LXXVI. THE PHOSPHATASE ACTIVITY OF SPLEEN EXTRACTS.

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THERE have been many references in the literature to the presence of enzymes, capable of synthesising and hydrolysing phosphoric esters, in mammalian tissues. Grosser and Husler [1912] and Forrai [1923] described such an enzyme in the intestine. Robison [1923] described similar enzymes in the bone, kidney, liver, spleen and pancreas and in this and a subsequent communication [Robison and Soames, 1924] suggested a possible function for the enzyme occurring in the bone in relation to bone formation. This suggestion has received support, and much evidence, which has been clearly summarised by Kay [1931], has accumulated in its favour.

Kay [1928, 1; 1931] has concluded that the phosphatases of the bone, kidney, intestinal mucosa and blood-plasma are identical, basing his claims upon the following evidence.

1. The optimum hydrogen ion concentration for the phosphatases of the bone, kidney, intestinal mucosa and the blood-plasma is the same. Thus for each of these tissues, for primary esters of phosphoric acid it is 8·9 [Kay, 1926; 1928, 1], for secondary esters it is 7·2–7·6 [Asakawa, 1929] and for pyrophosphoric acid it is 7·6 [Kay, 1928, 2].

2. All these enzymes hydrolyse β -glycerophosphoric acid more rapidly than the corresponding α -compound [Kay and Lee, 1931].

3. The dissociation constant of the enzyme substrate complex, using sodium β -glycerophosphate as substrate, is the same for all the enzymes under discussion [Michaelis and Menten, 1913, quoted by Kay, 1932].

4. Extracts of these tissues, *i.e.* bone, kidney and intestine, will synthesise phosphoric esters under identical conditions [Kay, 1926; Jenner and Kay, 1931; Roche, 1931].

5. Magnesium ions activate the phosphatases from these tissues under very similar conditions [Jenner and Kay, 1931].

Martland *et al.* [1924] reported the presence of a phosphatase in the red cells. Roche [1931], working in Robison's laboratory, further substantiated this claim by separating the plasma and red cells of whole blood and comparing the phosphatase properties of the two fluids under strictly comparable conditions. He demonstrated differences in the properties. Thus the optimum hydrogen ion concentration for the phosphatase of the red cell was found to be p_H 6·0 as compared with p_H 8·9 for the enzyme of the plasma. Moreover the red cell-enzyme hydrolysed α -glycerophosphoric acid more rapidly than the corresponding β -compound, while the plasma-enzyme hydrolysed the β -compound more rapidly.

Robison and Soames [1924] described experiments upon the p_H -activity of the bone, kidney, spleen, liver and pancreatic extracts. They interpreted their

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results as evidence in favour of the hypothesis that the spleen, liver and pancreas contained a phosphatase different from that of the bone and kidney.

In the present work this suggestion has been more fully investigated, and evidence is adduced to suggest that the liver and spleen contain a phosphatase which possesses properties somewhat different from those of both the bone- and the red cell-enzyme.

Technique.

The tissues used in the following experiments were generally from sheep or bullocks and were obtained from the public slaughterhouse immediately upon the death of the animal and used without delay in the majority of cases. Wherever delay occurred the tissue was stored at 0° until ready for use.

In the case of the dog tissue, I am indebted to Dr A. Hemingway, of the Physiology Department, of the University College, Cardiff, for his generosity in supplying me with tissues from the animals which he was using for other experiments. These animals were killed under anaesthetic, and the tissues were obtained immediately. There was no pre-death treatment which might invalidate the results here reported.

Extracts of tissue were prepared by a method similar to that of Kay [1928, 1]. The tissue was minced and then ground up with sand and chloroform water. In the case of the spleen and liver an active suspension was obtained by grinding up 1 part of tissue with 5 parts of chloroform water. After standing overnight at room temperature, the mixture was filtered through glass-wool and used without further purification.

The general method of the research consisted of buffering the extract with phthalate, borate or glycine buffers to a suitable p_H and then measuring the extent of hydrolysis of α - and β -glycerophosphoric acids at various p_H values and after heating at 37°. In order to control the p_H more efficiently it was found necessary to use three glycerophosphate solutions, the p_H of which had been previously adjusted for use over small acid, neutral or alkaline ranges. Even under these circumstances it was found that the buffer solutions did not accurately maintain solutions containing tissue extract at the nominal hydrogen ion concentration of the buffer used. Consequently it was always found necessary to carry out a p_H determination in conjunction with each phosphorus analysis; p_H was measured by the capillator method.

Inorganic phosphate was estimated by the method of King [1932]. Furthermore in view of the anomalies that glycerophosphate can cause in the determination of inorganic phosphorus [Davies and Cule-Davies, 1932], both the amount of substrate used and the time taken for colour development were carefully controlled as suggested by these authors.

