THE ACTION OF THE PHOSPHATASES OF HUMAN BRAIN ON LIPID PHOSPHATE ESTERS

BY

K. P. STRICKLAND*, R. H. S. THOMPSON, and G. R. WEBSTER

From the Department of Chemical Pathology, Guy's Hospital Medical School, London

Much work, using both histochemical and standard biochemical techniques, has been carried out on the phosphatases of peripheral nerve. It is known that this tissue contains both alkaline (Landow, Kabat, and Newman, 1942) and acid phosphatases (Wolf, Kabat, and Newman, 1943), and the changes in the levels of these enzymes in nerves undergoing Wallerian degeneration following transection have been studied by several groups of investigators (see Hollinger, Rossiter, and Upmalis, 1952).

Phosphatase activity in brain was first demonstrated by Kay (1928), and in 1934 Edlbacher, Goldschmidt, and Schläppi, using ox brain, showed that both acid and alkaline phosphatases are present in this tissue. Since that time the phosphatases have been studied in the brains of the rat, dog, sheep, rabbit, and man by a number of workers (Giri and Datta, 1936; Fleischhacker, 1938; Gordon, 1950; Reis, 1951; Naidoo and Pratt, 1951; Soullairac and Desclaux, 1951; McNabb, 1951; Pratt, 1953; Lowry, Roberts, Wu, Hixon, and Crawford, 1954). Few reports, however, are available on the distribution of the phosphatases in the grey and white matter of the brain. In dog brain McNabb (1951) has shown that both acid and alkaline phosphatases are more plentiful in grey matter than in white, but little work on their localization in human brain has so far been reported.

Furthermore, most of the studies on phosphatases have been carried out using either α - or β -glycerophosphate as substrate, or else with such unphysiological esters as phenyl phosphate, α -naphthyl phosphate, or phenolphthalein phosphate. Since, however, break-up of the fatty myelin sheath is one of the outstanding changes in the lesions of multiple sclerosis and the other demyelinating diseases, it would seem desirable to obtain more knowledge concerning the enzymic reactions associated with myelin synthesis and degradation. It was decided

therefore to study the action of the phosphatases in human brain on the "lipid phosphate esters", i.e., on the various monophosphate esters that occur in the sphingomyelins, cephalins, and lecithins. In addition to α - and β -glycerophosphate we have therefore used phosphoryl choline, phosphoryl ethanolamine, phosphoryl serine, and inositol monophosphate as substrates for the phosphomonoesterases, and have measured their rates of hydrolysis by brain preparations over the pH range 4.5 to 10.0.

Plimmer and Burch (1937) had earlier reported that phosphoryl choline and phosphoryl ethanolamine are hydrolysed by the phosphatases of both kidney and intestine, but the pH at which the hydrolyses were carried out was not given. In a later paper (Plimmer, 1941) it was reported that phosphoryl serine is hydrolysed by kidney and intestine phosphatase at pH 6.5. Roche and Bouchillon (1947) have also stated that phosphoryl choline and phosphoryl ethanolamine are split by both acid and alkaline phosphatases; they used dog liver and intestine and beef kidney as enzyme sources, and showed that at pH 9 these esters are split at about the same rate as glycerophosphate. At pH 5.6, and using dog liver, human prostate, and beef kidney as enzyme sources, they also obtained splitting of these substrates, but at a lower rate than glycerophosphate. We have been unable to find any account of the action of phosphatases of the nervous system on either phosphoryl ethanolamine or phosphoryl serine. Baccari (1948), however, has studied the hydrolysis of phosphoryl choline by preparations of both horse intestine and brain; he found that it was rapidly hydrolysed by the alkaline phosphatase (pH 10 to 11) but that it was unaffected by the acid phosphatase.

In addition to reporting on the action of the phosphatases of the human brain on these lipid phosphate esters, a few observations are also described on the distribution of acid and alkaline phosphatases in certain areas of the central nervous system in man.

* Post-doctorate Overseas Fellow, National Research Council of Canada.

Since it is not possible on present evidence directly to relate the chronic nerve degeneration (Barnes and Denz, 1953; Cavanagh, 1954) produced by tri-*ortho*-cresyl phosphate, diisopropyl-fluorophosphonate (D.F.P.), and certain other inhibitors of the cholinesterases to an inactivation of these enzymes in the nervous system (Davison, 1953; Thompson, 1954), it was felt that the action of these compounds should also be studied on other esterases. Although Webb (1948) had shown that the acid and alkaline phosphatases of rabbit kidney are inhibited only by high concentrations of D.F.P. (10^{-3} M or higher), it was thought desirable to investigate the action of tri-*ortho*-cresyl phosphate and D.F.P. on the phosphatases of brain. The effect of eserine has also been examined, although there is no evidence that this compound produces chronic nerve lesions.

