Biochem J. (1982) **208**, 845–850
Printed in Great Britain

Activation of glycerophosphocholine phosphodiesterase in rat forebrain by Ca^{2+}

Sheila SPANNER and G. Brian ANSELL*
Department of Pharmacology, University of Birmingham Medical School, Birmingham B15 2TJ, U.K.

(*Received 9 August 1982/Accepted 21 September 1982*)

The highest activity of glycerophosphocholine phosphodiesterase (EC 3.1.4.2) in subcellular fractions of rat forebrain was found in the microsomal fraction though significant amounts were found in fractions containing plasma membranes. With the use of Ca^{2+}/EGTA and Ca^{2+}/EDTA buffers it was shown that very low concentrations of free Ca^{2+} (EC_{50} approx. 10^{-9} M) could activate the enzyme.

The major catabolic route for phosphatidylcholine in animal tissues first entails the removal of fatty acids by the combined activity of phospholipase A_1 (EC 3.1.1.32), phospholipase A_2 (EC 3.1.1.4) and lysophospholipase (EC 3.1.1.5). The water-soluble *sn*-glycero-3-phosphocholine (glycerophosphocholine) so produced is then hydrolysed to *sn*-glycerol 3-phosphate and choline by glycerophosphocholine phosphodiesterase (EC 3.1.4.2; 'glycerophosphocholine diesterase') as was demonstrated in an extract of acetone-dried liver by Dawson (1956), in brain by Webster *et al.* (1957) and kidney by Ullrich & Pehling (1959). Of the rat tissues studied by Baldwin & Cornatzer (1968) the kidney showed the most activity and this was largely in the microsomal fraction from which they made a soluble preparation. Lloyd-Davies *et al.* (1972), on the other hand, found that the activity in rat liver was preferentially located in a plasma membrane fraction and that some activity was in the cytosol. The enzyme in secretions of the female reproductive tract is also a soluble one according to Wallace & White (1965).

Dawson (1956) found that the optimal activity for his preparation was at pH 7.4, as did Wallace & White (1965) for a soluble preparation from ewe uterus. However, most authors have found the optimum pH to lie between 8.5 and 9.0. There has been considerable uncertainty about the requirement for cations. Early observations (Dawson, 1956; Webster *et al.*, 1957; Baldwin & Cornatzer, 1968) showed that the activity of the enzyme was abolished by the chelating agent EDTA. Dawson (1956) noted that the dialysed liver enzyme was slightly activated by 10^{-3} M-Mg^{2+} but that higher concentrations tended to inhibit. Webster *et al.* (1957) could demonstrate no activation of dialysed brain homogenates by a variety of cations including Mg^{2+}, Zn^{2+} and Ca^{2+} but activation, particularly by Mg^{2+} or Mn^{2+}, occurred with dialysed preparations pre-treated with EDTA. Baldwin *et al.* (1969) confirmed for kidney the findings of Webster *et al.* (1957) and suggested that Zn^{2+} might be the active cation. Recently we noted that when a brain microsomal fraction was prepared in EDTA/sucrose, its glycerophosphocholine diesterase activity could be completely restored by washing the fraction with sucrose alone. This suggested that Ca^{2+} may be essential for activity at concentrations of the order of 10^{-6} M, and the present report is the first, as far as we are aware, in which the free Ca^{2+} levels have been controlled with Ca^{2+}/EGTA and Ca^{2+}/EDTA buffers.

* To whom correspondence and reprint requests should be addressed.

