XCI. THE POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION.

PART VII. THE BONE PHOSPHATASE.

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IN the earlier papers of this series [Robison and Soames, 1924] some account was given of the properties of the phosphatase which is present in ossifying cartilage, bones and teeth, and also in the kidney and intestinal mucosa. In the following we give the results of a more detailed study of some of its properties about which further knowledge was desirable in order to understand the part played by this enzyme and by phosphoric esters in the process of ossification.

OPTIMUM p_H .

It was previously stated that the optimum p_H of this enzyme lies on a flat curve between 8.4 and 9.4, the activity being nearly constant between these limits, but falling off rapidly on either side of them. This statement was based on results of 18-hour experiments, but later observations suggested that the flattening of the curve was in part due to the gradual inactivation of the enzyme at the higher p_H values and that over short periods of time the activity continues to increase with the alkalinity up to a p_H well above 8.4. More exact investigation of the course of hydrolysis has shown that these suppositions are correct. Fig. 1 shows the rate of hydrolysis of glycerophosphoric ester over a period of 5 hours at p_H between 7.25 and 9.4. Further measurements, not shown on the chart, were taken at the end of 24, 48 and 166 hours. The p_H was determined from time to time by a micro-colorimetric method (capillator) on 0.02 cc. liquid withdrawn from the reacting mixture. The addition of indicators to the bulk of the liquid was inadmissible on account of the subsequent colorimetric estimations of inorganic phosphate (Briggs). In this experiment the initial rate increased with the alkalinity up to 9.4, the highest p_H attempted, but at the end of 24 hours the amount hydrolysed at p_H 9.4 (59.8 %) was almost identical with that at 8.9 (59.1 %).

Fig. 2 shows the inactivation of the bone enzyme in 0.4 % solutions of sodium bicarbonate adjusted to different p_H . The rate of inactivation increases rapidly with the p_H above 9.0. At 9.55 over 60 % of the activity was lost in 24 hours.

On the acid side of p_H 6.0 inactivation occurs still more rapidly. In 2 hours at 38° 20 % of the activity was destroyed at p_H 5.2, 50 % at p_H 4.2, and 100 % at p_H 3.2.

In Fig. 3, Curve A, the rate of hydrolysis over a short initial period (1 hour) is plotted against the p_H .

Curve B gives the results of another experiment in which the acid range was more particularly investigated. Here, as in Curve A, a slight increase in the rate of hydrolysis is shown on the acid side of p_H 6.0, and this increase must have been greater but for the partial inactivation of the enzyme during the period of the experiment—in this case 2 hours. Correcting for this loss, Curve B would take approximately the course of the dotted line.

Fig. 1. Hydrolysis of glycerophosphoric ester by bone phosphatase at different p_H . Concentration of ester 0.1 *M*. Temp. 38.0° .

Our intention—to measure the initial velocities of the reaction—has not been completely realised in these experiments for, even in their short duration, the retarding effect of the liberated inorganic phosphate (see below) was appreciable, especially at the higher p_H . The true curve of initial velocities would, therefore, probably show a slightly steeper gradient than that of Curve A.

This activity- p_H curve resembles the dissociation curve of a weak acid, or the dissociation-residue curve of a weak base, and recalls those obtained by Michaelis and his co-workers [1911, 1920] for invertase, except that the slope is in the contrary direction. Michaelis at first explained his results on the assumption that invertase is an acid and that its undissociated molecule

combines with saccharose to form a compound whose decomposition takes place spontaneously as a monomolecular reaction. Subsequent investigation of the relation between the activity- p_H curve and the concentration of the substrate caused him to modify this conception in favour of the hypothesis

Fig. 2. Inactivation of the bone phosphatase at different p_H . Bone extract kept 24 hours in 0.4 % NaHCO_3 solutions adjusted to different p_H . Temp. 38.0° . Activity of enzyme measured by the hydrolysis of 0.1 M sodium glycerophosphate in $2\frac{1}{2}$ hours at p_H 9.1, and given as a percentage of the initial activity of the extract.

that invertase combines with the substrate to form a compound with acid properties and that it is the undissociated molecule of this compound which suffers spontaneous decomposition. On this assumption the parameter of the curve is identical with the dissociation constant of the saccharose-invertase acid.

By similar reasoning it may be supposed either (1) that the bone enzyme has the properties of a weak base whose undissociated molecule combines with the phosphoric ester to form a compound which thereupon breaks down into the hydrolysis products of the ester, or (2) that the enzyme combines with the ester to form a compound having the properties of a weak acid, whose anions suffer spontaneous decomposition¹.