The variations in the phosphatase activity of spleen and liver extracts at various hydrogen ion concentrations.

Table I shows the variation in the extent of hydrolysis of sodium β -glycerophosphate with extracts of spleen and liver at varying hydrogen ion concentrations.

Each tube contained 5.0 ml. of buffer solution, 1.0 ml. of tissue extract and 1.0 ml. of 1.5 % sodium β -glycerophosphate solution. The tubes were heated for 4 hours at 37° and then proteins were removed by the addition of 1.0 ml. of 20 % trichloroacetic acid. Aliquot portions of the protein-free filtrate were taken for analysis.

Table I. *The variation in phosphatase activity of tissue extracts under varying conditions of p_H .*

	mg. of P produced				
p_H	1.0 ml. of spleen extract	1.0 ml. of liver extract	1.0 ml. of kidney extract	2.0 ml. of plasma	2.0 ml. of lysed red cells
4.2	0.280	0.280	0.115	—	—
4.6	0.346	0.290	0.115	—	0.110
4.8	—	—	0.115	—	—
5.0	0.346	0.260	—	—	0.122
5.2	0.190	0.220	0.110	—	—
5.6	0.126	—	—	—	0.147
6.4	—	0.175	—	0.070	0.206
6.8	—	0.165	0.090	0.130	0.110
7.0	0.046	0.150	—	—	0.125
7.4	—	—	—	—	0.125
7.6	0.046	—	0.258	0.360	—
8.0	—	0.256	—	0.580	—
8.3	0.126	0.320	0.381	0.606	—
8.9	0.250	0.400	0.461	—	—
9.4	0.260	0.500	0.474	—	—
9.6	0.230	0.450	—	—	—

The above figures are corrected for the amount of P initially present in the enzyme preparation. The zero hour controls were spleen, 0.360 mg.; liver, 0.240 mg.; kidney, 0.075 mg.; plasma, 0.070 mg.; red cells, 0.052 mg. P per volume of tissue extract *etc.* indicated in the table.

The plasma and lysed cells were heated at 37° for 20 hours.

From p_H 4.4 to 5.0 extracts of spleen show an increasing activity reaching a maximum about p_H 4.8. At less acid reactions, the activity decreases fairly rapidly reaching an almost negligible value at p_H values near neutrality. From p_H 7.6 an increase of activity occurs and the rate of increase is fairly rapid. A second maximum is reached at or near p_H 9.0.

Liver extracts exhibit the same phenomenon. The activity increases to a maximum about p_H 4.7 and then decreases to a minimum at p_H 7.0, finally reaching a maximum again at p_H 9.0.

For purposes of comparison and control, similar series of observations were carried out with kidney extracts and red cells. These results are incorporated in Table I.

Kidney extract was prepared by the method of Kay [1926] in 5 % suspension. Plasma and cells were separated by centrifuging. The plasma was centrifuged a second time in order to ensure the removal of all traces of blood cells. The cells were washed three or four times with isotonic saline and then lysed with an equal quantity of distilled water. They were allowed to stand at room temperature for 12 hours in order to facilitate hydrolysis of naturally occurring esters of phosphorus.

Kidney extracts showed only one optimum of activity and that at p_H 9.0. Plasma exhibited phosphatase activity only on the alkaline side of neutrality. The red cell-enzyme, however, exhibited its maximum effect on the acid side of neutrality. These results confirm the findings of other workers [Kay, 1926; 1931; Asakawa, 1929; Roche, 1931].

These experiments demonstrate the fact that liver and spleen extracts exhibit two optima of hydrogen ion concentration for phosphatase activity. One occurring at p_H 9.0 and the other at p_H 4.5 to 5.0. This property is characteristic of liver and spleen but not of kidney, plasma or red cells.

The relative activities of spleen extracts at the two optima of hydrogen ion concentration.

The relative activities of spleen extracts at p_H 5.0 and 8.8 are not constant, either from animal to animal in the same species or from species to species (Table II).

Table II. *The relative activities of extracts of the spleen of various animals at p_H 5.0 and p_H 8.8.*

Ratio = $\frac{\text{Activity at } p_H \text{ 5.0}}{\text{Activity at } p_H \text{ 8.8}}$				
Animal				
Bullock	Calf	Pig	Dog	Lamb
2.30	1.00	9.00	2.00	2.32
2.80	2.70	4.00	2.90	2.09
2.70	4.50	6.00	—	1.48
0.80	4.30	3.20	—	2.26
2.20	1.40	5.50	—	—
1.50	—	1.40	—	—
—	—	5.50	—	—
—	—	3.80	—	—

The most consistent results were found with bullock, lamb and dog spleens. The average value found for 6 bullocks' spleens was 2.1, with maximum and minimum values of 2.8 and 0.8 respectively. The values found for 2 dogs' spleens were 2.0 and 2.9. The average value for the spleens of four lambs was 2.0, with the maximum and minimum values of 2.3 and 1.5.