Experimental

Materials

Glycerophosphocholine as its $CdCl_2$ complex was obtained from Sigma and freed from Cd^{2+} and Cl^- with a mixed-bed column of 2 volumes of Amberlite IRC-50 (H^+ form) and 4 volumes of Amberlite IR45 (OH^- form) as suggested by the manufacturer. Glycerophospho[1,2-^{14}C]choline (sp. radioactivity 1.42 μCi/μmol) was obtained from ICN Pharmaceuticals. Glycerophospho[*methyl*-^{14}C]choline was prepared as follows. [*methyl*-^{14}C]Phosphatidylcholine was prepared biosynthetically by growing yeast in the presence of [*methyl*-^{14}C]choline and isolating the lipid on a column of aluminium oxide as described by Hazlewood & Dawson (1975). The phosphatidylcholine was further purified by applying the crude lipid in chloroform to a column of silicic acid and eluting the phosphatidylcholine with chloroform/methanol (1:1, v/v). Glycerophospho-

0306-3283/82/120845-06$01.50/1

[*methyl*-^{14}C]choline was prepared from this lipid fraction by hydrolysis in methanolic LiOH and passing the hydrolysate through a column of Amberlite IRC-50 (H^+ form) (Dawson & Hemington, 1977). The effluent was concentrated to give an oily residue, taken up in methanol (1 ml/mg of P) and the glycerophosphocholine was precipitated by adding 25 vol. of diethyl ether and leaving the sample in the cold for several hours. The glycerophosphocholine was stored in ethanol/water (4:1, v/v). This material had a specific radioactivity of 0.42 μCi/μmol.

The labelled glycerophosphocholine was checked for purity before and after hydrolysis with HCl (Schmidt *et al.*, 1952). Both of the original compounds, with added carrier, and the hydrolysis products were chromatographed on t.l.c. plates (CEL 300-10) in butan-1-ol/ethanol/acetic acid/water, (8:2:1:3, by vol.). Acid hydrolysis yielded one radiolabelled spot which co-chromatographed with free choline.

All other chemicals were of AnalaR grade. Adult female rats of about 200 g body weight were used as a source of brain tissue.

Subcellular fractionation of brain tissue

The brains were removed immediately after death and the forebrain (the cerebellum was removed) was homogenized in 0.25 M-sucrose at 4°C. The fractions were prepared as previously described (Spanner & Ansell, 1979). Most of the experiments were carried out on the microsomal fraction which was prepared as follows. The forebrain was homogenized in 0.25 M-sucrose (for modification, see below), the homogenate was diluted to 10% (w/v) with more sucrose and centrifuged for 10 min at 5000 *g*. The supernatant was decanted and the pellet was re-homogenized in sucrose, the volume was re-adjusted to 10% (original wt./volume) and the sample was again centrifuged for 10 min at 5000 *g*. The two supernatant fractions were pooled and centrifuged for 20 min at 22000 *g*.

The resulting supernatant was carefully removed with a Pasteur pipette and the microsomal fraction was obtained by centrifuging this supernatant for 90 min at 100000 *g*. The microsomal pellet was homogenized in 0.25 M-sucrose and the homogenate was diluted so that 1 ml contained the equivalent of 0.1 g brain weight. After centrifuging this fraction for 90 min at 100000 *g* the resulting pellet was diluted in 0.25 M-sucrose, usually to a concentration equivalent to 200 mg of original tissue/ml.

In experiments where the microsomes were pretreated with EDTA, the initial homogenizations were carried out in 0.25 M-sucrose containing 1 mM-EDTA and the microsomal pellet was washed twice with 0.25 M-sucrose.

Assay of glycerophosphocholine diesterase

The enzyme activity was measured by incubating tissue samples in buffered media with glycerophospho[^{14}C]choline and measuring the [^{14}C]-choline released.

