CLVII. BRAIN PHOSPHATASE.

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Kay [1928] and King [1931] have shown that the brain of animals contains a phosphatase which hydrolyses glycerophosphate. Recently Edlbacher *et al.* [1934] have investigated the enzymes of brain and have come to the conclusion that the brain substance causes a slight cleavage of nucleic acid, magnesium hexosephosphate and sodium glycerophosphate. Except for the above findings very little is known about the phosphatases of the brain. Brain phosphatase is of interest since it is present in an organ rich in phosphatides. In the present work the nature and action of brain phosphatase and its identity with the phosphatases present in other organs have been investigated.

Experimental.

Preparation of material. Sheep's brain was used. The whole brain of each animal (weighing about 80–90 g.) was obtained just after the animal was killed, washed with distilled water and trimmed free from the outer membrane with capillaries containing blood. The tissue was ground in a mortar, the pulp stirred with 200 ml. of acetone for 15 min. and then filtered on a Büchner funnel. The process was then repeated, followed by washing with 200 ml. of ether. The dry powder was spread in a thin layer on filter-paper and left overnight at room temperature. The next day it was ground in a mortar to a fine powder and stored in a vacuum desiccator. The white, fat-free brain powder thus obtained amounted to about 10–12 g. This powder keeps its activity for several months.

Enzyme. The powder was treated with ten times the volume of distilled water containing toluene and allowed to remain at room temperature for 24 hours. A slightly opalescent extract was obtained after filtration on a Büchner funnel. The activity of this extract which was stored in a refrigerator at 0° remained intact for a considerable time.

Buffers. Walpole's acetic acid-acetate buffer was used for the acid range, veronal buffer of Michaelis for the p_H range 6·8–9·6 and Sørensen's glycine buffer for the p_H range 8·5–12·9. All solutions were adjusted to the desired p_H before mixing and the p_H of each mixture was determined by the capillator method.

Substrates. The substrates employed were Merck's sodium β-glycerophosphate and sodium hexosediphosphate.

The sodium hexosediphosphate solution was prepared from a pure sample of calcium hexosediphosphate obtained from Messrs British Drug Houses Ltd. A weighed amount was dissolved in water, treated with slightly less than the equivalent amount of pure sodium oxalate and centrifuged. After testing to ensure freedom from oxalate the solution was stored at 0°; fresh solutions were made up at frequent intervals.

Procedure. The hydrolysis was carried out as follows. A series of tubes was set up each containing 5 ml. of substrate (2% sodium glycerophosphate

solution, adjusted to the desired p_H), 10 ml. buffer, 5 ml. of the enzyme solution and water to make up the total volume to 25 ml. When other solutions such as $MgCl_2$, $6H_2O$ were added simple adjustments of the volume of buffer were made, the concentration being kept constant. Control tubes containing enzyme, added ions and buffer were incubated with the experimental tubes. It was found that in the control tubes containing the substrates no detectable amounts of inorganic P were present at any time.

The hydrolysis was carried out in a thermostat at 35° for exactly 4 hours. An aliquot portion of the reaction mixture was then removed, an equal volume of 10 % trichloroacetic acid added and the contents were filtered after 15 min. The filtrate was then analysed for inorganic phosphorus by a colorimetric method [Fiske and Subbarow, 1925]. The increase in inorganic P after deducting the control expressed in mg. P per 10 ml. of reaction mixture measures the activity of the enzyme.

Brain phosphatase activity and hydrogen ion concentration.

The activity of brain phosphatase at various hydrogen ion concentrations is plotted in Fig. 1. The brain extract is found to exhibit two optima for phosphatase activity, one at p_H 9·4–9·6 and the other at p_H 5·0. In this respect

