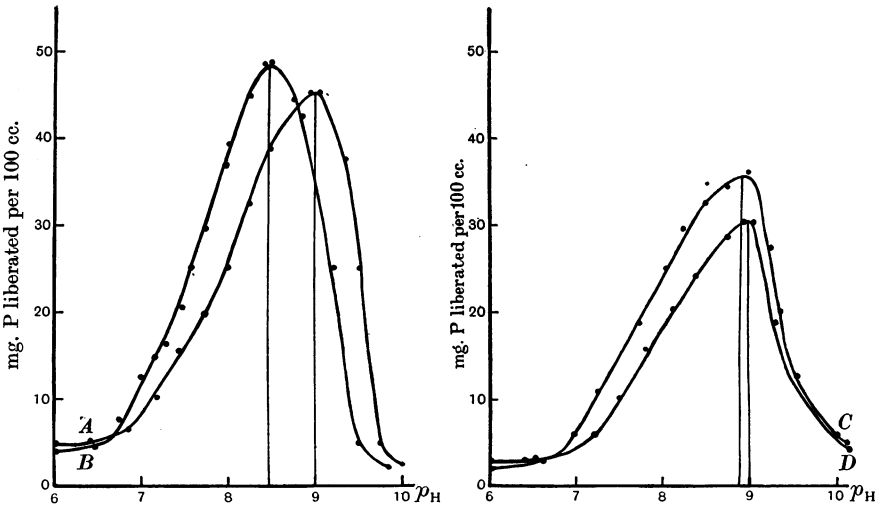


Fig. 4. p_H-activity curves for the phosphatase of the white cells. Substrates: *A*, glycerophosphate. *B*, phenylphosphate. *C*, glucosemonophosphate. *D*, fructosediphosphate.

phosphoric acid and it is possible that the observed differences are related to the differences between the dissociation constants of these esters. That the same optimum p_H was found for the hydrolysis of the blood-esters as for diphosphoglycerate is in agreement with the fact that this substance constitutes the chief part of the acid-soluble phosphorus compounds of the blood.

Such differences in the p_H optima are not so marked in the case of the serum and leucocyte phosphatases but it is possible, as was observed by Martland and Robison [1927] for the bone phosphatase, that spontaneous inactivation of the enzyme occurs at a p_H higher than 8·5, so that the apparent optimum will vary with the duration of the hydrolysis.

Specificity of the phosphatases of the blood and of other animal tissues.

The hydrolytic activity of the blood-phosphatases on various substrates was compared with that of other tissue phosphatases. The results of one typical experiment are shown in Table III. In each series of tests 1 cc. of the same enzyme preparation was allowed to act on an amount of ester equivalent to 1 mg. P at 37° and at the optimum p_H for the enzyme. The duration of the hydrolysis is shown in the Table.

The bone phosphatase was prepared according to the method of Martland and Robison [1929], and p_H 8·8 was adopted, as suggested by them, for tests with this enzyme. Intestinal and kidney phosphatases were prepared by Kay's

method [1926, 1928] and the optimum p_H 8·6, given by this author, was used in the tests.

Table III. *Hydrolysis of phosphoric esters by tissue phosphatases at their optimum* p_H *and 38° (mg. P/100 cc. of enzyme solution).*

	Source of phosphatase					
	Red cells	White cells	Serum	Bone	Intestine	Kidney
Duration of hydrolysis (hrs.)	16	10	16	1·4	1	2
Substrate (sodium salts)						
α-Glycerophosphate	38·8	32·7	27·9	47·1	38·4	15·9
β-Glycerophosphate	11·2	53·4	36·43	69·0	44·4	24·9
Glucosemonophosphate	33·7	58·2	36·9	65·2	37·9	32·8
Fructosemonophosphate	47·1	66·7	44·4	60·4	48·4	40·5
Fructosediphosphate	45·0	67·4	47·2	51·4	47·4	42·4
Monoethylphosphate	12·2	21·0	17·4	16·1	21·3	10·2
Diethylphosphate	0·1	0·1	0·1	0·2	0·2	0·1
Monophenylphosphate	75·6	68·4	63·4	75·2	48·4	39·4
Monopropylphosphate	14·4	19·7	17·1	—	—	—
Dipropylphosphate	0·1	0·2	0·1	—	—	—
Adenylate	8·2	22·1	15·1	22·2	14·0	16·9
Guanylate	9·6	20·4	16·1	25·1	15·2	14·4
Trehalosemonophosphate	5·5	22·1	16·4	20·0	6·1	18·8
Diphosphoglycerate	8·7	17·4	13·7	14·0	9·1	13·3
Pyrophosphate	6·2	31·2	7·0	39·0	35·2	15·2