Ca^{2+}/EGTA and Ca^{2+}/EDTA buffers. In order to control the free Ca^{2+} concentration during the incubation, Ca^{2+}/EGTA or Ca^{2+}/EDTA buffers were used. The composition of these buffers is shown in Table 1 and was in each instance calculated from the data of Raaflaub (1956). The stability constant of the Ca^{2+}–EGTA complex ($\log K_s$) is 11.0 and that of the Ca^{2+}–EDTA complex 10.59. The apparent stability constants (K_s) at pH 8.6 were calculated to be 5.09×10^9 for Ca^{2+}–EGTA and 8.32×10^8 for Ca^{2+}–EDTA, and the volumes of 1 mM-$CaCl_2$ required to be added to

Table 1. *Composition of Ca^{2+}/EGTA and Ca^{2+}/EDTA buffers at pH 8.6*

Ca^{2+}/EGTA			Ca^{2+}/EDTA		
Free [Ca^{2+}] (M)	Volume (ml) of 1 mM-EGTA in buffer pH 8.6	Volume (ml) of 1 mM-$CaCl_2$ in buffer pH 8.6	Free [Ca^{2+}] (M)	Volume (ml) of 1 mM-EDTA in buffer pH 8.6	Volume (ml) of 1 mM-$CaCl_2$ in buffer pH 8.6
10^{-11}	2	0.10	10^{-11}	2	0.016
3×10^{-11}	2	0.26	5×10^{-11}	2	0.080
10^{-10}	2	0.68	10^{-10}	2	0.154
3×10^{-10}	2	1.22	5×10^{-10}	2	0.588
10^{-9}	2	1.68	10^{-9}	2	0.908
3×10^{-9}	2	1.88	5×10^{-9}	2	1.612
10^{-8}	2	1.96	10^{-8}	2	1.780
	–	–	5×10^{-8}	2	1.950
10^{-7}	2	2.00	10^{-7}	2	1.980

2ml of 1mM-EGTA were calculated from eqn. (10) of Raaflaub (1956):

$$[Ca^{2+}]_F = \frac{1}{K_s \text{ at pH } 8.6} \times \frac{[Ca^{2+}]_T/[EGTA]_T}{1-[Ca^{2+}]_T/[EGTA]_T}$$

$$\therefore [Ca^{2+}]_T = \frac{K_s \text{ at pH } 8.6 \cdot [EGTA]_T \cdot [Ca^{2+}]_F}{1 + K_s \text{ at pH } 8.6 \cdot [Ca^{2+}]_F}$$

where $[Ca^{2+}]_T$ is the total calcium concentration, $[EGTA]_T$ or $[EDTA]_T$ are the total EGTA or EDTA concentrations, and $[Ca^{2+}]_F$ is the required free Ca^{2+} concentration.

Both the $CaCl_2$ and the EGTA and EDTA were made up in 0.05M-glycylglycine/NaOH buffer, pH8.6. The stability constant is not affected by the nature of the buffer (Owen, 1976) and measurements of the Ca^{2+} concentration in the micromolar range with a Ca^{2+}-specific electrode showed no effect of temperature between 20°C and 37°C. The calcium buffers and the glycylglycine/NaOH were made up on the day of the incubations: glycylglycine buffers in our hands did not retain their pH values for more than a day.

Incubation media. Each incubation was carried out in a medium containing 0.025M-glyclyglycine/NaOH buffer with or without Ca^{2+}/EGTA or Ca^{2+}/EDTA buffer, 10mM-glycerophospho[^{14}C]-choline (approx. 50d.p.s./μmol) and an amount of whole tissue or subcellular fraction equivalent to 0.5mg of protein for the microsomal fraction but up to 5mg of protein for other tissue fractions. After 30min the tubes were plunged into boiling water, left for 8min and, after cooling, 1ml of water was added.

