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# Acetylcholinesterase from Wistar Rat Brain *

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Acetylcholinesterase (E.C.3.1.1.7) was partially purified from rat brains stored in toluene. Extraction was performed using buffers containing non-ionic tensoactive detergents. Some properties of the enzyme were affected by the use of different activity measurement methods, such as the short-time radiometric or the long-time colorimetric method.

There were two zones of maximum activity in the range pH 7.5-8.0 and 8.0-8.6, respectively. There seems to be a histidine residue in the enzyme that participates in the catalytic process. Thermal denuration presented first order kinetics and different thermodynamic parameters were obtained on using different incubation periods.

On using the short-time activity measurement method there was activation at high substrate concentration, but with the long time method there was a marked inhibition produced by excess of substrate. However, if the enzyme was extracted from fresh rat brain, toluene untreated, these differences dissapeared. Gel filtration and disc electrophoresis showed the presence of multiple and interconvertible forms of the enzyme.

Acetylcholinesterase (E.C. 3.1.1.7) occurs at a high specific activity in brain and nervous tissue. Most studies have been carried out with the enzyme from the electrogenic organ of the «Electric Eel». In this source the enzyme exists in a number of multiple forms which can be separated on the basis of their charge and molecular weight. It has been suggested (3) that many of these forms are aggregates from a lower molecular monomer, and at present there is substantial knowledge about the number and nature of the multiple forms and their kinetic and thermodynamic behaviour. In addition, it has been established that this enzyme possesses a regulatory site different from the active site (6).

Relatively little is known about the enzyme from mammalian brain, though all the studies reveal a multiplicity of forms. The existence of regulatory site distinct from the active one has not been reported in this source.

The aim of this work is to determine,

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in rat brain, the thermodynamic and kinetic properties of the catalytic reaction and the number of molecular forms present in the homogenates in order to lead to a greater understanding of some of the molecular mechanisms involved in synaptic transmission.

## Materials and Methods

All reagents used were of analytical or spectral grade. Special chemicals: [1-$^{14}$C]-acetylcholine chloride (5-25 mci/mmol) was purchased from Radiochemical Centre (Amersham), Acetylcholine chloride (Ach) from Merck (Darmstadt), Amberlite CG-120 resin, sodium form, 200 mesh, was a product of BDH (Poole Dorset). Naphtalene was obtained from Eastman Organic Chemicals (Rochester). 2,5-diphenyloxazole (PPO), 1,4 bis-2-(5-phenyloxazolyl)- benzene (POPOP) and Triton X-100 were purchased from Intertechnique (France). Bovine serum albumine (BSA), standard proteins and α-naphtyl acetate were supplied by Sigma Chemical Company (St. Louis) and Sepharose 6B was a product of Pharmacia (Stockholm).

Wistar rats were obtained from «Jefatura Provincial de Sanidad» (Murcia, Spain). They were killed by decapitation and brains were stored, for at least one month, in a freezer with toluene dried by calcium chloride.

*Assay of acetylcholinesterase.* AChE activity was determined at 37° C and pH 7.6 by a colorimetric method (8); a titrimetric method using a pH-stat (12); and a radiometric method (17).

For some routine assays the colorimetric method of HESTRIN (8) was used. Measurements were made, at 540 nm, in a Spectronic colorimeter after the reaction was stopped by addition of hydroxylamine in alkaline medium. The reaction mixture was composed of 1 mM ACh, 0.1 M sodium phosphate buffer, pH 7.6, and enzyme preparation with a total volume of 10 ml. Sometimes, the titrimetric method (12) with a Radiometer pH-stat was used. The reaction mixture consisted of 0.15 M NaCl, 1.3 mM $MgCl_2$, 1 mM ACh and enzyme preparation, with a final volume of 8.0 ml.

Normally, we used the radiometric method of SIAKOTOS (17). The measurements were made in a Intertechnique scintillation spectrometer S.L. 31 and 0.1 ml of 0.1 M sodium phosphate buffer pH 7.6, 0.1 ml of enzyme preparation and 100 μl of $3 \times 10^{-3}$ M labelled substrate were incubated at 37° C for the required time. The reaction was stopped by adding 5 ml of dioxane-resin suspension, then diluted to 10 ml with dioxane and centrifuged at $1000 \times g$ for 1 minute. 5 ml of the clear supernatant was transfered to a scintillation vial and 10 ml of a modified «cocktail» (5) was added. The [$^{14}$C]-hydrolysis product was counted and considered as a measure of the enzyme activity. The yield of counting was 95 % and remained constant. In all cases, spontaneous hydrolysis was measured and discounted from the enzyme activity value.