The variation of this ratio was however most marked in calf and pig spleens. Thus the maximum and minimum values in the spleens of 5 calves were 4.5 and 1.0 and the average was 2.8. Its deviation was even greater with pigs' spleens. In this species the maximum and minimum values were 9.0 and 1.4 respectively, with an average of 6.0.

The effect of magnesium ions upon spleen extracts and red blood cells under varying conditions of hydrogen ion concentration.

Erdtmann [1927; 1928] and Jenner and Kay [1931] claim that magnesium ions exert an activating effect upon kidney- and bone-phosphatase and also upon the red cell-enzyme at p_H 7.6. The effect of magnesium ions upon spleen extracts and also upon the red cell-enzyme has been observed under different conditions of hydrogen ion concentration and compared with the corresponding effect on the bone-enzyme. The results are incorporated in Fig. 1 and demonstrate the effect of 0.006 *M* magnesium chloride upon spleen- and red cell-phosphatase. The broken line curves show the variation in activity of spleen extracts and also lysed red cells in the presence of added magnesium; the continuous line curves the behaviour in the absence of the added substance.

The observations with spleen-phosphatase yielded an unexpected result. The maximum relative effect occurred at p_H values near the neutral point. From p_H 4.5 to 5.5 a slight inhibition occurred and from p_H 6.0 to 9.5 a definite stimulation, although the maximum relative effect occurred between p_H 6.0 and 8.0. Table III illustrates this point.

Similar results were observed with red blood cells. The maximum effect was observed at p_H values near neutrality (see Fig. 1). Plasma, kidney and bone

Table III.

p_H	mg. P produced in the presence of added Mg	mg. P produced in the absence of added Mg
5.0	0.225	0.264
7.4	0.100	0.021
8.9	0.200	0.084

Fig. 1. The hydrolysis of β -glycerophosphate in the presence and absence of added magnesium, by spleen extracts and lysed red cells under varying conditions of hydrogen ion concentration.

●—● Hydrolysis in absence of added Mg; ○—○ Hydrolysis in presence of added Mg.

extracts all show marked stimulation by magnesium, particularly with dialysed preparations. The increase in activity varies with the p_H , exhibiting a maximum at the optimum p_H of the enzyme.

Both with blood and spleen, the effect of magnesium seems to be to move the optimum p_H of the enzyme to more physiological values and to facilitate the breakdown of organic phosphates at conditions near neutrality. Inasmuch as this is true, we have a fundamental difference between the enzyme of the blood and spleen on the one hand and that of the bone, kidney *etc.* on the other. The combined enzyme-magnesium complex of the bone-enzyme functions optimally at p_H 8.9, whilst that of the spleen and blood exhibits its maximum effect at or near neutrality.

The hydrolysis of α - and β -glycerophosphates by spleen extracts.

The rates of hydrolysis of α - and β -glycerophosphoric acids in the presence of the bone- and red cell-enzymes are different. The bone-enzyme hydrolyses β -glycerophosphoric acid more rapidly than the α -isomeride [Kay, 1926; Kay and Lee, 1931]. The red cell-enzyme however hydrolyses the α -compound the more rapidly [Roche, 1931].

Fig. 2 shows the variation in the rates of hydrolysis of α - and β -glycerophosphoric acids under various conditions of hydrogen ion concentration, using spleen extracts as the source of enzyme. The β -compound was used as the sodium salt: the α -isomeride was used as the ammonium salt. Solutions were prepared to contain the same concentration of phosphorus.

The α -isomeride was hydrolysed in a similar manner to the β -compound, in that the p_H -activity curve exhibited two optima at the same p_H values as with the β -compound as substrate. From Fig. 2 it will be seen that the ratio of

substrate hydrolysed at p_H 5.0 to that at p_H 9.0 varies with the substrate. Thus with the β -compound the ratio was 0.8 and with the α -compound, using the same extract and at the same time, the ratio was 1.7.

The relative rates of hydrolysis of the two compounds were however quite different. The β -compound was hydrolysed rather faster than the α -compound at alkaline p_H values. Near neutrality, the two substances were hydrolysed at

Fig. 2. The hydrolysis of α - and β -glycerophosphates at varying hydrogen ion concentrations, by spleen extracts and lysed red cells.

●—● α -Glycerophosphate; ○---○ β -Glycerophosphate.

nearly the same rate. On the acid side of neutrality however, the β -compound was hydrolysed about three times as fast as the α -compound. In order to control this result the experiment was repeated with red cells. Roche's results were substantiated and a marked difference in the properties of the two enzymes was emphasised (Fig. 2).

