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THE ESTIMATION OF THE ACTIVITY OF ACETYLCHOLINESTERASE AND OTHER ESTERASES IN THE RAT BRAIN BY AN AMPEROMETRIC METHOD

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Abstract—An amperometric method for the determination of acetylcholinesterase and other esterases in the rat brain has been described. Methyl mercuric iodide is used as a titrant against the —SH group available from the hydrolysis of the thioesters of choline. The end point is obtained when the current due to methyl mercuric iodide rises sharply. Kinetic constants determined by this method for crude rat brain extract and purified erythrocyte cholinesterase are presented.

INTRODUCTION

Since Koelle *et al.*[1] introduced the use of thioesters of choline for the histochemical localization of acetycholinesterase (AChE) and other cholinesterases, various biochemical methods for the determination of the rate of enzymic hydrolysis of these thioesters have been published. Meyer and Wilbrandt[2] introduced the iodometric titration method. Ellman *et al.*[3] used a colorimetric method and Guilbault *et al.*[4] published an electrochemical method. In the present study an amperometric titration method was used with methyl mercuric iodide (CH_3HgI) as titrant.[5] This reacts with — SH groups available from the hydrolysis of the thioesters of choline. Thus:

$$CH_3.COS.R \xrightarrow[H_2O]{AChE} CH_3COOH + RS^-H^+$$

$$RS^-H^+ + CH_3HgI \longrightarrow CH_3HgSR + HI$$

where R is equivalent to $(CH_3)_3\overset{+}{N}CH_2.CH_2-$

The diffusion current due to CH_3HgI rises sharply at the end point and this is used to determine the — SH concentration.

METHODS

An 'Electrochemical Laboratories' manual polarograph, model B MK2 was used together with a Pye Scalamp galvanometer. An enclosed, thermostatted glass titration vessel of 20 ml capacity was equipped with a dropping mercury electrode (DME), Ag/AgCl reference electrode, N_2 gas inlet and outlet, and a 2 ml microburette.

1. *Solutions*

Pure CH_3HgI was prepared according to the method of Lewis Maynard[6]. A 2×10^{-3} M solution was then prepared in 25 per cent v'/v dimethyl formamide in distilled water. The CH_3HgI was first dissolved in the dimethyl formamide and distilled water added.[5] This solution was stored in a dark bottle and used within a week.

2. *Substrates*

(a) Acetylthiocholine iodide.
(b) Butyrylthiocholine iodide.
(c) Proprionylthiocholine chloride.

(a) and (b) were obtained from Mann Research Laboratory N.Y. and (c) from Light and Co.

Appropriate amounts of these substrates were dissolved in 0·1 M Tris–Buffer containing 0·15 M NaCl and 0·04 M $MgCl_2$ and adjusted to pH8 with dilute HCl. These solutions were stored in the refrigerator and used within a week.

3. *Crude preparation of rat brain esterases*

A number of albino rats (4 to 10) were decapitated and the brains were quickly and carefully removed, washed in ice-cold water and then homogenized in 20% w/v ice-cold water in a Potter–Elvehjem homogeniser. The homogenates were combined and frozen in liquid air or dry ice. The preparation was then freeze-dried and stored in small 5 ml ampoules filled with dry N_2 gas and sealed off for future use. Purified acetylcholinesterase from bovine erythrocytes was supplied by Sigma Biochemical Company. Both enzyme preparations (3 and 4) were dissolved in a suitable volume of TRIS-Buffer at pH 8.

5. *Inhibitor*

The cholinesterases were inhibited immediately after incubation by 10^{-2} M Eserine Sulphate in distilled water.