*Protein.* Protein concentration was measured with the methods of LOWRY *et al.* (11), and NAKAO (13), using crystalline BSA as standard.

*Enzyme solubilization.* The solubilization procedure was performed on the rat brains after toluene was removed. 1 g of brain cortex was excised and then homogenized in a mortar with sand and 50 ml of 0.1 M sodium phosphate buffer, pH 7.6, containing 1 % Triton X-100. The homogenate was centrifuged at $16,000 \times \times g \times 1$ h and the supernatant considered as the enzyme preparation.

*Gel filtration chromatography.* The enzyme preparation obtained after centrifugation gave an enzyme activity of 35,000 cpm by the radiometric method. 2 ml of this solution was placed on the

top of a chromatographic column (56 × × 2.6 cm) containing Sepharose 6B or Sephadex G-200 gels. The column was eluted with sodium phosphate buffer 0.1 M, pH 7.6, and 2.5 ml fractions were collected. Each fraction was assayed for acetylcholinesterase activity and protein. Molecular weight estimation of the peaks of enzyme activity was carried out by calibration of the columns with markers: chymotrypsin (25,000), ovalbumin (45,000), bovine serum albumin (67,000), aldolase (147,000), catalase (240,000), ferritine (540,000) and Blue dextran (>2,000,000), in accordance with ANDREWS (2).

*Disc Electrophoresis.* Before establishing a standard condition for the electrophoresis on rods of polyacrylamide, three buffers were investigated, namely: Tris-ClH, sodium phosphate and sodium barbitone-barbituric acid. In each case different pH and ionic strength were assayed (pH 7.4-9.1 and 0.2, 0.1, 0.05 and 0.025 M). 7 % and 5 % (W/V) poliacrylamide gels were used and the best patterns were obtained when the pH of the cathodic compartment was more acidic than that of the anodic one.

The sample (50-100 μl) was mixed with sucrose and aplied to the surface of 7 % polyacrylamide gels, using 0.1 M Tris-ClH, pH 8.1, buffer in the cathodic compartment and 0.1 M Tris-ClH, pH 8.7, buffer in the anodic one, with 7 mA/tube intensity for a 3 hours period. Staining for acetylcholinesterase was carried out with α-naftil acetate as substrate and fast blue RR as the diazonium reagent, according to PAUL and FOTTRELL (15). Gels were scanned at 540 nm in a Vitraton Spectrophotometer.

## Results

*Extraction procedure.* Acetylcholinesterase from Wistar rat brain could be extracted by homogenization with buffers containing non-ionic tensoactive detergents (Triton X-100 or Lubrol WX 1 % W/V) but results were very poor if ionic tensoactive detergents were used. Factors such as pH of medium (in a wide range), ionic strength or the presence of $Na^+$ and $Mg^{+2}$ ions did not ostensibly change the yield of the extraction.

The extract prepared with 0.1 M sodium phosphate buffer pH 7.6 containing 1 % W/V of Triton X-100, possessed a specific activity of 15-20 μmol of acetylcholine per hour and per mg of protein, at 37° C, using $10^{-3}$ M substrate. Similar results were obtained with the colorimetric, radiometric and pH-Stat methods.

*Optimum pH for activity measurement.* Different buffers were used in the pH range 4.5-10.0 and results were obtained using the colorimetric and the radiometric methods (fig. 1). In both cases 2 peaks of maximum activity were observed, the first one between pH 7.5-8.0 and the second between pH 8.0-8.6. The height of the second peak was specific and severely reduced in the colorimetric method (long time period of incubation) as compared to the radiometric method (short time period of incubation).

*Variation of $K_M$ with pH.* Plotting of $pK_M$ against pH (5.3-9.4) (fig. 2), revealed

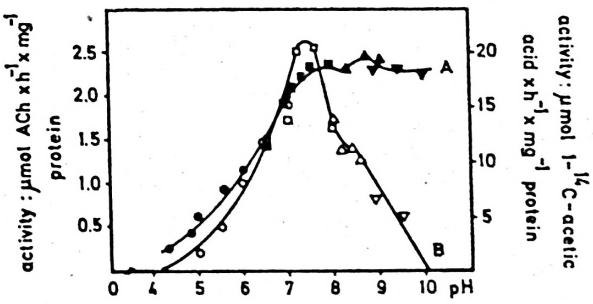


Fig. 1. *Enzyme activity plotted against pH of measurement media.*
*A*) Radiometric method. *B*) Colorimetric method. ●—●, O—O 0.1 M citrate-phosphate buffer. ■—■, □—□ 0.1 M phosphate buffer. ▲—▲, △—△ 0.1 M barbital buffer. ▼—▼, ▽—▽ 0.1 M glycine-NaOH buffer.

the presence of an ionizable group at pH 6.3, which could pertain to a histidine residue (pKa 6.52) in a way similar to other AChE from several sources.