Experimental

Enzyme Preparations.—Human brain and spinal cord, obtained as soon as possible after necropsy, were used for the enzyme preparations.

Dialysed Homogenates.—Homogenates (1 g. brain/3 ml. H_2O) of cortical grey matter, basal ganglia, sub-cortical white matter, corpus callosum, and spinal cord (lumbar region) were prepared as described by Webster (1954). These homogenates were dialysed in sausage skin sacs against distilled water at 0 to 4° C. for 20 to 24 hours. The dialysed homogenates were then either used directly for phosphatase assays or were acetone dried.

Acetone-dried Preparations.—Dialysed homogenates of cortical grey and subcortical white matter were treated with 4 volumes of ice-cold acetone. The precipitate was centrifuged down and resuspended in ice-cold acetone. The precipitate was filtered off under suction and dried in air with the aid of a small volume of ether. This dried powder was dispersed in water (a weight equivalent to 250 mg. fresh wt./ml.) 15 minutes before it was to be used as a source of phosphatase.

Buffers.—All buffers were adjusted to the desired pH with the Cambridge pH meter.

0.2M Acetate buffer was used for the pH range 4.5 to 5.9.

0.2M Glycylglycine. This buffer was used for the pH range 6.5 to 8.0.

0.2M Carbonate-bicarbonate. This buffer was used for the pH range 8.0 to 10.0.

Substrates.—Stock solutions (0.15M) of each substrate were prepared. For phosphatase assays 0.2 ml. of this stock solution was added; for a final volume of 3 ml. this gives a concentration of 10^{-2} M.

The following substrates were used :—

Sodium phenyl phosphate (British Drug Houses Ltd.)

Sodium α -naphthyl phosphate (Hopkin & Williams Ltd.)

Sodium phenolphthalein diphosphate (L. Light & Co. Ltd.)

Sodium α -glycerophosphate (L. Light & Co. Ltd.)

Sodium β -glycerophosphate (British Drug Houses Ltd.)

Phosphoryl Choline.—This substrate was prepared as the calcium salt by the method of Riley (1944). Calcium was removed from a solution of this salt by the addition of sodium oxalate.

Phosphoryl Ethanolamine.—The barium salt of this compound was prepared according to the method of Outhouse (1937). Barium was removed from a solution of this salt by adding Na_2SO_4 until no further precipitation of $BaSO_4$ occurred.

Phosphoryl Serine.—The barium salt of phosphoryl serine was prepared essentially according to the method of Plimmer (1941). It was found necessary to use 100% H_3PO_4 in the preparation. This was obtained by removing the water from syrupy H_3PO_4 (s.g. 1.75) *in vacuo* at 145° C., aided by a soxhlet thimble, filled with P_2O_5 and asbestos fibre, suspended over the acid. On cooling long, plate-like crystals formed; this crystalline H_3PO_4 was dried over P_2O_5 . As for phosphoryl ethanolamine, barium was removed in the form of the insoluble sulphate.

Inositol Monophosphate.—A crude preparation of free inositol monophosphate was obtained using the method outlined in *Biochemical Preparations* (McCormick and Carter, 1952).

Inhibitors.—A fresh solution of each of the cholinesterase inhibitors was prepared of sufficient strength that 0.2 ml. of this solution in a final volume of 3 ml. gave the desired concentration. The following inhibitors were used : eserine sulphate (British Drug Houses Ltd.); D.F.P., kindly provided by the Ministry of Supply, Experimental Station, Porton, Wilts; and tri-*ortho*-cresyl phosphate (T.O.C.P.) (Geigy Pharmaceutical Laboratories, Ltd.).

Incubation and Sampling.—For each experiment stoppered tubes were set up containing 1.5 ml. of buffer, 0.5 ml. of enzyme preparation, 0.1 ml. 0.03M $MgCl_2$, and 0.7 ml. water (0.9 ml. water for tubes containing no substrate). The tubes were warmed to 38° C. and then 0.2 ml. of substrate was added to each tube. One-millilitre samples were taken immediately following addition of the substrate and after 60 minutes incubation with shaking at 38° C. Each sample was added to 1.5 ml. 8.3% trichloroacetic acid in order to stop the enzyme reaction and to precipitate the protein.