5 ml of the incubation medium containing appropriate amounts of substrate, enzyme and tris-Buffer (pH 8) were maintained at a temperature of 37·5°C for the necessary period of time. 1 ml of inhibitor solution was added immediately after incubation. The amperometric titration with the DME (drop rate 20/min) was carried out at 25° and a constant voltage of − 0·7 V. The titration vessel contained a total volume of 17 ml made up from:

2·0 ml of eserine sulphate solution; final concentration. $1{\cdot}2 \times 10^{-3}$ M (This inhibits all enzymic activity[7]) a known volume of the incubation solution and the appropriate volume of distilled water and/or tris-Buffer. The background electrolytes used correspond to those recommended by Nachmansohn[8] to give maximum cholinesterase activity. Care was also taken to maintain these background electrolytes (NaCl mainly) at a constant level of 0·02–0·03 M. N_2 saturated with water vapour was bubbled through the solution for 10–15 min. to remove 0_2 prior to the titration. This procedure was also repeated after each addition of titrant until a constant current was obtained.

Prior to the assay proper, a blank titration on the rat brain extract in the absence of substrate indicated a small but significant − SH concentration. This was subsequently subtracted from the total − SH concentration after incubation. Corrections

were also made for non-enzymic hydrolysis of the substrate, which was quite appreciable at high concentrations. Polarographic current-voltage curves were plotted for the enzyme preparations, each of the different substrates, and eserine sulphate. None of these had a current plateau at −0·7 V and consequently the diffusion current measured during the amperometric titration was due wholly to CH_3HgI.

RESULTS

The results obtained by amperometric titration using both rat brain extract and purified enzyme showed the characteristic kinetics of enzyme-substrate interaction. Figure 1 shows the rate of hydrolysis of acetylthiocholine (AcThCh) by rat brain

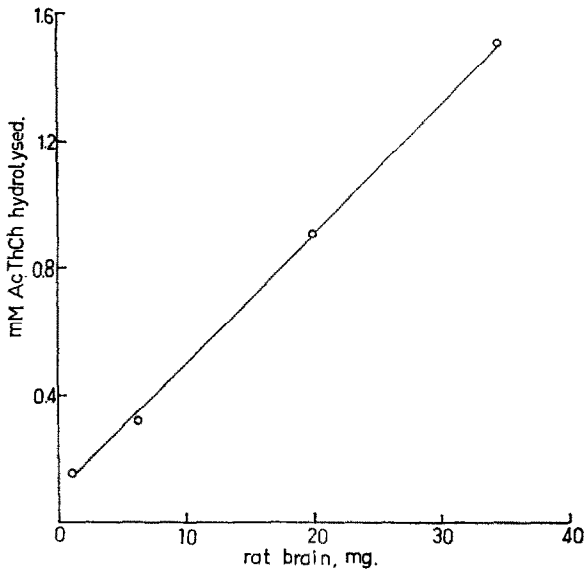

FIG. 1. The rate of hydrolysis of AThCh plotted as a function of enzyme concentration. The activity of the enzyme preparation was expressed as μM AThCh hydrolyzed per 30 min.

esterases as a function of enzyme concentration. The acitvity of the crude enzyme preparation was expressed in μM AcThChI hydrolysed per 30 min, and the total concentration of the crude enzyme in mg/5 ml. A linear function of enzyme concentration verses activity was obtained. The activity of the crude enzyme, expressed inμM AcThChI hydrolyzed per 6 mg rat brain extract, was plotted as a function of time in Fig. 2. The information obtained from this and similar experiments was used to determine the interval during which the initial reaction velocity was linear with respect to time (Fig. 2). The Michaelis Constants, K_M, for both rat brain extract and purified enzyme were obtained by graphic plots of the reciprocal of the initial rate of hydrolysis versus the reciprocal of substrate concentration as predicted by the kinetic expression:

$$\frac{1}{v} = \frac{K_M}{v}\left(\frac{1}{S}\right) + \frac{1}{V}$$

These plots are illustrated in Figs. 3 and 4.