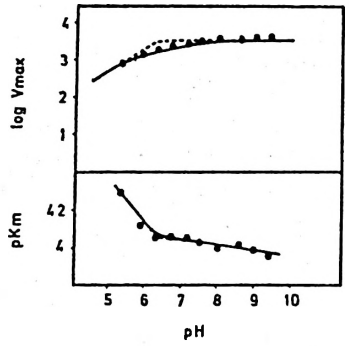


Fig. 2. *Ionizable groups of the enzyme.*

*Arrhenius' plot and activitation energy.* The activity of the enzyme preparation was determined using 1 mM ACh in the temperature range of 5° C to 80° C. Results can be seen in figure 3. There were, at least, 2 peaks when activities were plotted against temperature, and the Arrhenius' plot showed two straight lines with an intersection at 23° C. The activation energies calculated from these 2 different straight lines were 6.4 Kcal × × $mol^{-1}$ (at temperatures higher than 23° C) and 3.8 Kcal × $mol^{-1}$ (at temperatures lower than 23° C), respectively.

*Thermodynamic parameters for the thermal enzyme denaturation.* Thermal denaturation was studied with enzyme preparation preincubated during different time at several temperatures from 50° C to 56° C. In figure 3 the results show that there is a first order kinetic for thermal denaturation, but with different slopes, depending on incubation time (5, 20 and 60 minutes). In table I different values of velocity constants for the denaturation are represented. From these values and using the expression

$$\Delta G = RT \ln \frac{K \cdot T}{k \cdot h}$$

in which K = Boltzmann's constant, h =

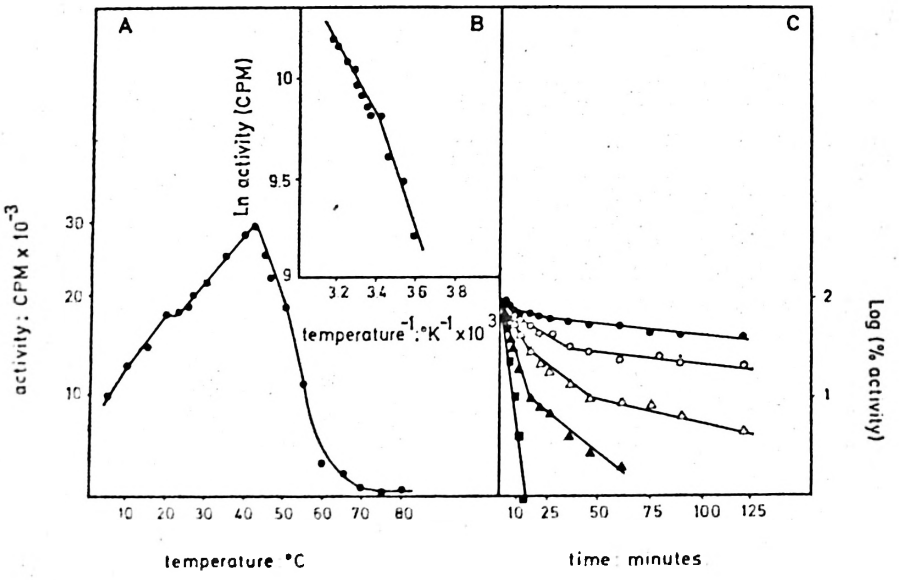


Fig. 3. *Effect of temperature on the enzyme.*
*A)* Optimum temperatures for the actuation of ACh. *B)* Arrhenius' representation for calculation of activation energies. *C)* Thermal denaturation of the enzyme at different temperatures and times.