Separation and measurement of choline. Choline was separated from other water-soluble choline-containing compounds on Zeolit 225 (Na^+ form, 52–100 mesh) (Mann, 1975). The 2ml sample obtained above was applied to a column 0.9cm in diameter containing 3ml of resin. The tube was washed out with 2 x 2ml of water which were added to the column followed by a further 16ml of water. The effluent contained all the labelled glycerophosphocholine and any phosphocholine formed. [In the course of this study no evidence was found for the presence of glycerophosphocholine cholinephosphodiesterase (EC 3.1.4.38) which hydrolyses glycerophosphocholine to yield glycerol and phosphocholine and was reported to be present in brain by Abra & Quinn (1975).] The [^{14}C]choline remaining on the column was eluted with 15ml of 0.5M-KCl and the eluate was taken to dryness on a rotary evaporator under reduced pressure. The dry sample was dissolved in 1.0ml of water and 0.8ml was taken for counting after the addition of 10ml of scintillation fluid (FisoFluor 1; Fisons Ltd., Loughborough, Leics., U.K.). A sample of the glycerophospho[^{14}C]choline used as substrate in each set of incubations was also counted and assayed for phosphorus to determine its specific radioactivity. From this value and the radioactivity in the choline samples after incubation, the enzyme activity could be determined.

Other analytical procedures

Phosphorus was determined by the method of Fiske & Subbarow (1925) and protein either by the method of Lowry *et al.* (1951) or that of Sedmark & Grossberg (1977). There was absolute agreement between the two methods.

Results

Preliminary assays of glycerophosphocholine diesterase on homogenates of rat forebrain under optimal conditions indicated a value of 15.5 ± 1.0 ($n = 8$)μmol/h per g of tissue. Little variation was found in six different rat brain areas and rat forebrain was routinely used for the experiments described below. However, it was noted that the subcellular distribution was different in rat forebrain as a whole to that of the striatum. The subcellular distribution in rat forebrain is shown in Table 2, from which it can be seen that the microsomal fraction contains the highest activity/mg of protein and also the highest relative specific activity compared with the homogenate. The relatively high concentration in the crude myelin fraction (P_2A) is probably due to the large amount of plasma membranes recovered in this fraction (Spanner, 1972). When rat striatum was fractionated, however, all the glycerophosphocholine diesterase was recovered in the microsomal fraction with a relative

Table 2. *Distribution of glycerophosphocholine diesterase activity in subcellular fractions of rat forebrain*

The nomenclature is that of Spanner & Ansell (1979). Relative specific activity is activity/mg of protein in each fraction compared with that in the original homogenate. Results are given ± S.D. with number of experiments in parentheses.

	Glycerophosphocholine diesterase activity		
Brain fraction	(μmol/h per g original tissue)	(nmol/h per mg of protein)	Relative specific activity
Total homogenate	15.5 ± 1.0 (8)	117 ± 7.5 (8)	1.00
P_1	2.82 ± 0.31 (8)	128 ± 14.1 (8)	1.09
P_2A	2.84 ± 0.93 (4)	214 ± 70.1 (4)	1.83
P_2B	1.52 ± 0.36 (4)	188 ± 44.5 (4)	1.61
P_2C	0.90 ± 0.08 (4)	99 ± 8.8 (4)	0.84
P_3	7.42 ± 0.79 (8)	322 ± 34.3 (8)	2.75
S_3	0.10 (2)	<0.5 (2)	<0.03