Fig. 3. Activity- p_H curve of the bone phosphatase. Activity measured by the hydrolysis of 0.1 M sodium glycerophosphate and given as the amount of ester hydrolysed (mg. P) per cc. in 1 hour. Temp. 39.5°.

Whichever possibility is the correct one, the p_K of this basic or acidic compound would be equal to the p_{OH} or p_H respectively at the point of 50 % dissociation, that is, where the velocity of hydrolysis is half the maximum. The uncertainty connected with the experimental determination of this maximum, which lies in the region where rapid inactivation of the enzyme

¹ There are other possibilities which seem less credible, *e.g.* that the enzyme-substrate complex has the properties of a base, the concentration of whose undissociated molecule governs the rate of the reaction.

occurs, precludes more than a rough guess at the value of this constant. This appears to lie in the neighbourhood of p_H 8.2.

On the second supposition, the acid would thus be weaker than either glycerophosphoric or phosphoric acid, for which the values of p_{K_2} are 6.33 and 6.81 [Meyerhof and Suranyi, 1926].

Our present data are insufficient to decide between these alternatives and some doubt must even be admitted as to whether the interpretation of the graph as a dissociation curve is justified. The possibility of some loss of activity occurring even in short periods at p_H above 9.2 is difficult to eliminate and might account for the flattening of the curve in this region. Further experiments are, however, in progress.

EFFECT OF THE CONCENTRATION OF SUBSTRATE ON THE RATE OF REACTION.

Michaelis and Menton [1913] found that the rate of hydrolysis of saccharose by invertase was dependent on the concentration of the substrate and that the "activity- p_S " graph, like that showing the relationship between activity and p_H , had the character of a dissociation curve.

We have been unable to demonstrate any corresponding variation of activity of the bone phosphatase with concentrations of ester between 0.003 M and 0.3 M . Table I gives the results of two experiments lasting 15 minutes and 1 hour respectively. Such small differences as were observed in the amounts hydrolysed are irregular and cannot be attributed to changes in the substrate concentration.

Table I. *Effect of concentration of substrate on the rate of reaction.*

Concentration of ester (glycerophosphate)	Amount hydrolysed					
	<i>Exp. 1</i>			<i>Exp. 2</i>		
	p_H 8.2.	Temp. 38°.	Time 15 mins.	p_H 8.5.	Temp. 38°.	Time 1 hr.
	mg.	P per cc.	% of ester	mg.	P per cc.	% of ester
0.3 M	0.041	—	0.45	0.272	—	3
0.2 M	—	—	—	0.250	—	4.5
0.1 M	0.039	—	1.3	0.265	—	9
0.05 M	—	—	—	0.238	—	16
0.03 M	0.043	—	4.8	0.274	—	30
0.01 M	0.051	—	17	0.248	—	80
0.003 M	0.039	—	43	—	—	—

EFFECT OF HYDROLYSIS PRODUCTS ON THE RATE OF THE REACTION.

If the values for $k = \frac{1}{t} \log \frac{a}{a-x}$, the velocity constant for a monomolecular reaction, are calculated from the data of hydrolysis experiments such as those quoted on p. 666, these values are found to fall throughout the experiment, and more rapidly than can be accounted for by inactivation of the enzyme. An investigation of the effect of the hydrolysis products on the velocity of the reaction showed that a great part of this retardation is due to the inorganic phosphate, and very little, if any, to the glycerol. Table II gives values of k for the hydrolysis of sodium glycerophosphate with and

without added inorganic phosphate, showing the effect of varying amounts of the latter on the rate of the reaction. With no added inorganic phosphate the values of k fall from 0.0133 to 0.0076 during the hydrolysis of 59.8 % of the ester. The initial value of k is reduced by about 20 % in presence of 0.017 M inorganic phosphate, and by more than 50 % when the concentration of phosphate is increased to 0.033 M . With 0.1 M phosphate the results for the first 5 hours appear to indicate no hydrolysis, and although definite increase of inorganic phosphate was observed after 24 and 48 hours the amount of hydrolysis was less than one-fifth of that found in the control. Results of another experiment with different concentrations of organic and inorganic phosphate are shown graphically in Fig. 4.

Fig. 4. Effect of inorganic phosphate on the rate of hydrolysis of glycerophosphoric ester by the bone phosphatase. Concentration of ester 0.03 M . Concentration of added inorganic phosphate as shown on each curve. Temp. 38.0°.

Table III gives the results of experiments in which glycerol was added, and shows that this hydrolysis product has very little effect on the rate of the reaction in concentrations up to 0.1 M . The more pronounced effect of glycerol in high concentrations (25 % and over) is discussed in the next section.

THE SYNTHETIC ACTION OF THE ENZYME.