The precipitate was centrifuged and a portion of the supernatant was used for the inorganic phosphate analysis.

Estimation of Inorganic Phosphate.—Inorganic phosphate (ortho) was estimated according to the method of Ernster, Zetterström, and Lindberg (1950). One millilitre of the above supernatant was added to a stoppered tube containing 5 ml. 1 : 1 isobutanol : benzene, 3.5 ml. 5% trichloroacetic acid, and 0.5 ml. 60% perchloric acid. Then 0.5 ml. of 10% ammonium molybdate was added and the tube stoppered and shaken vigorously for 30 seconds; 2 ml. of isobutanol : benzene layer containing the phosphomolybdic acid complex were then diluted to 6 ml. with acid alcohol (32 ml. concentrated H_2SO_4 /1. absolute ethanol). The blue colour was developed by

FIG. 1

FIG. 2

FIG. 3

adding 0.5 ml. of dilute SnCl_2 (stock solution of 10 g. $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}/25$ ml. concentrated HCl diluted 200 times with $\text{N H}_2\text{SO}_4$). This colour was read after 30 minutes on a spectrophotometer at 730 m μ .

Phosphatase Activity.—Phosphatase activities have been expressed in terms of $\mu\text{g. inorganic P released/g. fresh tissue/hour}$. In all cases, small corrections have been made for inorganic phosphate released from the tissue and for non-enzymic hydrolysis of the substrate.

Results

The rates of hydrolysis of these various substrates by acetone-dried preparations of human cerebral cortical grey matter over the pH range 4.5 to 10.0 are shown in Figs. 1 to 3. It will be seen (Fig. 1) that the three aromatic phosphate esters are each hydrolysed by both the acid and alkaline phosphatases. There does not appear to be any great difference in the pH optima for these three substrates; values ranging from 5.5 to 5.8 for the acid phosphatase and from 9.0 to 9.4 for the alkaline phosphatase being found.

The results shown in Fig. 2 indicate that under our conditions α - and β -glycerophosphate are hydrolysed by the alkaline phosphatase of human brain at approximately the same rate as that at which the aromatic esters are split. The glycerophosphates are also split by the acid phosphatase, but at a considerably lower rate than the aromatic esters. The pH optima for the glycerophosphates are in the neighbourhood of 5.1 to 5.5 and 8.7 to 9.1, for the acid and alkaline phosphatases respectively.

The phosphate esters of choline, ethanolamine, and serine are also each hydrolysed rapidly by the alkaline phosphatase of brain, the rates being of the same order as those for the aromatic and glycerol esters. In contrast to these other esters, however, the choline, ethanolamine, and serine esters do not appear to be hydrolysed to any significant extent by the acid phosphatase.

The relative levels of acid and alkaline phosphatase activity in different areas of grey and white matter from the brain, and also in the spinal cord, are shown in Table I.

There does not seem to be any noticeable difference in the acid or alkaline phosphatase activity

FIG. 1.—Hydrolysis of phenyl phosphate, α -naphthyl phosphate, and phenolphthalein phosphate by acetone-dried preparations of human cerebral cortical grey matter.

FIG. 2.—Hydrolysis of sodium α - and β -glycerophosphate by acetone-dried preparations of human cerebral cortical grey matter.

FIG. 3.—Hydrolysis of phosphoryl choline, phosphoryl ethanolamine, and phosphoryl serine by acetone-dried preparations of human cerebral cortical grey matter.

of different areas of grey (cortical grey matter and basal ganglia) or white matter (subcortical white and corpus callosum). However, as can be observed from the ratios of 2.1 and 4.6 for the means of the acid : alkaline phosphatase activity of grey and white matter respectively, there is much more acid phosphatase than alkaline phosphatase in each tissue. The results for spinal cord are very similar to those for white matter, there being a much more active acid phosphatase than alkaline phosphatase. At pH 8.9, mean activities of 726 for grey matter and 261 for white matter were obtained using phosphoryl ethanolamine. These values are very similar to those obtained for phenyl phosphate (Table I).

