Glycerylphosphorylcholine Diesterase Activity of Nervous Tissue

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(Received 19 July 1956)

A study of the demyelinating diseases of man reveals at once the need for more knowledge of the enzymic processes that may be specifically concerned with the metabolism and maintenance of the myelin sheath of the nerve fibres. Experimental nerve degeneration and an accompanying break-up of the myelin sheath can be produced in some species of animal, notably the hen, by the administration of certain organophosphorus anticholinesterase compounds, and we have already described the changes in the activity of the cholinesterases and of other enzyme systems in animals poisoned with these substances (Earl & Thompson, 1952; Earl, Thompson & Webster, 1953). Since, on present evidence, it is not possible directly to relate the nerve degeneration produced by these compounds to an inhibition of either true or pseudo-cholinesterase activity in the nervous system (Davison, 1953; Thompson, 1954), we decided to study more fully the other esterases and related enzymes that are present in this tissue.

Because of the complex nature of the phospholipids of nervous tissue, and the various types of esters contained in these compounds, it was decided to begin this study with an examination of the action of brain extracts on component parts of these lipids. The action of the phosphatases of brain on phosphorylcholine, phosphorylserine and phosphorylethanolamine, components of the lecithins, sphingomyelins and cephalins, has already been reported (Strickland, Thompson & Webster, 1956*a, b*).

Since, however, the phospholipids are diesters of phosphoric acid, the action of brain preparations on the water-soluble α -glycerylphosphorylcholine was next investigated. Little work appears to have been done on the enzymic hydrolysis of this compound, although Hayaishi & Kornberg (1954) reported the presence of an adaptive glycerylphosphorylcholine diesterase in a strain of *Serratia plymuthicum*. The present study has, for reasons given above, centred round the nervous system, and a preliminary statement of some of our early results has already appeared (Thompson, 1956). While this work was in progress, Dawson (1956) described the presence of a glycerylphosphorylcholine diesterase in various rat tissues; certain differences, however, appear to exist between the enzyme studied by him in rat liver and our own findings in rat brain.

EXPERIMENTAL

Enzyme preparations. Aqueous homogenates (1 g. of tissue in 3 ml. of water) of the various tissues were prepared with a modified Potter-Elvehjem type of homogenizer. These homogenates were used either fresh or after dialysis in sausage-skin sacs for 20–24 hr. at 0–4° against approx. 100 vol. of water.

The following tissues were studied. Human: cerebral cortical grey matter, subcortical white matter, caudate nucleus, cerebellum, lower thoracic and lumbar spinal cord, and sciatic nerve. Dog and rabbit: grey and white matter (cerebrum). Hen: whole fore-brain, cerebellum, spinal cord and sciatic nerve. Rat and guinea pig: whole fore-brain, cerebellum and spinal cord. In the experiments with hen sciatic nerve, weighed amounts of the fresh minced tissue were used; human sciatic nerve was homogenized in a Waring Blendor and strained through muslin.

Buffer solutions. All the buffer solutions were adjusted to the required pH, a Cambridge pH meter being used. Acetate buffer was used for the range pH 4.5–5.5, phosphate for pH 6.5–7.5, and carbonate-bicarbonate for pH 8.5–10.5. The concentrations of the buffers are stated in the Results section.

For the manometric experiments, 0.025M-NaHCO₃, gassed with N₂ + CO₂ (95:5, v/v), was used.

Substrate. A specimen of crystalline α -glycerylphosphorylcholine (GPC), prepared from o-oleicthoin, was kindly provided by Professor C. S. McArthur. A small amount of the crystalline CdCl₂ complex of GPC was also prepared in this laboratory according to the method of Tattrie & McArthur (1955), and was used, after removal of the cadmium on an ion-exchange resin (Zeo-Karb 225, Na form), in some of the later experiments described in this paper. The rate of enzymic hydrolysis of this latter preparation was very slightly less than that obtained with the crystalline free ester, under comparable conditions; this difference was shown not to be due to traces of cadmium remaining in solution, but may have been associated with the increased ionic strength of the reaction mixture owing to the presence of NaCl in the substrate solution as a result of the ion-exchange treatment (see Results).