The depression of the velocity of hydrolysis by inorganic phosphate observed in the previous experiments suggested that the reverse reaction, synthesis of phosphoric ester, was proceeding simultaneously at an appreciable

rate. We were, however, unable to obtain conclusive evidence of synthesis starting from inorganic phosphate and glycerol in the concentrations (0.01 *M*–0.1 *M*) used in these experiments.

Table II. *Effect of inorganic phosphate on the rate of hydrolysis of glycerophosphoric ester by bone phosphatase.*

Concentration of ester 0.1 *M*, *p*_H 8.05, temp. 37°.

Concentration of added inorganic phosphate as shown below.

Hrs. hydrolysed	Inorg. P = 0		Inorg. P = 0.017 <i>M</i>		Inorg. P = 0.033 <i>M</i>		Inorg. P = 0.1 <i>M</i>	
	% of ester	$\frac{1}{t} \log \frac{a}{a-x}$	% of ester	$\frac{1}{t} \log \frac{a}{a-x}$	% of ester	$\frac{1}{t} \log \frac{a}{a-x}$	% of ester	$\frac{1}{t} \log \frac{a}{a-x}$
1	3.02	0.0133	2.48	0.0109	1.28	0.0051	0	0
2	5.67	0.0121	4.62	0.0097	2.67	0.0061	0	0
5	12.11	0.0103	9.17	0.0071	4.98	0.0035	0	0
24	38.97	0.0083	34.05	0.0062	23.58	0.0050	7.73	0.0015
48	59.80	0.0076	50.38	0.0060	32.87	0.0024	11.77	0.0008

Table III. *Effect of glycerol on the rate of hydrolysis of glycerophosphoric ester by bone phosphatase.*

Concentration of ester 0.1 *M*, *p*_H 8.4, temp. 38°.

Concentration of added glycerol as shown below.

Hrs.	Percentage of ester hydrolysed		
	Glycerol = 0	Glycerol = 0.01 <i>M</i>	Glycerol = 0.1 <i>M</i>
1	9.7	9.7	9.2
2	15.3	17.5	16.5
5	30.3	31.3	32.2

When the concentration of the glycerol was considerably increased (and the active mass of the water thereby reduced) the synthetic action of the enzyme became very evident, up to 25 % of the inorganic phosphate being converted into phosphoric ester. Synthesis of phosphoric esters was also observed when glycerol was replaced by other polyhydric alcohols, glycol, mannitol, glucose and fructose, but in 50 % ethyl alcohol neither synthesis nor hydrolysis (of glycerophosphate) occurred to an appreciable extent.

The experiments were carried out by adding the alcohols and solid K_2HPO_4 to the bone extract and keeping the mixture at 38°, withdrawing 0.2 cc. into 10 cc. 3 % trichloroacetic acid at intervals. Duplicate determinations of total P and inorganic phosphate were carried out on the protein-free filtrates. No attempt was made to determine the *p*_H of these concentrated solutions, but the presence of the alkaline phosphate was relied on to maintain a suitable reaction.

The results of some of the experiments are set out in Table IV, the amount of phosphoric ester synthesised being calculated as a percentage of the inorganic phosphate added.

Table IV. *Synthesis of phosphoric esters by the bone phosphatase.*

				Concentration of K_2HPO_4	Amount of P-ester formed calculated as % of inorganic P added					
Alcohol					M	1 day	7 days	14 days	21 days	28 days
Glycerol	50 % by vol.	...	...	0.07	6.0	22.7	—	24.5	—	
"	"	...	...	0.2	1.8	12.3	19.3	—	22.6	
"	"	...	...	0.5	0.8	3.9	5.4	—	10.6	
"	66 %	...	...	0.12	—	7.0	12.3	—	16.4	
Glycol	25 % by vol.	...	...	0.1	3.8	11.9	17.3	—	16.3	
"	50 %	...	...	0.1	6.9	17.2	22.4	23.1	—	
"	"	...	...	0.1	—	18.3	24.8	23.5	—	
Alcohol	50 %	...	...	0.1	0	0	—	—	—	
Mannitol, sat. solution, about 30 %		...	...	0.16	—	8.5	9.8	8.3	—	
Glucose	60 %	...	...	0.12	1.8	2.8	5.8	5.3	—	
Fructose	60 %	...	...	0.12	—	3 days 4.0	—	—	—	

THE EFFECT OF HORMONES ON THE RATE OF HYDROLYSIS AND OPTIMUM p_H OF THE ENZYME.

In view of the work of Langfeld [1921], who claimed to have demonstrated that the optimum p_H of liver diastase was shifted in presence of various glandular extracts, a series of experiments was performed to find whether such an effect could be obtained with the bone phosphatase. The relative rates of hydrolysis were measured at various p_H ranging from 7.0 to 9.3 with additions of the following hormones.