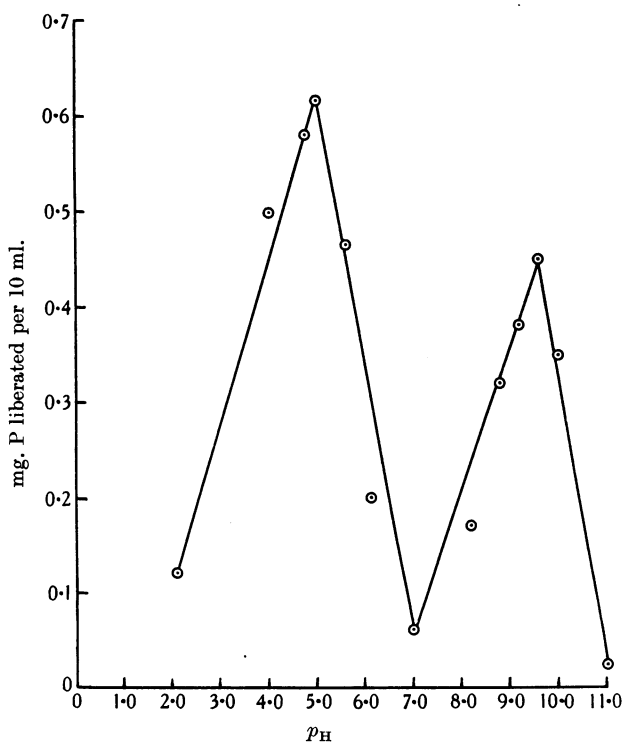

Fig. 1. Effect of p_H on the hydrolysis of sodium β-glycerophosphate by the brain phosphatase.

brain phosphatase resembles spleen, liver and kidney phosphatases, which have been shown by Davies [1934] and Bamann and Riedel [1934] respectively, to contain both alkaline and acid phosphatases.

Presence of two phosphatases in brain.

Sheep's brain was found to contain appreciable quantities of both the acid and alkaline phosphatases. In Table I are given the activities at p_H 5·0 and 9·4 of both the phosphatases of several brain extracts prepared from acetone-treated brain. The ratio of the two phosphatases $\frac{\text{acid phosphatase } (p_H\ 5{\cdot}0)}{\text{alkaline phosphatase } (p_H\ 9{\cdot}4)}$ was very nearly the same in most of the brains examined except in two cases where a much higher value was obtained. Further, the results show that the individual variation in the activity of the phosphatases, particularly the acid phosphatase, is not considerable, so that the quantitative variation in the phosphatase activity of brain under normal and abnormal conditions could be studied without any serious error.

Table I. *The relative activities of the acid and alkaline phosphatases of brain.*

	Activity (mg. P in 10 ml. of reaction mixture)		Ratio $\frac{\text{Activity of the acid phosphatase } (p_H\ 5{\cdot}0)}{\text{Activity of the alkaline phosphatase } (p_H\ 9{\cdot}4)}$
Brain No.	Acid phosphatase	Alkaline phosphatase	
1	0·460	0·348	1·32
2	0·460	0·350	1·31
3	0·542	0·404	1·34
4	0·428	0·396	1·10
5	0·378	0·244	1·50
6	0·390	0·284	1·37
7	0·534	0·176	3·00
8	0·520	0·186	3·30

The activity of brain phosphatase as compared with the other tissue phosphatases.

Brain, liver and kidney of the same animal (sheep) were similarly treated with acetone and ether and dried in a desiccator. The dry powders were extracted with water in the same manner as described for brain. The activities of the extracts were determined and the results are presented in Table II.

Table II. *Phosphatase activities of brain, liver and kidney.*

Tissue	The weight of fresh tissue taken for acetone treatment (g.)	The weight of the dry powder obtained after acetone treatment (g.)	Activity (mg. P in 10 ml. reaction mixture after 4 hours' hydrolysis)	
			p_H 9·2	p_H 5·0
Brain	85	12	0·244	0·378
Liver	80	23	0·348	—
Kidney	65	12	0·994	—

Purification of the enzyme.

The crude extract from the dry fat-free brain powder can be purified by the following methods.

Isoelectric precipitation of the inert material at p_H *4·8.* Purification was effected by isoelectric precipitation of the inert material at p_H 4·8 by adding 0·2 *M* acetic acid-acetate buffer to three times the volume of the extract and centrifuging after keeping the mixture at 0° for 30 min. A water clear extract obtained in this manner was found to be quite active. The extract can be further purified by dialysis in a collodion bag for 24 hours.

Dialysis. Short-period dialysis (24 hours) of the crude extract removed the phosphate almost completely without any loss of activity of the enzyme. On continuing the dialysis for about 15 days however the activity was almost completely lost and could be regained by addition of magnesium.