= Planck's constant, T = absolute temperature and k = velocity constant, $\Delta G$ values were deduced and other thermodynamic parameters were obtained from the expressions

$$\Delta H = Ea - RT; \quad \Delta S = \frac{\Delta H - \Delta G}{T}$$

Values corresponding to Ea, $\Delta H$, $\Delta G$

Table I. *Velocity constants ($sec^{-1} \times 10^6$) for the thermal denaturation of the enzyme.*

| Temperature (°C) | Time (min) 5 | 25 | 60 |
|---|---|---|---|
| 323.0 | 802 | 315 | 189 |
| 324.5 | 1,215 | 577 | 390 |
| 326.0 | 1,512 | 1,145 | 676 |
| 327.5 | 3,365 | 1,790 | 1,085 |
| 329.0 | 4,966 | — | — |

Table II. *Thermodynamic parameters for the enzyme denaturation.*

| Time (min) | Temperature (°K) | ΔG (cal) | ΔH (cal) | $E_a$ (cal) | ΔS cal × °K⁻¹ |
|---|---|---|---|---|---|
| 5 | 323.0 | —6,024 | 62,618 | 63,260 | 212.3±0.2 |
| | 324.5 | —6.217 | 62,615 | | |
| | 326.0 | —6,484 | 62,612 | | |
| | 327.5 | —6,901 | 62,609 | | |
| | 329.0 | —7,315 | 62,607 | | |
| 25 | 323.0 | —5,424 | 80,025 | 80,667 | 264.5±0.3 |
| | 324.5 | —5,836 | 80,022 | | |
| | 326.0 | —6,304 | 80,019 | | |
| | 327.5 | —6,491 | 80,016 | | |
| 60 | 323.0 | —5,096 | 80,496 | 81,138 | 265.0±0.3 |
| | 324.5 | —5,584 | 80,493 | | |
| | 326.0 | —5,963 | 80,490 | | |
| | 327.5 | —6,165 | 80,487 | | |

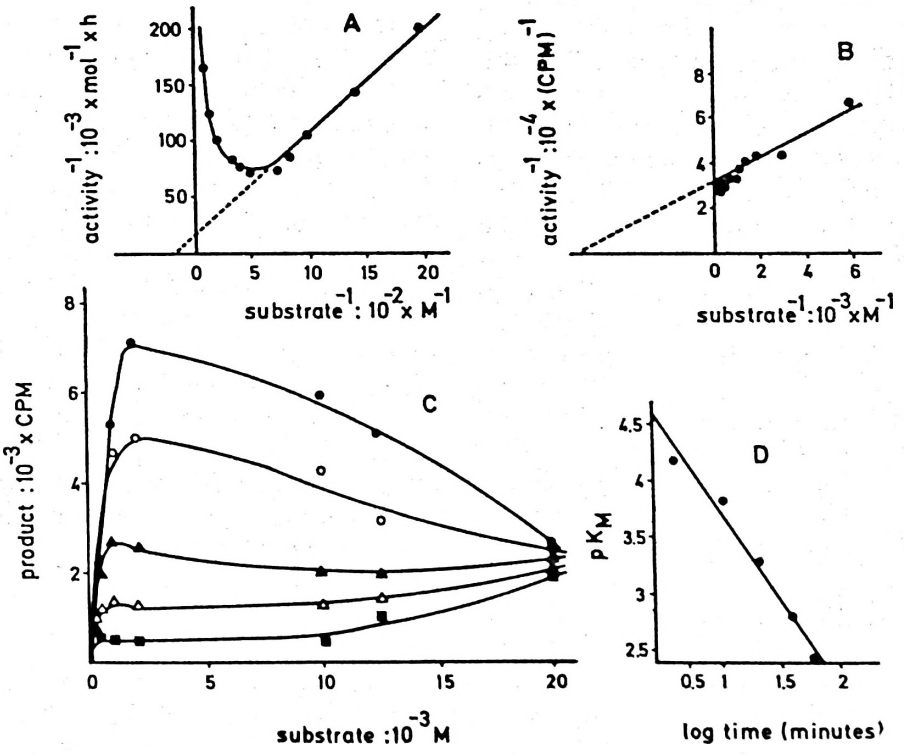


Fig. 4. *Kinetics of acetylcholinesterase.*
*A*) Lineweaver-Burk representation (colorimetric method). *B*) Lineweaver-Burk representation (radiometric method). *C*) Radiometric method. Product plotted against substrate concentration at different times: ●–● 60 min; ○–○ 40 min; ▲–▲ 20 min; △–△ 10 min; ■–■ 4 min. *D*) $pK_M$ platted against log time.

and $\Delta S$ are presented at each of the three different incubation periods considered (table II).