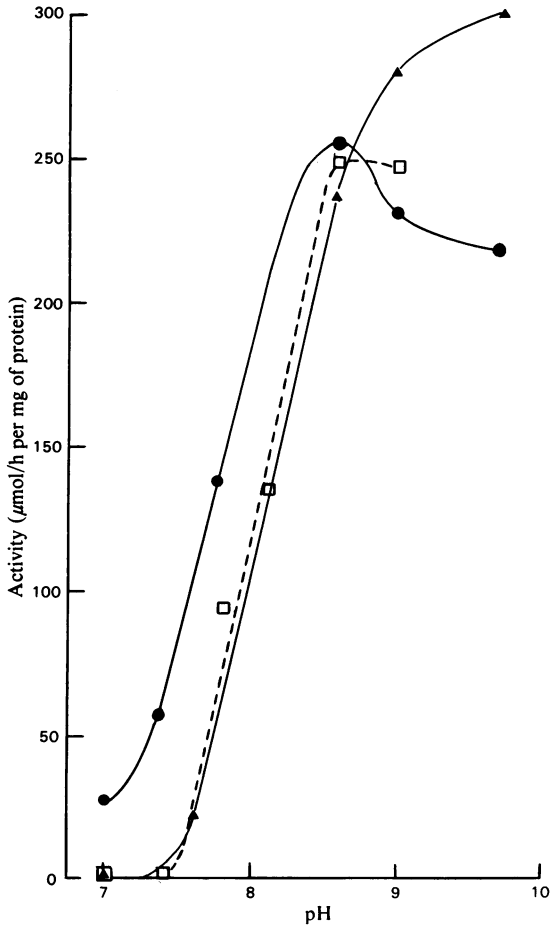

Fig. 1. *Activity of glycerophosphocholine diesterase in the microsomal fraction as a function of pH*

●, 0.025 M-Tris/HCl; ▲, 0.025 M-glycylglycine/NaOH; □, 0.025 M-sodium veronal. The molarities indicate the final concentration of each buffer in the incubation medium. Each point is the mean of four separate experiments.

specific activity of 3.8 and a similar observation was made on bovine caudate nucleus.

pH optimum

Three different buffers were used to establish the pH optimum of the enzyme: glycylglycine/NaOH, Tris/HCl and sodium veronal. It can be seen from Fig. 1 that with Tris/HCl and sodium veronal buffers the pH optimum appeared to be between pH 8 and 8.6. Though the activity at pH 8.6 was the same with glycylglycine/NaOH buffer, maximum activity was not reached until the buffering capacity of this buffer was exceeded. It therefore seemed reasonable to use a pH value of 8.6, especially as most authors have found optimum activity at or around that value.

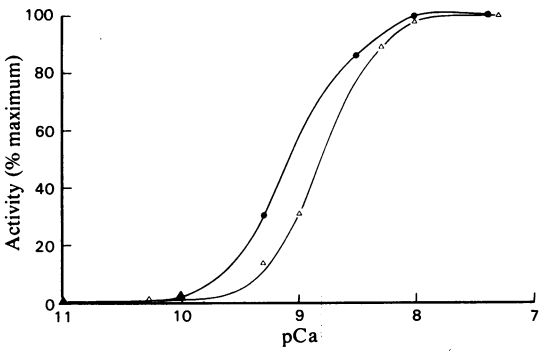

Fig. 2. *Activity of glycerophosphocholine diesterase in microsomes from rat forebrain as a function of free Ca^{2+} concentration (pCa)*

Incubations were carried out in 0.025 M-glycylglycine buffer (pH 8.6) and Ca^{2+}/EGTA (●) or Ca^{2+}/EDTA (△) buffers (see the text). Each point is the mean of four separate assays (each in duplicate) and the variation in activity/mg of protein was <10%.

Linearity with concentration

Assays were carried out over a range of microsomal protein concentrations from 0.5 to 10 mg of protein, i.e. equivalent to 50–1000 mg original tissue weight. The activity was proportional to enzyme (protein) concentration up to 5 mg of protein and then began to fall off, probably due to lack of substrate.

Cation requirement

The presence of 5×10^{-6} M-EDTA completely inhibited the enzyme activity, so the requirement for a bivalent cation was confirmed.

Since the same concentration of the more specific Ca^{2+}-chelating agent EGTA also caused total enzyme inhibition it seemed likely that the enzyme was Ca^{2+}-dependent. This was reinforced by the finding that, when microsomes prepared in EDTA/sucrose were washed twice with sucrose alone, activity was totally restored. This indicated a very low requirement for Ca^{2+}. The Ca^{2+} in the incubation media which had been prepared from Ca^{2+}-free solutions was found to be around 1.4×10^{-5} M when measured with a Ca^{2+}-specific electrode, consistent with a significant elution of Ca^{2+} from the glassware.