Ultrafiltration. Purification of the extract by high pressure ultrafiltration was found to be quite satisfactory. Ultrafiltration was carried out through cellophane sheet No. 300 under a pressure of 70 kg. per cm.^{2} The extract was kept stirred electromagnetically during filtration. This prevented clogging of the membrane and facilitated rapid filtration. The cellophane sheet was soaked in water for 24 hours before use. Under these conditions the filtration was effected in a very short time about 10–15 ml. being filtered in 1 hour. The residue obtained after the ultrafiltration was found to have retained intact the activity of the acid phosphatase and to have lost almost completely the activity of the alkaline phosphatase which however could be restored by addition of the ultrafiltrate. This method affords a very convenient means of obtaining a preparation of the acid phosphatase only.

The results of a typical experiment are shown in Table III. 40 ml. of the crude extract were filtered and the residue on the filter after complete filtration was removed by washing repeatedly with distilled water and made up to the original volume. The activities of the alkaline and acid phosphatases of the ultrafiltered enzyme extract thus obtained were determined in presence and absence of the ultrafiltrate. For activity determination 5 ml. of the ultrafiltered enzyme extract and 5 ml. of the ultrafiltrate were used.

Table III. *Ultrafiltration of the aqueous extract of acetone-treated brain.*

	Activity (mg. P in 10 ml. of reaction mixture)	
	Alkaline phosphatase (p_H 9·2)	Acid phosphatase (p_H 5·0)
1. Ultrafiltered enzyme	0·02	0·153
2. Ultrafiltrate	0	0
3. Ultrafiltered enzyme *plus* ultrafiltrate	0·178	0·158

Acetone precipitation. The enzyme was also obtained in the form of a dry powder by precipitation from the aqueous extract with twice the volume of acetone after isoelectric precipitation of the impurities. The precipitate was separated by centrifuging, washed with absolute alcohol and ether and dried in a desiccator. A white powder was obtained which was found to exhibit very little activity of the alkaline phosphatase; activity could be restored by addition of magnesium. The preparation retained however the activity of the acid phosphatase.

Effect of magnesium on brain phosphatase.

The influence of magnesium on the activity of brain phosphatase was studied by using both dialysed and undialysed extracts of the acetone-treated brain. Dialysis of the crude extract was carried out in collodion bags against distilled water for 3 days. The activity of the alkaline phosphatase of both crude and dialysed extracts in presence of varying concentrations of $MgCl_2$, $6H_2O$ was determined at p_H 9·4 at 35°. The results are shown in Table IV.

It is evident that the percentage activation of the phosphatase by magnesium is greater in dialysed preparations. Thus an increase of activity of about 600 % is shown by the dialysed extract, whilst the activity of the crude extract increased

Table IV. *Effect of magnesium on the activity of brain phosphatase.*

Added Mg concentration (*M*)	Crude extract: Activity (mg. P in 10 ml. of reaction mixture)	Crude extract: Activation %	Dialysed extract: Activity (mg. P in 10 ml. of reaction mixture)	Dialysed extract: Activation %
0	0·127	—	0·083	—
0·0004	0·222	75	0·412	395
0·001	0·264	108	0·506	510
0·002	0·258	103	0·555	568
0·010	0·228	80	0·606	628
0·020	—	—	0·597	618
0·040	—	—	0·588	607

by only 100 % at optimum concentration of magnesium. The concentration of added magnesium for maximum activation of dialysed enzyme extract is found to lie between 0·01 and 0·02 *M*, while for the crude extract it is only 0·001–0·002 *M*. This is in conformity with the observations of Jenner and Kay [1931]. A possible explanation of this finding is that probably the crude extract contains magnesium in an amount to account for partial activation of the enzyme.

The acid phosphatase of the brain extract behaves differently from the alkaline phosphatase towards magnesium. It does not show any increased activity in presence of magnesium at p_H 5·0. It loses much of its activity on dialysis which cannot be restored by the addition of magnesium. That the loss of activity of the acid phosphatase during dialysis is due to autolysis and not to the removal of any activator is shown by the fact that the activity of the dialysed extract cannot be increased by adding boiled brain extract. It appears from these results that the acid phosphatase of brain is identical with the acid phosphatases of liver and spleen which according to Bamann and Riedel [1934] and Davies [1934] respectively are not activated by magnesium.

Time course of the action of the acid and alkaline phosphatases of brain.