Action on α- and β-glycerophosphate. Kay [1926] and Kay and Lee [1931] have shown that the kidney and plasma phosphatases attack β-glycerophosphate more readily than the α-isomeride, and the results of my experiments prove the same to be true also for the phosphatases of the leucocytes, bone and intestine and lead me to conclude that this property is common to the various tissue phosphatases for which the optimum p_H is 8·5–9·0. The phosphatase of the red cells, on the other hand, hydrolyses the α-glycerophosphate more rapidly than the β-form. It will be interesting to find whether this holds good also for the muscle enzyme which, according to Kay and Robison [1924], has an optimum p_H close to 7·0.

Action on hexosephosphoric esters. These substrates were readily hydrolysed by all the phosphatases here studied. The hydrolysis of the fructosemono- and di-phosphates was somewhat more rapid than that of glucosemonophosphate except in the case of the bone enzyme, where the relationship was reversed. It is possible that this peculiarity of the bone phosphatase may be due to the relative purity of this enzyme preparation, which contained very little protein.

Action on mono- and di-substituted phosphoric esters. Monophenylphosphate was the most rapidly hydrolysed of all the substrates that were tested. The marked effect of the substituting group on the rate of hydrolysis of the ester is shown by the difference between the results for monophenyl-, monopropyl- and monoethyl-phosphate. Another property common to all the phosphatases was their inability to hydrolyse disubstituted esters such as diethyl- and dipropyl-phosphate. The latter observation is in accordance with the previous findings of Martland and Robison [1927] for bone phosphatase, but in disagreement with those of Neuberg and Wagner [1926], who found that kidney

extract was able to hydrolyse diethylphosphate. This inability to hydrolyse compounds in which two hydroxyl groups of phosphoric acid have been esterified may, as suggested by Martland and Robison, be due to the necessity of two free hydroxyl-groups being present in the substrate in order that it may combine with the enzyme.

Action on other phosphoric esters. The purine nucleotides, trehalosemonophosphate and diphosphoglycerate were hydrolysed much more slowly than the hexosephosphates by all phosphatases.

With pyrophosphate very different rates of hydrolysis were obtained with the different enzyme preparations, which suggests that a specific pyrophosphatase may be present. It may be noted that all phosphatases were found to have some hydrolytic power on the compounds which represent the chief part of the acid-soluble phosphorus of the blood, namely, diphosphoglyceric acid [Greenwald, 1925; see also Macheboeuf, 1927], adenylic acid [Jackson, 1924; Hofmann, 1925], pyrophosphoric acid [Engelhardt, 1930; Roche, 1930] and hexosephosphoric acids [Roche, 1930]. Kay and Robison [1924] found that only a fraction, from 14 to 36 %, of these blood-esters was hydrolysed by the bone phosphatase, a result which may, perhaps, be explained by the relatively slow action of the enzyme on certain of these esters, by the inhibiting effect of inorganic phosphate or by the spontaneous inactivation of the phosphatase during the prolonged experiments.

Factors affecting the rate of hydrolysis of glycerophosphates by the phosphatases of the blood.

Martland and Robison [1927], studying the hydrolysis of glycerophosphate by bone phosphatase, observed that the velocity of this reaction was strongly diminished in presence of inorganic phosphate in concentration as low as 0·005 *M*, while the presence of the second hydrolysis product, glycerol, in concentrations up to 0·1 *M*, had no appreciable effect. Variations in the concentrations of the substrate between 0·003 *M* and 0·3 *M* likewise had no effect on the initial rate of hydrolysis. It would appear that the affinity of the enzyme for inorganic phosphate is greater than for the other constituents of the ester, and it was of interest to find whether this character is shared by the other phosphatases.

The effects of inorganic phosphate and glycerol on the rate of hydrolysis of glycerophosphate (mixed α- and β-forms) by phosphatases of the red cells and of serum are shown in Table IV.