Enzyme assay. For each experiment stoppered tubes were set up containing 1 ml. of brain homogenate ($\approx$ 250 mg. of fresh brain), 1.5 ml. of buffer and 0.3 ml. of water (or of a solution of activator or inhibitor). The tubes were placed in a water bath at 38°, and after 10 min. 0.2 ml. of the substrate solution was added to each tube, to give a final concn. of 0.02M. Samples (1 ml.) were then immediately removed and transferred into tubes containing 1.5 ml. of 8.3% (w/v) trichloroacetic acid. After 60 min. at 38° further samples were treated in the same way. After filtering off the precipitated proteins and phospholipids, 0.2 ml. portions of

each filtrate were taken for estimation of free choline, and 1 ml. samples for estimation of inorganic phosphate.

Estimation of choline. Free choline was estimated by the method of Appleton, La Du, Levy, Steele & Brodie (1953), modified by the use of CHCl_3 instead of ethylene dichloride as the solvent for the choline periodide (Webster, 1956).

Estimation of inorganic phosphate. Inorganic phosphate was estimated by the method of Ernster, Zetterström & Lindberg (1950), according to the procedure outlined by Strickland *et al.* (1956*a*).

RESULTS

Evidence for hydrolysis of GPC by brain

Initial experiments, to determine whether GPC is actively hydrolysed by brain, were carried out by estimating free choline and inorganic phosphate in the reaction mixture, after incubating GPC with homogenates of dialysed rat brain. In order to follow acid production, these experiments were carried out at pH 7.4 in 0.025M- NaHCO_3 buffer, and the CO_2 evolution was simultaneously measured by the Warburg technique. The results are summarized in Table 1.

It will be seen that at pH 7.4 GPC is rapidly hydrolysed by dialysed homogenates of rat brain. Further, it will be noted that approximately 6 moles of choline are liberated for each mole of inorganic phosphate set free. This would suggest that choline is first split off from the substrate, with a subsequent smaller release of inorganic phosphate from α -glycerophosphate, rather than that phosphorylcholine is first formed, secondary hydrolysis of which would, of course, release free choline and inorganic phosphate in equimolar proportions. It must be remembered also that it has been shown by Strickland *et al.* (1956*a, b*) that at pH 7.4 both α -glycerophosphate and phosphorylcholine are attacked only slowly by the phosphomonoesterases of brain. It should also be pointed out that under the conditions used here phosphorylcholine is not estimated by the periodide method (Appleton *et al.* 1953; Webster, 1956).

Table 1. Free choline, inorganic phosphate and acid production from GPC by dialysed rat-brain homogenates

pH 7.4, in 0.025M bicarbonate buffer, and in the presence of mM- Mg^{2+} ions.

Expt. no.	$\mu\text{moles liberated/g. of moist tissue/hr.}$			Molar quotient Choline/phosphate
	CO_2	Choline	Inorganic phosphate	
1	9.6	27.2	4.1	6.6
2	17.1	27.0	4.2	6.4
3	8.0	22.1	4.3	5.2
4	25.1	35.8	6.0	6.0
Average	15.0	28.0	4.6	6.0

Although the amount of choline liberated showed some variation in different experiments, it will be seen (Table 1) that the CO_2 evolution varied much more widely, ranging from 8.0 to 25.1 $\mu\text{moles/g./hr.}$ This variability of measured CO_2 output can probably be attributed to the high pK of the α -glycerophosphoric acid formed, and will be discussed more fully below.

It was found that dialysis had a negligible effect on the activity of these brain homogenates, although subsequent acetone-drying of the dialysed preparations resulted in about 36 % loss of activity, even in the presence of mM- Mg^{2+} ions.

Effect of pH

The pH-activity curve for the hydrolysis of GPC by rat-brain homogenates is shown in Fig. 1. In these experiments the final buffer concentration was 0.06M, acetate being used for the range pH 4.5-5.5, phosphate for pH 6.5-7.5, and carbonate-bicarbonate for pH 8.5-10.5. It will be seen that under our conditions the enzyme shows maximal activity at about pH 9.5, although also showing considerable activity at the physiological pH.

It will be noted, however, that the rate of hydrolysis at pH 7.5 in 0.06M phosphate buffer (Fig. 1) is approximately 40 % lower than the rate at pH 7.4 in 0.025M- NaHCO_3 buffer (Table 1), and it seemed possible that variations in the ionic strength of the incubation medium might affect the apparent activity of the enzyme. Experiments were therefore carried out to test this point. Thus addition of NaCl to incubation media, initially 0.025M with respect to carbonate-bicarbonate buffer, to give a final total $[\text{Na}^+]$ and $[\text{Cl}^-]$ equivalent to 0.05 and 0.025M respectively, at pH 8.9 caused 19 % inhibition of activity, whereas twice this amount of added NaCl caused a 27 % fall. Further, increasing the strength of the carbonate-bicarbonate buffer from 0.025 to 0.1M caused a 27 % decrease in apparent activity.