In an attempt to find the concentration of Ca^{2+} required by the enzyme, Ca^{2+}/EGTA buffers were prepared as described in the Experimental section, so as to yield free Ca^{2+} in the range 10^{-7}–10^{-11} M. As can be seen in Fig. 2, the effective concentration giving 50% maximum activity (EC_{50}) was 8×10^{-10} M. At the very low concentration of 10^{-11} M-Ca^{2+}, neither Mg^{2+} up to 1 mM nor Zn^{2+} at 10^{-5} M concentrations were able to restore the activity,

Table 3. *Effect of cations and two cation-chelating agents on the activity of glycerophosphocholine diesterase of rat forebrain microsomes*

Preparation of microsomes	Incubation conditions (all in glycylglycine buffer pH 8.6)	Activity (nmol/h per mg of protein)
0.25 M-sucrose	Buffer alone	290 ± 26 (21)
	Ca^{2+}/EGTA (free [Ca^{2+}] 10^{-8} M)	279 ± 31 (11)
	Ca^{2+}/EDTA (free [Ca^{2+}] 10^{-7} M)	106 ± 13 (9)
	10^{-5} M-EGTA	25 ± 5 (10)
	10^{-5} M-EDTA	0 (9)
	10^{-5} M-EGTA + 10^{-3} M-Mg^{2+}	0 (8)
	10^{-5} M-EGTA + 10^{-3} M-Zn^{2+}	102 (2)
	10^{-5} M-EGTA + 10^{-5} M-Zn^{2+}	0 (2)
0.25 M-sucrose containing 10^{-3} M-EDTA followed by two washes in 0.25 M-sucrose	Buffer alone	1000 (2)
	Ca^{2+}/EGTA (free [Ca^{2+}] 10^{-8} M)	1190 ± 110 (4)
	Ca^{2+}/EDTA (free [Ca^{2+}] 10^{-8} M)	67 (2)

though at high concentrations both cations partially restored the activity (Table 3). Using Ca^{2+}/EDTA buffers the EC_{50} was very similar to that with Ca^{2+}/EGTA, i.e. 1.7×10^{-9} M (Fig. 2). However, with Ca^{2+}/EDTA the final maximum activity was significantly lower than with Ca^{2+}/EGTA (Table 3). Ca^{2+}/EGTA buffers at pH 7.4 rather than at pH 8.6 gave a similar response curve, but the values were very much lower as would be expected from the pH–activity curve.

One strange result was that, if the microsomes were prepared in sucrose containing EDTA and the EDTA was washed out (see the Experimental section), there was a four-fold stimulation of enzyme activity compared with microsomes prepared in sucrose alone and the microsomes incubated with Ca^{2+}/EGTA (free Ca^{2+} 10^{-8} M). Although the enzyme activity was affected by Ca^{2+} concentration, the typical sigmoid response curve was now absent. When Ca^{2+} levels were controlled by Ca^{2+}/EDTA, however, no such stimulation was found, (Table 3).

Discussion

The last stage in the catabolism of phosphatidylcholine is the production of free choline by the action of the enzyme glycerophosphocholine diesterase which hydrolyses glycerophosphocholine, the water soluble product of phospholipases A_1 and A_2 and lysophospholipase to glycerophosphate and choline. Glycerophosphocholine diesterase in brain has a high K_m of 2.4 mM (Dross, 1975) and is optimally active at pH 8.6 (confirmed in this paper). Previous work on the enzyme described in the introduction indicated that it required a bivalent cation for activity, but the nature of that cation was unknown and several bivalent cations were shown to restore activity after inhibition with EDTA. Baldwin & Cornatzer (1968), however, showed for the enzyme of kidney that, after inhibition with EDTA, the activity could be fully restored by simple dialysis. In the present study we demonstrated that when brain microsomes were prepared in sucrose/EDTA and then washed twice with sucrose alone the activity was restored (Table 3). This suggested that Ca^{2+} might well be the active cation, especially since the highly Ca^{2+}-selective chelating agent EGTA could inhibit the enzyme.