*Acetylcholinesterase kinetics.* The kinetic behaviour of the enzyme against substrate concentration was checked by both the colorimetric and radiometric method. In the first one, optimum activity was obtained at 2 mM substrate concentration, and above this value inhibition by excess of substrate was observed (figure 4A). From the Lineweaver-Burk plot a $K_M$ of 6.2 mM was calculated. In the radiometric-method no inhibition by excess substrate was demonstrated (figure 4B); on the other hand, activation occurred when high substrate concentrations were assayed. $K_M$ value was 75 $\mu$M. In order to ascertain if this phenomenon was caused by the variation in the incubation time (one hour for colorimetric method against 5 minutes for the radiometric one) we have represented the progress curves, using the radiometric method at different times of incubation (figure 4C) of the product formed in relation to the concentration of substrate and the Lineweaver-Burk plots, also at different incubation times. It can be seen that during long periods of incubation the behaviour was similar to that of the colorimetric method: inhibition by a substrate excess, and the $K_M$ values were dependent on incubation times. In figure 4D $pK_M$ are brotted against long time, showing a linear relationship.

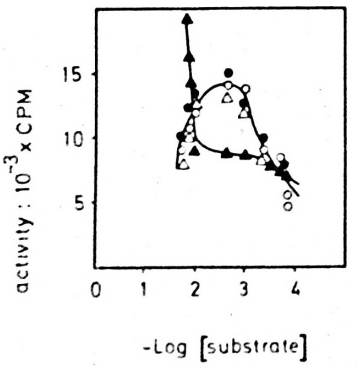


Fig. 5. *Kinetics of acetylcholinesterase from different sources.*
●–● pig brain; ○–○ bovine erytrocytes; △–△ electric eel; ▲–▲ rat brain.

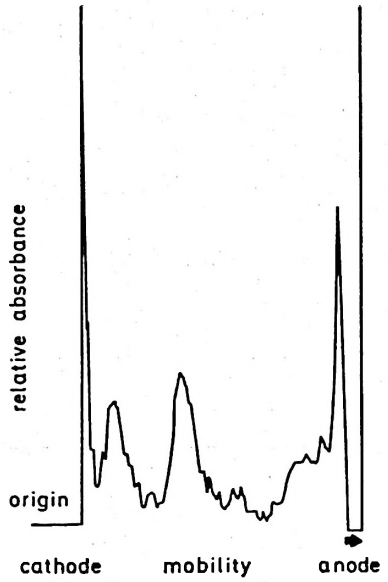


Fig. 6. *Disc electrophoresis of the enzyme.*

To compare acetylcholinesterase from toluene-stored rat brains with the enzyme from other sources, similar experiments were carried out (figure 5) using acetylcholinesterase extracted from pig brain, bovine erythrocytes and electric eel. In all cases the behaviour of acetylcholinesterase was different from that of rat brain, showing the expected inhibition by excess of substrate, with maximum activities when substrate concentration was close to 2 mM.

*Gel filtration.* Good separation was reached wich Sepharose 6B, and at least 8 different forms could be distinguished the elution pattern. The first three peaks have molecular weights near 1,000,000, and the others were calculated as: IV: 560,000; V: 350,000; VI: 180,000; VII: 120,000; VIII: 50,000. When a fraction, such as number VII, was rechromatographied on Sepharose 6B, a pattern very

similar to that of the original enzyme was obtained.

*Disc Electrophoresis.* Disc electrophoresis of the enzyme also showed several bands as indicated in figure 6.

## Discussion

The best extraction for acetylcholinesterase was obtained by the use of nonionic detergents, and the combination of detergents and high salt concentration did not increase the yield or the extraction in contrast with PLUMMER *et al.* results in pig brain (16). A possible explanation could be that these authors used fresh porcine brain whereas we have used toluene stored rat brain and in this case toluene could partially break the membrane involved in binding with the enzyme.

The two peaks present in the pH-activity profile could be explained as a result of the participation of different forms of the enzyme with different optimum pH. A similar situation was reported by SILMAN and KARLIN (18), working with electric eel acetylcholinesterase. In our case, the form with a more acidic optimum pH seems to be more stable than the other forms as deduced from the relative heights of the peaks in the representations using the radiometric (short time) or the colorimetric (long time) method for the activity measurement.