Fig. 1. pH-Activity curve for hydrolysis of GPC by rat-brain homogenates. Buffers: pH 4.5-5.5, 0.06M acetate; pH 6.5-7.5, 0.06M phosphate; pH 8.5-10.5, 0.06M carbonate-bicarbonate. (Results expressed as μmoles of choline/g. of fresh tissue/hr.)

Activation by metals

In view of the known activation of many of the phosphomonoesterases by Mg^{2+} ions, the sensitivity of this glycerylphosphorylcholine diesterase to metal ions was studied.

Rat-brain homogenates, dialysed for 24 hr., showed no significant activation by mM- Mg^{2+} ions either at pH 7.4 or at pH 8.9. Equivalent concentrations of Mn^{2+} , Ca^{2+} , Co^{2+} , Cu^{2+} and Zn^{2+} ions also failed to show any significant activating or inhibitory effects. Since it was possible that this period of dialysis might have been insufficient to remove relatively firmly bound metals, it was decided to study next the effect of addition of ethylenediaminetetraacetic acid (EDTA). It was found that mM EDTA caused 100% inhibition both of free choline release and of liberation of inorganic phosphate.

In view of this abolition of activity by EDTA, the possible activating effect of various metal ions on the EDTA-treated enzyme was next examined. For this purpose a brain homogenate was dialysed for 6 hr. at 0–4° with four changes of water (each of about 100 vol.). EDTA was then added to give a final concentration of 10^{-2} M, and dialysis was continued for a further 65 hr. at 0–4°, with two changes of water at 24 and 48 hr. respectively. The results of two such experiments are summarized in Table 2.

Table 2. *Effect of addition of various metal ions on enzymic hydrolysis of GPC by rat-brain preparations treated with EDTA and dialysed*

For details of treatment see text. Final concn. of added metal ions was mM. Results are expressed as μ moles of choline/g./hr. Numbers of experiments are given in parentheses.

	Activity	% of untreated value
Untreated (4)	36.4	—
No activator (2)	3.9	11
Mn^{2+} (2)	29.8	82
Mg^{2+} (2)	26.6	73
Ca^{2+} (2)	22.0	60
Co^{2+} (2)	16.6	46
Zn^{2+} (2)	9.6	26
Cu^{2+} (2)	6.2	17

The effect of EDTA treatment followed by dialysis was to reduce the activity from a mean level of 36.4 μ moles of choline/g./hr. to 3.9 μ moles of choline/g./hr. A very considerable degree of reactivation was brought about by the addition of either Mn^{2+} or Mg^{2+} ions; Ca^{2+} and Co^{2+} ions caused somewhat less reactivation, whereas with Zn^{2+} and Cu^{2+} ions the effect was small.

Effect of inhibitors

The complete inhibition of activity brought about by mM EDTA has already been mentioned.

Since the substrate used in this work is a choline ester it was of interest to discover whether recognized inhibitors of the cholinesterases had any effect on this diesterase; it was found that eserine (mM), diisopropyl phosphorofluoridate (mM) and tri-*o*-cresyl phosphate (18 mM) had no significant effect.

The enzyme was also fluoride-insensitive, and was not affected by the presence of mM cysteine hydrochloride.

Distribution in nervous tissue

Preliminary experiments were carried out with homogenates prepared from different regions of the nervous system, and also from different species, to discover whether any marked variations in the level of enzymic activity were present in different areas. The results of these experiments are shown in Table 3. It will be seen that the central nervous system of the rat is considerably more active than nerve tissue from any of the other species studied. Although the areas studied and the observations made are too few to warrant any firm conclusions, it would appear that, in general, the whole fore-brain and spinal cord show higher levels of activity than the cerebellum, cerebral grey and white matter, caudate nucleus or sciatic nerve.

DISCUSSION

The results described above indicate that GPC is rapidly hydrolysed by preparations of human, rat, guinea pig, dog and hen nervous tissue. With rat

Table 3. *Glycerylphosphorylcholine diesterase activity of different areas of nerve tissue from different species*

Expressed as μ moles of choline/g./hr. Measurements carried out with undialysed homogenates or, in the hen sciatic nerve, with minced tissue, at pH 8.9 in 0.025 M- $NaHCO_3$ buffer. Numbers of determinations are given in parentheses.