The retarding influence of inorganic phosphate on the rate of hydrolysis is very marked with both these phosphatases, while glycerol also has a considerable inhibitory effect, especially in the case of the red cell phosphatase. The degree of retardation increases with increasing concentration of both hydrolysis products.

Table V shows the effect of variations in the concentration of the substrate on the rate of hydrolysis.

Table IV. *Effect of inorganic phosphate and glycerol on the hydrolysis of glycerophosphate by phosphatases of red cells and serum.*

Initial concentration of ester 0·05 *M*. $t = 37°$. Hydrolysis calculated as mg. P per 100 cc.

Red cells, p_H 6·3	Initial concentration of inorganic phosphate (*M*)					
Hours	*0·03*	*0·015*	*0·01*	*0·005*	*0·003*	*0·002*
1½	2·1	3·3	4·5	7·7	9·7	11·1
3	5·2	7·0	7·8	11·3	13·6	16·0
4½	8·5	10·6	11·0	14·6	18·1	20·1
8	13·3	14·6	16·1	20·8	24·7	27·9
Serum, p_H 8·6	*0·025*	*0·013*	*0·007*	*0·003*	*0·002*	
4½	5·3	6·7	7·5	8·5	8·9	
10	7·9	9·5	10·4	11·7	14·0	
Red cells, p_H 6·3	Initial concentration of glycerol (*M*)					
	0·1	*0·02*	*0·01*	*0·005*	*0*	
3	6·1	10·5	16·2	17·0	19·1	
6½	11·2	16·0	25·1	26·3	31·5	
Serum, p_H 8·6						
4	1·7	2·2	3·4	5·0	6·1	
8	4·3	4·4	8·1	9·1	11·3	

Table V. *Effect of concentration of substrate on the rate of hydrolysis of glycerophosphate.*

Red cells		Serum	
Concentration of ester (*M*)	Hydrolysis in 2 hrs. at p_H 6·3 and 37° (mg. P per 100 cc.)	Concentration of ester (*M*)	Hydrolysis in 6 hrs. at p_H 8·6 and 37° (mg. P per 100 cc.)
0·0075	1·4	0·006	5·9
0·015	2·9	0·013	6·9
0·030	9·4	0·032	7·0
0·075	23·0	0·064	10·2
0·150	37·0	0·128	11·8
—	—	0·320	14·9

With both enzymes the rate of hydrolysis was increased by raising the concentration of substrate between the limits shown in Table V, but, whereas in the case of the serum phosphatase the effect was comparatively small, with the red cell phosphatase the rate of hydrolysis was nearly proportional to the initial concentration of ester. The difference between the behaviour of the serum and bone phosphatases may be due to the specific characters of these enzymes or to the very different state of purity of the preparations used.

The synthetic action of the blood-enzymes.

The synthetic action of bone phosphatase has been demonstrated by Martland and Robison [1927] and that of kidney phosphatase by Kay [1928]. In Table VI the results of a number of experiments are set out, showing synthesis of phosphoric esters effected by the phosphatases of the red cells and of serum in presence of various alcohols. Sodium fluoride in 1 % concentration had no significant effect on such synthesis.

Table VI. *Synthesis of phosphoric esters by blood-phosphatase.*

Alcohol	Inorganic phosphate (mg. P per 100 cc.) Days 0	2	5	10	18	Synthesis (mg. P per 100 cc.)
Serum phosphatase (p_H 8·5: t. 37°)						
Glycol 45 %	159·2	137·2	116·2	115·4	115·4	43·8
Glycerol 40 %	300·2	300·0	296·9	284·9	273·4	26·8
,,	244·4	238·4	—	219·6	199·8	44·6
,,	125·4	117·2	110·2	106·4	108·4	17·0
Sorbitol (saturated)	157·6	156·3	—	—	152·2	5·4
Dulcitol (saturated)	160·1	159·4	—	—	157·4	2·7
Erythritol (saturated)	158·4	160·1	159·2	158·4	157·7	0·7
Glucose 50 %	138·4	139·2	116·9	110·2	109·4	29·0
,, 50 % + 1 % NaF	134·9	134·2	114·4	109·2	106·4	28·5
Fructose 50 %	115·1	112·4	107·6	100·4	92·4	22·7
,, 50 % + 1 % NaF	112·1	113·4	110·4	113·2	92·2	19·9
Glyceric acid 30 %	220·2	218·2	199·9	194·6	192·9	27·3
Red cell phosphatase* (p_H 6·3: t. 37°)						
Glycol 45 %	158·1	144·2	—	130·4	124·4	23·7
Glycerol 40 %	298·7	304·4	—	294·4	270·8	27·9
,,	239·2	244·4	226·4	194·4	190·6	49·6
,,	128·3	130·2	—	122·1	120·1	8·2
Sorbitol (saturated)	158·4	164·4	—	—	160·2	—
Dulcitol (saturated)	159·7	162·4	—	159·4	159·1	—
Erythritol (saturated)	155·4	160·2	—	159·8	160·0	—
Glucose 50 %	127·2	130·6	124·4	118·4	113·4	13·8
,, 50 % + 1 % NaF	121·1	124·6	120·3	114·4	110·4	10·7
Fructose 50 %	121·4	123·4	120·2	116·4	115·3	6·1
,, 50 % + 1 % NaF	127·4	128·9	126·4	120·2	120·1	7·3
Glyceric acid 30 %	195·1	203·2	196·4	—	193·4	1·7