The experiment was carried out at 35° using 20 ml. of the enzyme, 20 ml. of 2 % sodium glycerophosphate solution, 40 ml. of buffer (acetic acid-acetate buffer of p_H 5·0 and glycine buffer of p_H 9·4 respectively) and sufficient water to bring the volume up to 100 ml. 5 ml. of this solution were analysed at once for P after the enzyme had been added; subsequent samples (5 ml.) were analysed for P at known intervals of time. The rate of hydrolysis of sodium glycerophosphate by extracts of acetone-treated brain at p_H 5·0 and 9·4 are plotted in Fig. 2. The broken lines indicate the time course of the action of dialysed extracts with and without the addition of magnesium. All the curves can be considered to be linear till about 10–12 % of the substrate is hydrolysed. Subsequently the rate of reaction diminishes. The course of the action of the phosphatase is therefore of zero order. For reaction times up to 6 hours the amount of hydrolysis affords a good measure of the initial reaction velocity. 4-hour period hydrolysis was therefore adopted for all experiments on the determination of the activity of the enzyme.

Relation between substrate concentration and brain phosphatase activity.

According to Michaelis and Menten [1913] an enzyme combines with its substrate to form a compound and the speed of the reaction depends directly upon the concentration of the enzyme-substrate compound. The theory which

was originally founded on experiments with the invertase-sucrose system has been extended to other enzyme systems. To decide whether the concentration of the enzyme-substrate compound determines the rate of the reaction, it is

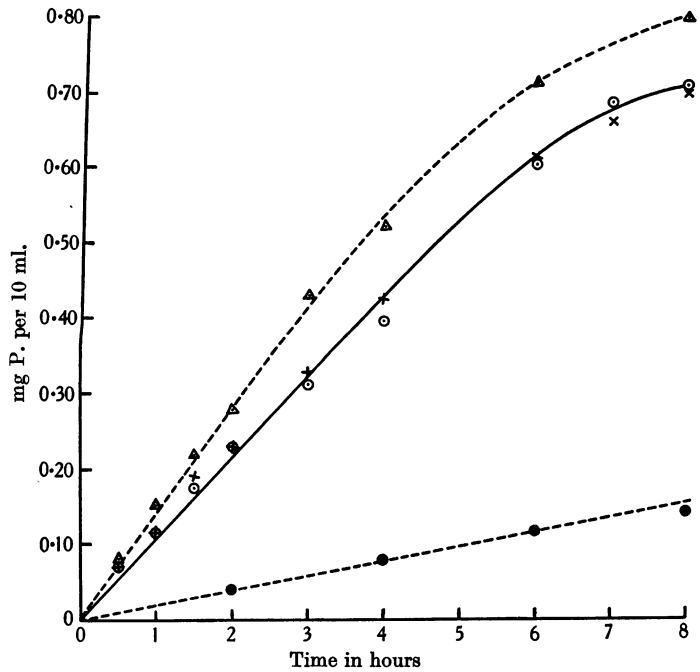

Fig. 2. Rate of hydrolysis of glycerophosphate, by the brain phosphatase.

⊙—⊙ Alkaline phosphatase (undialysed).
×—× Acid phosphatase (undialysed).
●—● Alkaline phosphatase (dialysed).
◬—◬ Alkaline phosphatase (dialysed) activated by 0·01 *M* Mg^{++}.

necessary to examine the applicability of Michaelis and Menten's equations to the enzyme system concerned. The observed initial reaction velocity v is given by the equation (*cf.* Michaelis and Menten)

$$v = V_{max}{}^{(S)}/[K_s + (S)],$$

where v is the initial velocity of hydrolysis. V_{max} is a numerical constant representing the maximum velocity obtained when the enzyme E exists completely in the form ES [$V_{max} = K\,(E_{total})$]. S is the substrate concentration and K_s (the Michaelis constant) is the dissociation constant of the enzyme-substrate compound. The value of the dissociation constant is given by the substrate concentration at half-maximum velocity. In order to decide whether combination between enzyme and substrate according to the mass law actually takes place the calculation of the substrate concentration at half-maximum velocity is not enough. Further examination of the results is essential to show that the enzyme system concerned behaves according to Michaelis and Menten's theory. The possibility of the application of this theory to brain phosphatase was tested. The constants V_{max} and K_s were evolved by the graphical method described by Lineweaver and Burk [1934].