TABLE I

ACTIVITY OF ACID (pH 5.5) AND ALKALINE (pH 8.9) PHOSPHATASES IN DIFFERENT AREAS OF HUMAN BRAIN AND SPINAL CORD *

	Activity		Acid : Alkaline Ratio
	Acid	Alkaline	
<i>Grey matter</i>			
Cortical grey	1,775	662	
"	1,163	1,128	
Corpus striatum	1,920	574	
"	1,613	717	
Mean	1,618	770	2.1
<i>White matter</i>			
Subcortical	1,267	152	
"	1,007	294	
Corpus callosum	1,201	185	
"	1,143	368	
Mean	1,155	250	4.6
<i>Spinal cord</i>			
Lumbar	1,193	269	
"	1,151	257	
Mean	1,172	263	4.5

* Dialysed homogenates of each area; phenyl phosphate as substrate. Activity expressed as $\mu\text{g. P/g. fresh tissue/hr.}$

It will further be seen that there is very much less alkaline phosphatase in the spinal cord or the white matter of the brain than in grey matter. The acid phosphatase activity of white matter, on the other hand, is only slightly less than that of the grey. These relative proportions are summarized in Table II.

In view of the presence of phosphate esters of inositol in the diphosphoinositide component of the cephalins (Folch, 1949), the action of the brain phosphatases has been studied, in a preliminary experiment, on a crude preparation of inositol monophosphate. It was found that, using an acetone-dried powder of cortical grey matter, this substrate at pH 8.9 was hydrolysed at a rate of 315 $\mu\text{g. P/g. fresh tissue/hour}$, but showed no significant hydrolysis at pH 5.5. This preliminary

TABLE II

RATIOS OF ACTIVITY OF PHOSPHATASES IN GREY AND WHITE MATTER OF HUMAN BRAIN (DIALYSED HOMOGENATES)

pH	Substrate	Activity of Grey Matter : White Matter
5.5	Phenyl phosphate	1.4
8.9	Phenyl phosphate	3.1
8.9	Phosphoryl ethanolamine	2.8

result, therefore, indicates that the pattern of hydrolysis of this substrate by brain phosphatases is similar to that shown by the other lipid phosphate esters.

The effects of the two "demyelinating" anticholinesterases and of eserine on the acid and alkaline phosphatases of human brain, using both phenyl phosphate and phosphoryl ethanolamine as substrates, are shown in Table III. It will be seen that none of these inhibitors exerted any significant effect on either type of phosphatase.

TABLE III

EFFECT OF CHOLINESTERASE INHIBITORS ON ACID AND ALKALINE PHOSPHATASE OF ACETONE-DRIED HUMAN CEREBRAL CORTICAL GREY MATTER *

Substrate	pH	Phosphatase Activity in Presence of			
		No Inhibitor	Eserine (10 ⁻⁴ M)	D.F.P. (10 ⁻³ M)	T.O.C.P. (1.8 x 10 ⁻⁴ M)
Phenyl phosphate ..	8.9	1,350	1,402	1,252	1,382
Phosphoryl ethanolamine ..	8.9	647	676	661	632
Phenyl phosphate ..	5.5	1,147	1,315	1,235	1,189

* Expressed as $\mu\text{g. P/g. fresh tissue/hr.}$

Discussion

The experiments described above confirm the earlier findings of Gordon (1950) and Reis (1951) that both acid and alkaline phosphatases are present in human brain. It is clear, however, that the observed levels of each of these enzyme activities depend very markedly on the substrate used for their measurement. This is particularly so in the case of the acid phosphatase; thus, while the glycerophosphates are split more slowly than the aromatic esters, the lipid phosphate esters are hardly attacked at all. These latter esters would, therefore, seem to be selective substrates for the alkaline phosphatase of nervous tissue. As pointed out earlier, Baccari (1948) described a similar inactivity of the acid phosphatase of horse brain towards phosphoryl choline, and we have shown that, with human brain, this applies also to the ethanolamine and serine esters, and probably also to inositol monophosphate.

It will be seen that our study of the distribution of the phosphatases in different areas of the human

brain has been limited to a very few regions. Nevertheless, we have not found any obvious difference between the two areas of grey or of white matter which we have investigated. From this preliminary survey it is apparent that our finding of a higher acid : alkaline phosphatase ratio in white matter than in grey supports the conclusion reached by Hollinger and others (1952) for cat sciatic nerve that the alkaline phosphatase is mainly neuronal in situation whereas the acid phosphatase is a sheath component, and suggests that in the central nervous system the latter enzyme is possibly associated with glial structures.