The effect of magnesium upon the rate of hydrolysis of α -glycerophosphoric acid was the same as upon that of the β -acid. The relative difference in the rates of hydrolysis was unaltered.

DISCUSSION.

The identity of enzymes cannot be determined exactly as long as one enzyme is mixed with others of the same group. If it is possible to separate the group into its components, then the problem becomes much simpler and the results more definite. The identity of the phosphatases occurring in mammalian tissues cannot be accurately defined, because although results have been quoted in favour of the identity or otherwise of the various phosphatases from animal sources, such results have been based upon the evidence of experiments in which either crude tissue extracts or mixtures of enzymes have been used.

Up to the present, all the reliable evidence suggests the existence of but two distinct and separate phosphatases. (i) The bone-enzyme and (ii) the red blood cell-phosphatase. Roche's claim that the red cell-enzyme is a different mechanism

from that of the bone-phosphatase is a strong one inasmuch as he was able to effect a separation of the enzymes of bone and the red cell and to demonstrate a difference between their properties.

In the present communication experiments have been described which demonstrate the fact that spleen extracts are capable of exhibiting phosphatase activity upon both the acid and alkaline sides of neutrality. The interpretation of such results is difficult because it has not yet been possible to purify the extracts to any great degree, nor has it been possible to separate the extracts into fractions which contain two distinct enzymes. Even so the assumption that spleen extracts contain two distinct and separable enzymes, each capable of hydrolysing phosphoric esters, may be made, in so far as it explains all the facts at our disposal.

Thus such a hypothesis explains the existence of two optima of p_H , the fact that the ratio of the activities of the extracts at the two optima is such a variable quantity (if we are dealing with the same mechanism over the whole range of hydrogen ion concentration studied, then it is reasonable to believe that the ratio should be a constant one); and lastly it explains the effect of magnesium.

The two enzymes may be described for the sake of clarity as the "acid" and "alkaline" phosphatases of the spleen. A comparison of the properties of these two enzymes with those of the bone and blood cells shows some interesting similarities and differences (Table IV).

Table IV. *Comparison of properties of the phosphatases of spleen, bone and red cells.*

Property	Source of enzyme			
	Bone	Red cell	Spleen, acid	Spleen, alkaline
Optimum p_H	8.9	6.0	4.5-5.0	9.0
Effect of magnesium at optimum p_H	Stimulation	Stimulation	Slight inhibition	Stimulation
Effect of magnesium at p_H 7.6	Slight stimulation	Maximum stimulation	Maximum stimulation	Slight stimulation
Relative rates of hydrolysis of α - and β -glycerophosphates	Hydrolyses β -compound 30 % faster than α	Hydrolyses α -compound 300 % faster than β	Hydrolyses β -compound 300 % faster than α	Hydrolyses β -compound about 30 % faster than α

As a result it is possible to identify the alkaline enzyme of the spleen with the bone-enzyme. Thus they both possess the same optimum p_H . They also hydrolyse α - and β -glycerophosphates at the same relative rates, and the effect of magnesium is the same upon both, at both the optimum p_H and at p_H 7.6. The acid enzyme of the spleen however, exhibits markedly different properties from either the alkaline enzyme or the bone-enzyme (Table IV). It resembles the red cell-phosphatase but also differs from this chiefly in two respects; it possesses a different optimum p_H , and it hydrolyses α - and β -glycerophosphates at different rates from the red cell-phosphatase.

The fact that the acid enzyme of the spleen differs from the red cell-phosphatase in just those properties which Kay used for grouping the phosphatases of the bone, kidney *etc.*, suggests the possibility that the two mechanisms may be different. Since, however, little is known of the effect of naturally occurring stimulants and retardants to phosphatase activity it must be remembered that possibly the differences here described may be due to the effect of such substances.

SUMMARY.

1. Extracts of spleen and liver show two optima of hydrogen ion concentration for phosphatase activity. Extracts of bone and intestine and blood-plasma and red cells show only one optimum.

2. The two optima for spleen and liver extracts occur at p_H 4.5–5.0 and at p_H 8.9.

3. There is no relationship between the phosphatase activity at the two optima either from animal to animal or from species to species.

4. Magnesium ions stimulate spleen-phosphatases from p_H 6.0 to 9.5. Below p_H 6.0 a slight inhibition is produced.

5. The maximum relative effect of magnesium ions is found at p_H values near neutrality.

6. Spleen extracts hydrolyse β -glycerophosphoric acid at p_H 5.0 about three times as rapidly as the corresponding α -compound.

7. It is suggested that the spleen contains two separate and distinct phosphatases, one which is identical with the bone-enzyme and the other which possesses properties different from those of the enzyme of either bone or the red cell.

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