Arrhenius plot of acetylcholinesterase showed a biphasic behaviour, with a transitional point at 23° C and activation energies of 6.4 and 3.8 Kcal $\times$ mol$^{-1}$; a similar situation to that described by BLOJ *et al.* (4) in enzyme from rat erythrcytes, with activation energies and breakpoint temperatures changing with the solubilization of the enzyme and the essential fatty acid deficiency of the membrane. PLUMMER *et al.* (16) reported that the membrane AChE from pig brain showed a break in the Arrhenius plot with a transition temperature of 27° C and activation energies of 1.99 and 9.36 Kcal $\times$ mol$^{-1}$. The break was abolished if the enzyme was solubilized with Triton X-100. The existence of such breakpoint in Arrhenius plots in the case of membrane bound enzymes is likely due to phase changes in the lipid portion of the lipoprotein structure. This interpretation could ve valid for pig brain AChE behaviour. However, some soluble or solubilized enzymes exhibit breakpoints in their Arrhenius plots, probably due to temperature-dependent conformational changes of the protein molecules. HILLMAN and MAUTNER (9) and WILSON and CABIB (21) claimed that the nonlinear temperature dependence of AChE must be ascribed to the two-step mechanism of the acetylcholine hydrolysis, each of these steps having a different activation energy. Another possible explanation could be the existence of multiple forms with different behaviour.

Similarly, a nonlinear temperature dependence was found when thermal denaturation of the enzyme was studied, with three defined activation energies of 63.2, 80.6 and 81.1 Kcal $\times$ mol$^{-1}$ corresponding at 5, 25 and 30 minutes of incubation respectively. Entropy changes were high enough to assume true denaturation of the enzyme, which, lost its orderly conformation.

The kinetic behaviour of AChE extracted from toluene stored rat brains was different from that AChE of other sources which normally show inhibition by high substrate concentration. In rat brain AChE the inhibition was present only during short incubation times. On the other hand, at higher incubation times there was a clear activatory effect producet at high substrate concentration. $K_M$ values were similar to that of other sources only if incubation times were short. NOLAN and SCHNITZERLING (14) have found no excess substrate inhibition for AChE from acaricide-resistant strains of the Cattle Tick *Boophilus microplus*, and they assume

possible advantages if physiological concentrations of the transmitter were of the order of $10^{-2}$ M. DESIRE and BLANCHET (7), have shown that the enzyme from bovine erythrocytes presented an activation by excess of substrate only when high salt concentration were added to the incubation medium.

The possibility of two different catalytic forms of the enzyme was established by WERMUTH and BRODBECK (19, 20), for one of the multiple forms of *Electrophorus Electricus*. However, our last results indicates that AChE extracted from fresh rat brains presents inhibition at high substrate concentration. Other properties, such as activation energies and elution pattern on Sephadex G-200 were also different to those obtained using AChE from toluene-stored rat brains. It might be hypothesized that kinetics properties of the enzyme seems to be closely related to its lipid enviroment which is altered by the toluene treatment. In the present, we are studying the comparative behaviour of both forms of AChE.

Gel filtration showed the presence of several molecular forms of AChE of different molecular weights with one form having a very small molecular weight of 50.000. In adult mouse brain (1) there is evidence that the native form is a monomer with a molecular weight of about 74.000. Our results concerning the existence of an equilibrium between the different multiple forms support other reports about AChE from calf brain (10) and from mouse brain (1). 3 active forms of the enzyme can be detected by specfic substrate staining after electrophoresis in polyacrylamide gel, a similar pattern to that obtained by WRIGHT and PLUMMER (21), with AChE from human erythrocytes.

## Resumen

Se purificó parcialmente acetilcolinesterasa de cerebro de rata Wistar, conservado en tolueno. Para su extracción se emplearon diversas disoluciones tampón conteniendo detergentes de naturaleza no iónica. Ciertas propiedades del enzima resultaron afectadas cuando se usaron diferentes métodos para determinar la actividad, concretamente, según se utilizara un método radiométrico (tiempos de incubación breves) o colorimétrico (tiempos de incubación largos).

Se obtuvieron dos zonas de máxima actividad en el rango de pH 7,5-8,0 y 8,0-8,6, respectivamente. Un resto de histidina parece estar implicado en el proceso catalítico. Al estudiar la desnaturalización térmica del enzima, se obtuvo una cinética de primer orden, así como una dependencia entre los valores de los parámetros termodinámicos y el tiempo de incubación.

Al emplear un método radiométrico para medir la actividad, apareció activación por altas concentraciones de sustrato; sin embargo, cuando se empleó el colorimétrico — largos tiempos de incubación —, se encontró fuerte inhibición por exceso de sustrato. No obstante, si el enzima se extraía de cerebro fresco, no conservado en tolueno, desaparecían estas diferencias. El empleo de filtración en gel y electroforesis de disco demostró la presencia de formas múltiples del enzima que sufrían fenómenos de interconversión.

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