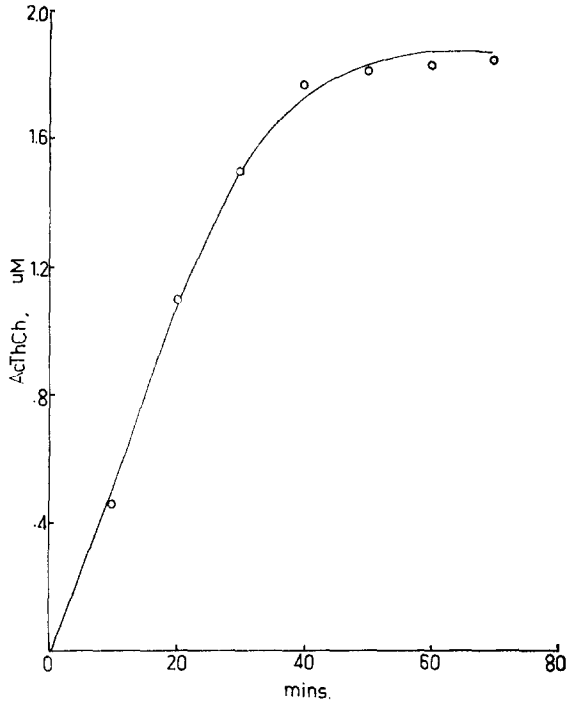

Fig. 2. The hydrolysis of AThCh expressed as μM AThCh/6gm extract plotted as a function of time

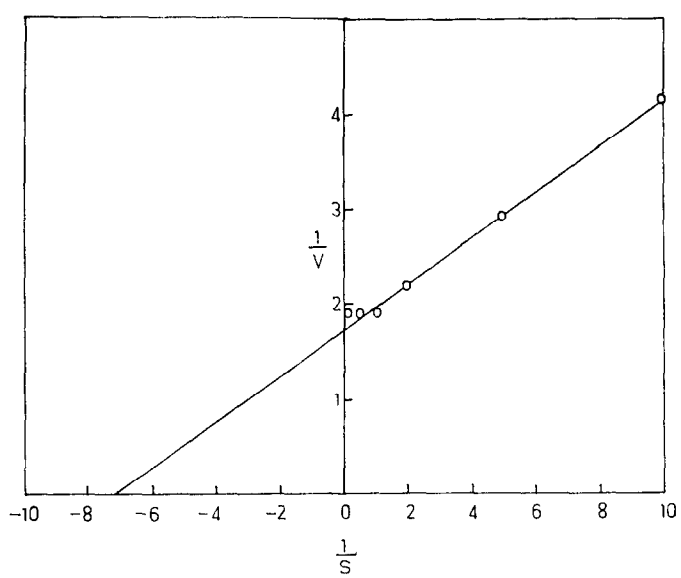

Fig. 3. The Lineweaver–Burke plot of $^1/v$ against $^1/S$ for purified AChE.

Table 1 gives the K_M values for the crude enzymes of the rat's brain and for purified acetylcholinesterase (AChE). The K_M obtained for the purified AChE with AcThChI as substrate is in good agreement with the published data. It may be noted in the Lineweaver–Burke[12] plots that the curve obtained deviates slightly from linearity at concentration above 5×10^{-3} M in both the experiments using rat brain extract and

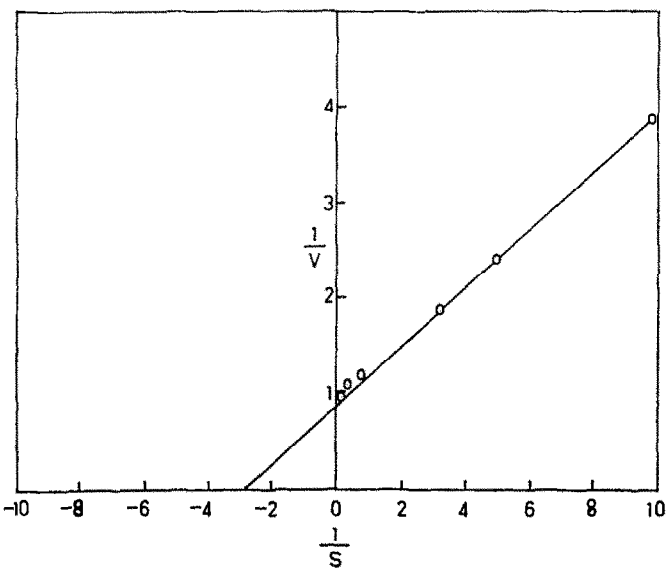

FIG. 4. The Lineweaver–Burke plot of $1/v$ against $1/S$ for crude brain extract.