* The slight increase in inorganic phosphates after 2 days' experiment is probably due to the hydrolysis of blood-esters.

Summary.

1. The phosphatases present in serum and in the red and white cells of the blood have been studied and some of their properties compared with those of the bone, kidney and intestinal phosphatases.

2. The phosphatase of the red cells is quite distinct from all the other enzymes. It differs from them in having an optimum p_H on the acid side of neutrality, 6·0–6·8, according to the substrate, and in acting more energetically on α- than on β-glycerophosphate.

3. The phosphatases of the serum and of the white cells appear to be identical, while the small differences observed between the properties of these enzymes and of the bone phosphatase may probably be attributed to differences in the state of purity of the preparations examined. Kidney and intestinal phosphatases are also probably identical with these.

4. The relative rates of hydrolysis of various substrates by these phosphatases have been measured. All monosubstituted phosphoric esters examined were acted on by each enzyme, but the rate of hydrolysis varied with the nature of the substituting group. Disubstituted esters were not appreciably hydrolysed by any of these phosphatases.

5. The hydrolysis of glycerophosphate by each of the blood-phosphatases is diminished by the presence of inorganic phosphate and also by glycerol.

6. The phosphatases of the serum and red cells are able to synthesise phosphoric esters from inorganic phosphate and various alcohols, including glycol, glycerol, and hexoses.

I wish to express my thanks to the Director of the Lister Institute and to Prof. A. Harden for the hospitality of the Biochemical Department and to Dr R. Robison for his interest and helpful criticism in this work. I wish also to thank the Rockefeller Foundation for the Fellowship which enabled me to carry out this research.

REFERENCES.

Demuth (1925). *Biochem. Z.* **159**, 415.
Engelhardt (1930). *Biochem. Z.* **227**, 16.
Greenwald (1925). *J. Biol. Chem.* **63**, 339.
Hofmann (1925). *J. Biol. Chem.* **63**, 675.
Jackson (1924). *J. Biol. Chem.* **59**, 529.
Kay (1926). *Biochem. J.* **20**, 791.
—— (1928). *Biochem. J.* **22**, 855.
—— (1929). *Brit. J. Exp. Path.* **10**, 253.
—— (1930, 1). *J. Biol. Chem.* **89**, 235.
—— (1930, 2). *J. Biol. Chem.* **89**, 249.
—— and Lee (1931). *J. Biol. Chem.* **91**, 135.
—— and Robison (1924). *Biochem. J.* **18**, 1139.
Macheboeuf (1927). Thèse Med. Paris.
Martland, Hansman and Robison (1924). *Biochem. J.* **18**, 1152.
—— and Robison (1926). *Biochem. J.* **20**, 847.
—— —— (1927). *Biochem. J.* **21**, 665.
—— —— (1929). *Biochem. J.* **23**, 237.
Neuberg (1918). *Biochem. Z.* **88**, 432.
—— and Wagner (1926). *Biochem. Z.* **171**, 485.
Robison (1923). *Biochem. J.* **17**, 286.
—— and Soames (1924). *Biochem. J.* **18**, 740.
Roche (1930). *Bull. Soc. Chim. Biol.* **12**, 636.
—— and Roche (1929). *Bull. Soc. Chim. Biol.* **11**, 549.