	Whole fore-brain	Spinal cord	Cerebellum	Cerebrum		Caudate nucleus	Sciatic nerve
				Grey	White		
Rat	36.4 (4)	58.0 (2)	25.0 (2)	—	—	—	—
Guinea pig	19.8 (2)	12.1 (2)	12.0 (2)	—	—	—	—
Hen	13.4 (2)	9.5 (2)	2.8 (2)	—	—	—	1.6 (2)
Human	—	9.4 (2)	3.1 (3)	2.2 (5)	2.9 (4)	3.3 (3)	5.2 (3)
Rabbit	—	—	—	2.0 (2)	2.0 (2)	—	—
Dog	—	—	—	10.3 (2)	12.4 (2)	—	—

tissues it was found that dialysis of the brain homogenates had little effect on the final activity. Acetone-dried preparations of dialysed rat brain showed about 60% of the original activity. It is of interest to point out that the fore-brain of the rat appears to be, under our conditions, more active than the preparations extracted from rat liver by Dawson (1956).

It has been stated by Schmidt, Greenbaum, Fallot, Walker & Thannhauser (1955) that GPC is not hydrolysed by either the acid phosphatase of the prostate or the alkaline phosphatase of the intestine, and we have concluded from our observations that the liberation of choline from GPC by brain is also brought about by a diesterase distinct from the phosphomonoesterases which are active against phosphorylcholine, and which are known to be present in this tissue. Our reasons for this conclusion are: (i) approximately 6 moles of choline are liberated for each mole of inorganic phosphate set free; (ii) although the optimum pH for splitting is at about pH 9.5 there is considerable activity at pH 7.4, in contrast with the phosphomonoesterases of brain which show only negligible activity against phosphorylcholine at this pH; (iii) the presence of mM cysteine hydrochloride has no effect on the release of choline from glycerylphosphorylcholine, although causing approximately 60% inhibition of hydrolysis of phenyl phosphate or of phosphorylethanolamine (Strickland *et al.* 1956b).

Although in the earlier experiments we attempted to follow the course of the hydrolysis of GPC by measuring liberation of carbon dioxide from a bicarbonate buffer at pH 7.4, we found large variations in the amounts of carbon dioxide released, relative to the amounts of choline and inorganic phosphate set free. This variability, we believe, is due to the fact that the splitting of choline from GPC will release a monosubstituted phosphoric acid which will be only partially dissociated at pH 7.4, so that only a fraction of the theoretical amount of carbon dioxide will be liberated, and this fraction will vary widely with any slight shift of the pH of the medium.

The influence of pH on the activity of this diesterase in brain, determined under our conditions, is markedly different from that noted by Dawson (1956) for the enzyme in rat liver. Whereas in the rat-liver preparations optimum activity was observed at about pH 7.5, the brain enzyme shows an optimum at about pH 9.5. This latter pH optimum and also the overall shape of the pH-activity curve of the brain enzyme are very similar to the findings of Hayaishi & Kornberg (1954) with the glycerylphosphorylcholine diesterase of *Serratia plymuthicum*.

The influence of substrate concentration on enzymic activity was also examined. The approximate Michaelis constant calculated from the slowly rising substrate concentration-activity curve appears to be about 2.7 mM. Hayaishi & Kornberg (1954) obtained a K_m of 2.5 mM for their bacterial diesterase. In agreement with these workers and also with Dawson (1956), the brain diesterase is completely inhibited by mM EDTA, although in contrast to the earlier findings with the bacterial and liver enzymes, we have not found the presence of zinc ions to be inhibitory to the brain enzyme.

SUMMARY

1. The hydrolysis of α -glycerylphosphorylcholine (GPC) by preparations of nervous tissue from various species has been studied.
2. GPC is rapidly hydrolysed to α -glycerophosphate and choline by a diesterase present in these tissues.
3. Although active at pH 7.4, maximal splitting of GPC was observed at pH 9.5.
4. The addition of mM ethylenediaminetetraacetic acid caused complete inhibition of activity, which could be largely restored by the addition of Mn^{2+} , Mg^{2+} , or Ca^{2+} ions.
5. The diesterase is not inhibited by fluoride, cysteine, eserine, *diisopropyl* phosphorofluoridate or *tri-o-cresyl* phosphate.

Our thanks are due to Professor C. S. McArthur for the gift of crystalline α -glycerylphosphorylcholine, and to the Medical Research Council for a grant to one of us (E. A. M.).

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