The activity of brain phosphatase prepared by extraction of the acetone-treated brain was determined in the presence of substrate concentrations varying from 0·0032 to 0·640 *M* at 35°. The p_H of each digest was adjusted to 9·2 and 5·0 for alkaline and acid phosphatases respectively. The activities of the two phosphatases were determined by the procedure already described. Typical results are presented in Table V.

Table V. *Relation between substrate concentration and brain phosphatase activity.*

	Alkaline phosphatase activity at p_H 9·2		Acid phosphatase activity at p_H 5·0	
Glycerophosphate concentration (S) *M*	Observed mg. P per 10 ml. reaction mixture after 4 hours' hydrolysis	Calculated from the equation $v=\frac{0·66\,(S)}{(S)+0·012}$	Observed mg. P per 10 ml. reaction mixture after 4 hours' hydrolysis	Calculated from the equation $v=\frac{1·66\,(S)}{(S)+0·029}$
0·0032	0·134	0·139	0·178	0·165
0·0064	0·230	0·229	0·300	0·300
0·0128	0·350	0·341	0·504	0·508
0·032	0·430	0·480	0·766	0·860
0·064	0·350	0·555	1·120	1·140
0·128	0·290	0·603	1·142	1·360
0·320	0·280	0·639	1·620	1·520
0·640	—	—	0·992	1·590

It is evident that there is a close agreement between the observed and calculated values. The initial velocity of hydrolysis can therefore be predicted by the theory that glycerophosphate enters into combination with the phosphatase. Recently Folley and Kay [1935] have shown that the phosphatases of kidney and mammary gland behave in accordance with the theory of Michaelis and Menten, the Michaelis constant being the same for each. In the present case, the two phosphatases of brain differ in their behaviour towards the substrate the Michaelis constants of the two phosphatases being different; 0·012 was obtained for the alkaline phosphatase, and 0·029 for the acid phosphatase showing that the latter has less affinity for the substrate than the former. Further the results indicate that in the range of concentration above 0·032 *M* and 0·320 *M*, for alkaline and acid phosphatases respectively, the observed velocity was always less than the calculated. This is possibly due to a decrease in the relative water concentration. Further work is however necessary to elucidate this point.

Action on different substrates.

The hydrolytic activities of the two phosphatases of brain on sodium β-glycerophosphate and sodium hexosediphosphate were next examined. In each test the enzyme was allowed to act on an amount of ester equivalent to 10 mg. P at 35° and at the optimum p_H for the enzyme. The total P was determined by the method of King [1932].

Table VI. *Action on different substrates.*

Substrate	Activity of the alkaline phosphatase at p_H 9·2 mg. P in 10 ml. of reaction mixture	Activity of the acid phosphatase at p_H 5·0 mg. P in 10 ml. of reaction mixture
Sodium glycerophosphate	0·174	0·215
Sodium hexosediphosphate	0·240	0·310

The results show that sodium hexosediphosphate is more easily hydrolysed than sodium β-glycerophosphate by the alkaline and acid phosphatases of brain.

SUMMARY.

1. Brain phosphatase exhibits two optima: one at p_H 9·4–9·6 and the other at p_H 5·0.

2. The ratio between the activities of the two phosphatases is almost constant for brains of animals of the same species.

3. The activity of acetone-treated brain extracts is about as high as that of liver of the same animal.

4. The phosphatase can be purified by (*a*) isoelectric precipitation of the inert material at p_H 4·8, (*b*) ordinary dialysis, (*c*) ultrafiltration through cellophane membrane and (*d*) acetone precipitation.

5. Magnesium ions activate the alkaline phosphatase while the acid phosphatase remains unaffected. The percentage activation is considerably enhanced when the enzyme extract has been previously purified by dialysis.

6. The time course of the reaction of the alkaline and acid phosphatases is linear in character in the early stage of hydrolysis irrespective of the purity of the enzyme and presence or absence of magnesium.

7. The initial rate of hydrolysis by the alkaline and acid phosphatases varies with the substrate concentration in a manner predictable by the theory of Michaelis and Menten.

8. Sodium hexosediphosphate is more easily hydrolysed than sodium glycerophosphate by the two phosphatases.

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