When free Ca^{2+} concentrations were controlled with Ca^{2+}/EGTA buffers, very low concentrations of the cation were shown to activate the enzyme (EC_{50} approx. 10^{-9} M) (Fig. 2). At concentrations of Ca^{2+}/EGTA at which free Ca^{2+} was $<10^{-10}$ M and enzyme activity negligible, 1 mM-Mg^{2+} had no effect and 1 mM-Zn^{2+} had only a slight effect (Table 3). Although Baldwin *et al.* (1969) produced good evidence that low concentrations of Zn^{2+} activated the enzyme from kidney, this would seem unlikely for the brain enzyme from both our own observations and those of Webster *et al.* (1957).

When brain microsomes were prepared in EDTA/sucrose and washed with sucrose before incubation with Ca^{2+}/EGTA buffer, the enzyme activity produced by a free Ca^{2+} concentration of 10^{-8} M was four times higher than if a Ca^{2+}/EDTA buffer was used. This is difficult to explain but suggests that the requirements of the enzyme are complex and that further purification of the enzyme may be necessary for the appropriate experiments to be done.

The activity of the enzyme (15.5 μmol/h per g fresh wt.) in the rat forebrain was somewhat lower than the value given by Webster *et al.* (1957) and Dross (1975) for whole brain. Illingworth & Portman (1973) found a very low value of 0.7 μmol/h per g of brain for monkey brain cortex, suggesting significant species differences.

The subcellular distribution in rat forebrain (Table 2) indicates that the greatest activity is in the microsomal fraction though significant amounts were found in the crude myelin fraction. This suggests a significant level in the plasma membranes

of neurons and glia which migrate with myelin in the system used (Spanner, 1972). Lloyd-Davies *et al.* (1972) found significant amounts in the plasma membranes of liver, but also 42% of the total tissue activity in the soluble fraction. No activity was found in the soluble fraction of brain (Table 2) though Mann (1975) reported that 17% of the enzyme from whole rat brain was in this fraction. It is clear, however, from separate observations on the striatum (included in the forebrain for most of this study) that there may be regional differences in the subcellular distribution of this enzyme in brain.

The results presented in this paper indicate that the glycerophosphocholine diesterase in the microsomal fraction of rat forebrain is apparently dependent for activity on a very low concentration of Ca^{2+}, comparable with the amount normally found free in cells ($<10^{-7}$M). Other phosphodiesterases are activated by low concentrations of Ca^{2+}, e.g. the polyphosphoinositide phosphodiesterase of erythrocytes (Allan & Michell, 1978) which is not dependent on calmodulin (Downes & Michell, 1982) and the 3′:5′-cyclic nucleotide phosphodiesterase (EC 3.1.4.17) of brain (Wang *et al.*, 1980) which is dependent on calmodulin. Whether brain glycerophosphocholine diesterase requires calmodulin is unknown.

The function of glycerophosphocholine diesterase in brain tissue is presumably related to the turnover of phosphatidylcholine in the brain which occurs in a number of compartments, two of which have half-lives in the rat of 1.5 and 20 days (Horrocks *et al.*, 1976). The brain, however, readily assimilates choline lipids, particularly lysophosphatidylcholine, from the plasma (Illingworth & Portman, 1972) and this may well be the major source of brain choline (for discussion see Ansell & Spanner, 1979). Recent experiments by Jope & Jenden (1979) have indicated that lysophosphatidylcholine can enter the brain and readily give rise to glycerophosphocholine, so that glycerophosphocholine diesterase in brain may well be responsible for the release of choline from exogenous choline glycerophospholipids though such choline is rapidly re-metabolized. It has been noted in the present paper that levels of glycerophosphocholine diesterase are evenly distributed in gross regions of the brain, but this may not be true for its substrate since Jope & Jenden (1979) showed that glycerophosphocholine levels in rat striatum were 0.8 μmol/g fresh wt. compared with 0.3 for cortex. It may be noted that post-mortem hydrolysis of endogenous glycerophosphocholine in whole cortex at 37°C proceeds at a minimal rate of 0.6 μmol/h per g and in striatum at 1 μmol/h per g (Jope & Jenden, 1979).