Adrenaline	$\frac{1}{25,000}$	—	$\frac{1}{2,500,000}$	(dilution in total volume)
Pituitrin	$\frac{1}{25}$	—	$\frac{1}{2,500}$	(" ")
Thyroid	$\frac{1}{400}$	—	$\frac{1}{40,000}$	(" ")

It is not necessary to give in detail the results of the numerous experiments carried out, but in no case was any definite effect detected on the rate of hydrolysis or the optimum p_H . Similar negative results have also been reported by Demuth [1925].

THE ACTION OF THE BONE PHOSPHATASE ON VARIOUS PHOSPHORIC ESTERS.

Table V shows the action of the bone phosphatase on mono- and diethyl phosphoric esters, which were kindly given to us by Prof. R. H. A. Plimmer and Mr W. J. N. Burch. The enzyme had very little action on the diethyl ester but the monoethyl ester was readily hydrolysed. The enzyme was also found to be without action on a fully methylated derivative of hexosediphosphate prepared by Mr W. T. J. Morgan [1927] whereas the methylhexosediphosphate was readily hydrolysed. These differences may perhaps be attributed simply to the steric hindrance of the adjacent methyl or ethyl groups, but in view of the much greater affinity of the enzyme for inorganic phosphate than for phosphoric esters, and its very small affinity for glycerol, it is an interesting speculation as to whether the combination of

enzyme and substrate takes place through the hydroxyl groups attached to the phosphorus, the presence of at least two such groups being required.

Table V. *Action of bone phosphatase on mono- and di-ethyl phosphates.*

Ester	Percentage of ester hydrolysed Hours					
	1	2	3	5	24	48
Potassium monoethyl phosphate 0.05 <i>M</i>	8.0	11.7	18.9	26	57	69
Potassium diethyl phosphate 0.08 <i>M</i>	0.1	0.3	0.3	0.4	0.5	0.7

The discovery of the marked effect of inorganic phosphate on the rate of hydrolysis furnished an explanation of a difficulty encountered in attempting to use the bone phosphatase for the hydrolysis of hexose mono- and di-phosphoric esters and the investigation of their sugar component.

When the ester was used in such concentration as would give a reasonable amount of the sugar, hydrolysis rapidly came to an end, although the enzyme was shown to be still active. The difficulty was overcome by carrying out the hydrolysis in presence of barium ions by which the free phosphate was precipitated in the form of $\text{Ba}_3(\text{PO}_4)_2$. As the formation of this salt involved a fall in the p_{H} sufficient baryta was added at frequent intervals to maintain a p_{H} of about 8.4 to 8.8. In this way quantities of 0.5 g. barium hexosediphosphate can be almost completely hydrolysed in a few days. The drawback of this method for use with the free hexosephosphoric esters lies in the necessary use of an alkaline reaction and the consequent intramolecular transformation of the sugars which may be anticipated. This objection does not apply to methylhexosides of these hexosephosphates, and the application of this method to these compounds may furnish useful information as to the nature of the sugar [*v. Morgan*, 1927]. The method is in fact analogous to the case of calcification in the body where any increase of inorganic phosphate in the cartilage due to the activity of the enzyme would normally be followed by the precipitation of this excess as calcium phosphate, leaving the activity of the enzyme unimpaired.

SUMMARY AND CONCLUSIONS.

1. The activity of the bone phosphatase increases with the alkalinity up to p_{H} 9.4, but above 8.4 this is offset by the gradual inactivation of the enzyme.

In the body the optimum p_{H} may therefore be taken as about 8.4.

2. The activity- p_{H} curve has the form of the dissociation curve of a weak acid, p_{K} about 8.2, or the dissociation-residue curve of a weak base.

3. Variations in the concentration of the substrate between 0.003 *M* and 0.3 *M* do not affect the initial rate of hydrolysis.

4. The rate of hydrolysis of glycerophosphoric ester is greatly retarded by inorganic phosphate even in low concentrations, but is not appreciably affected by similar concentrations of the second hydrolysis product—glycerol.

5. In high concentrations of glycerol and in presence of inorganic phosphate, synthesis of phosphoric ester takes place, as much as 25 % of the inorganic phosphate being esterified under certain conditions.

6. The synthetic action of the enzyme has likewise been demonstrated with mannitol, glycol, glucose and fructose.

7. No effect on the rate of hydrolysis or the optimum p_H of the enzyme has been detected as a result of adding adrenaline, pituitrin or thyroid.

8. The action of the enzyme on various types of phosphoric ester is discussed.

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