The experiments with the anti-cholinesterases indicate that levels of D.F.P. and T.O.C.P. higher than those that are likely to be found in the nervous system of animals poisoned by "demyelinating" doses of these compounds produce no inhibition of either type of phosphatase. This would seem to rule out any inactivation of brain phosphatases as a cause of the nerve degeneration produced by these anti-cholinesterases.

Summary

The effect of brain phosphatase preparations on a number of "lipid phosphate esters" has been studied.

It has been found that at pH 8.9 the alkaline phosphatase of human brain hydrolyses phenyl phosphate, α -naphthyl phosphate, phenolphthalein phosphate, α - and β -glycerophosphate, and the phosphate esters of choline, ethanolamine, and serine at approximately the same rate.

The acid phosphatase of human brain, on the other hand, while strongly active against the aromatic phosphate esters, hydrolyses α - and β -glycerophosphate relatively slowly and has virtually no effect on the lipid phosphate esters.

A crude preparation of inositol monophosphate

has also been used as a substrate; like the other lipid phosphate esters this compound appears to be hydrolysed only by the alkaline phosphatase.

The acid : alkaline phosphatase ratio is considerably higher in white matter than in grey.

High concentrations of two "demyelinating" anti-cholinesterases (diisopropylfluorophosphonate and tri-*ortho*-cresyl phosphate) have been shown to cause no inhibition of either acid or alkaline phosphatase of human brain.

We wish to thank the Medical Research Council for a grant towards the expenses of this work. Our thanks are also due to Mr. D. B. Mays for technical assistance.

REFERENCES

- Baccari, V. (1948). *Arch. Sci. biol. (Napoli)*, **32**, 391.
 Barnes, J. M., and Denz, F. A. (1953). *J. Path. Bact.*, **65**, 597.
 Cavanagh, J. B. (1954). *Journal of Neurology, Neurosurgery and Psychiatry*, **17**, 163.
 Davison, A. N. (1953). *Brit. J. Pharmacol.*, **8**, 212.
 Edlbacher, S., Goldschmidt, E., and Schlappi, V. (1934). *Hoppe-Seyl. Z. physiol. Chem.*, **227**, 118.
 Ernster, L., Zetterström, R., and Lindberg, O. (1950). *Acta chem. scand.*, **4**, 942.
 Fleischhacker, H. H. (1938). *J. ment. Sci.*, **84**, 947.
 Folch, J. (1949). *J. biol. Chem.*, **177**, 505.
 Giri, K. V., and Datta, N. C. (1936). *Biochem. J.*, **30**, 1089.
 Gordon, J. J. (1950). *Ibid.*, **46**, 96.
 Hollinger, D. M., Rossiter, R. J., and Upmalis, H. (1952). *Ibid.*, **52**, 652.
 Kay, H. D. (1928). *Ibid.*, **22**, 855.
 Landow, H., Kabat, E. A., and Newman, W. (1942). *Arch. Neurol. Psychiat.* (Chicago), **48**, 518.
 Lowry, O. H., Roberts, N. R., Wu, M., Hixon, W. S., and Crawford, E. J. (1954). *J. biol. Chem.*, **207**, 19.
 McCormick, M. H., and Carter, H. E. (1952). *Biochem. Prep.*, **2**, 208.
 McNabb, A. R. (1951). *Canad. J. med. Sci.*, **29**, 208.
 Naidoo, D., and Pratt, O. E. (1951). *Journal of Neurology, Neurosurgery and Psychiatry*, **14**, 287.
 Outhouse, E. L. (1937). *Biochem. J.*, **31**, 1459.
 Plimmer, R. H. A. (1941). *Ibid.*, **35**, 461.
 —, and Burch, W. J. N. (1937). *Ibid.*, **31**, 398.
 Pratt, O. E. (1953). *Ibid.*, **55**, 140.
 Reis, J. L. (1951). *Ibid.*, **48**, 548.
 Riley, R. F. (1944). *J. Amer. chem. Soc.*, **66**, 512.
 Roche, J., and Bouchilloux, S. (1947). *C.R. Soc. Biol. (Paris)*, **141**, 1068.
 Soulaïrac, A., and Desclaux, P. (1951). *Rev. neurol. (Paris)*, **85**, 81.
 Thompson, R. H. S. (1954). *Chem. and Industry*, p. 749.
 Webb, E. C. (1948). *Biochem. J.*, **42**, 96.
 Webster, G. R. (1954). *Ibid.*, **57**, 153.
 Wolf, A., Kabat, E. A., and Newman, W. (1943). *Amer. J. Pathol.*, **19**, 423.