The authors are extremely grateful to Miss Norma Hemington of the Department of Biochemistry, A.R.C. Institute of Animal Physiology, Babraham, Cambs., U.K. for preparing [*methyl*-^{14}C]choline-labelled glycerophosphocholine. They also wish to thank Dr. R. M. C. Dawson, the Head of that Department, for allowing one of us (S.S.) to use some of the facilities.

References

Abra, R. M. & Quinn, P. J. (1975) *Biochim. Biophys. Acta* **380**, 436–441

Allan, D. & Michell, R. H. (1978) *Biochim. Biophys. Acta* **508**, 277–286

Ansell, G. B. & Spanner, S. (1979) in *Nutrition and the Brain*, vol. 5 (Barbeau, A., Growdon, J. H. & Wurtman, R. J., eds.), pp. 35–46, Raven Press, New York

Baldwin, J. J. & Cornatzer, W. E. (1968) *Biochim. Biophys. Acta* **164**, 195–204

Baldwin, J. J., Lanes, P. & Cornatzer, W. E. (1969) *Arch. Biochem. Biophys.* **133**, 224–232

Dawson, R. M. C. (1956) *Biochem. J.* **62**, 689–693

Dawson, R. M. C. & Hemington, N. (1977) *Biochem. J.* **162**, 241–245

Downes, C. P. & Michell, R. H. (1982) *Biochem. J.* **202**, 53–58

Dross, K. (1975) *J. Neurochem.* **24**, 701–706

Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400

Hazlewood, G. P. & Dawson, R. M. C. (1975) *J. Gen. Microbiol.* **89**, 163–174

Horrocks, L. A., Toews, A. D., Thompson, D. K. & Chin, J. Y. (1976) in *Function and Metabolism of Phospholipids in the Central and Peripheral Nervous System* (Porcellati, G., Amaducci, L. & Galli, C., eds.), pp. 37–54, Plenum Press, New York

Illingworth, D. R. & Portman, O. W. (1972) *Biochem. J.* **130**, 557–567

Illingworth, D. R. & Portman, O. W. (1973) *Physiol. Chem. Phys.* **5**, 365–373

Jope, R. S. & Jenden, D. J. (1979) *J. Neurosci. Res.* **4**, 69–82

Lloyd-Davies, K. A., Michell, R. H. & Coleman, R. (1972) *Biochem. J.* **127**, 357–368

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mann, S. P. (1975) *Experientia* **31**, 1256–1258

Owen, J. D. (1976) *Biochim. Biophys. Acta* **451**, 321–325

Raaflaub, J. (1956) in *Methods in Biochemical Analysis*, Vol. 3 (Glick, D., ed.), pp. 301–325, Interscience Publishers, New York

Schmidt, G., Hecht, L., Fallot, P., Greenbaum, L. & Thannhauser, S. J. (1952) *J. Biol. Chem.* **197**, 601–609

Sedmark, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–552

Spanner, S. (1972) in *Glycolipids, Glycoproteins and Mucopolysaccharides of the Nervous System* (Zambotti, V., Tettamanti, G. & Arrigoni, M., eds.), pp. 195–207, Plenum Press, New York

Spanner, S. & Ansell, G. B. (1979) *Biochem. J.* **178**, 753–760

Ullrich, K. J. & Pehling, G. (1959) *Biochem. Z.* **331**, 98–102

Wallace, J. C. & White, I. G. (1965) *J. Reprod. Fertil.* **9**, 163–176

Wang, J. H., Sharma, R. K., Huang, C. Y., Chau, Y. & Chock, P. B. (1980) *Ann. N.Y. Acad. Sci.* **356**, 190–204

Webster, G. R., Marples, E. A. & Thompson, R. H. S. (1957) *Biochem. J.* **65**, 374–377