the purified enzyme. This may be due to substrate inhibition of AChE at high levels of AcThCh. Such observations have been reported by Ellman *et al.*[3] and Guilbault *et al.*[4] The rate of hydrolysis of the substrates Acetyl-Proprionyl- and butyryl-thiocholine esters by purified AChE as well as rats' brain extract have been plotted as a function

TABLE 1.

Enzyme	Substrate Concentration	K_M Obtained	K_M Published
Purified AChE	$1 \times 10^{-2} - 1 \times 10^{-4}$	$1{\cdot}4 \times 10^{-4}$ M	$1{\cdot}4 \times 10^{-4}$M [3,4,11] $1{\cdot}2 \times 10^{-4}$M [10]
Rat Brain Extract	$5 \times 10^{-3} - 1 \times 10^{-4}$M	$4{\cdot}04 \pm 0{\cdot}19 \times 10^{-4}$M	

The K_M value for rat brain is given $\pm$ S.E. of the mean of 5 observations.

of the increasing acyl-C chain length of these esters in Fig. 5. It is clear that when allowance is made for the greater activity of the purified enzyme preparation, there is a significant difference in the slope of the plot between the two preparations. This is largely due to the greater rate of hydrolysis of the butyl ester by the brain preparation and indicates the presence of significant amounts of pseudoesterases in the brain.

DISCUSSION

The recent literature has contained many references to new and specific methods for determining AChE activity. Older methods which relied upon acetic acid release following hydrolysis of ACh were open to objections such as the possibility of non-specific pH changes in the tissue contributing to the result. Further, the change in pH would of itself alter the enzyme activity; ideally kinetic measurements of this type should

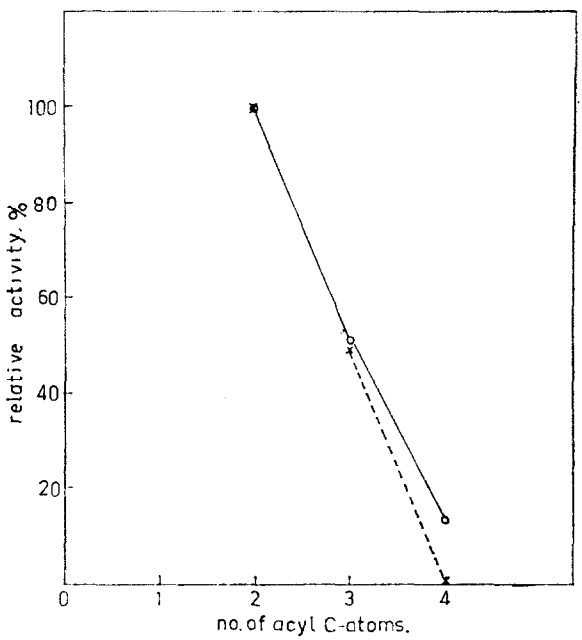

FIG. 5. The relative activity of the purified enzyme and the rat brain extract in the hydrolysis of three esters of choline has been plotted as function of the number of acyl carbon atoms in the molecule. The hydrolysis of AThCh has been taken as 100 per cent in both preparations.

be carried out at constant pH[9]. These difficulties are obviated in the amperometric technique described in the present publication, where the appearance of sulphydryl groups following AThCh hydrolysis is specifically determined by the methyl mercuric iodide titration. The method is therefore specific and capable of a high degree of accuracy. It may be noted that the K_M value for the rat brain extract differs significantly from that found with the purified enzyme preparation. It may well be that brain extract contains significant amounts of non-specific esterases, which would contribute to this value. We were however interested in determining the K_M value for brain esterases, as the method is to be used to determine the inhibitory potency of a series of centrally acting drugs.

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