KINETICS OF INHIBITION OF CHOLINESTERASES BY 1,2,3,4-TETRAHYDRO-9-AMINOACRIDINE *in vitro*

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The kinetics was studied of the inhibition of acetylcholinesterase and butyrylcholinesterase by 1,2,3,4-tetrahydro-9-aminoacridine *in vitro*. It has been shown that this compound reacts with both enzymes in the same manner and that one inhibitor molecule forms an inactive complex with one catalytic enzyme unit. The site of interaction is the "hydrophobic domain" of the enzyme molecule, in acetylcholinesterase the so-called γ-anionic site. The bond between the inhibitor and the enzyme is very strong and most likely brings about conformational changes in the enzyme molecule. The character of inhibition of acetylcholinesterase is noncompetitive with respect to the substrate whereas the inhibition of butyrylcholinesterase is competitive – noncompetitive (mixed) with respect to the substrate. The effect was investigated of temperature and concentration of Na^+- and Ca^{2+}-ions on the interaction of the inhibitor with acetylcholinesterase and some thermodynamic constants were determined.

1,2,3,4-Tetrahydro-9-aminoacridine (THA) was prepared first by Albert and Gledgill[1]. It was shown that unlike most of the acridine derivatives THA is lacking antibacterial properties but inhibits cholinesterases *in vitro*[2,3]. The affinity of THA for butyrylcholinesterase is higher than its affinity for acetylcholinesterase[4]. The inhibition of both enzymes *in vivo* was demonstrated later in experiments with dogs[4] and rats[5]. The mechanism of the inhibitory effect of THA on the cholinesterases has not been elucidated. An effort to explain this mechanism in more detail has been made in this study.

EXPERIMENTAL

Chemicals. 1,2,3,4-Tetrahydro-9-aminoacridine hydrochloride was prepared in this Institute[6] as a colorless crystalline product, m.p. 284–287°C (reported[7] m.p. 283–284°C). The base melted at 180–182°C (reported[7] m.p. 183–184°C). The pK of the amine group was 10·3 (reported[8] value 10·0). The partition coefficient of the hydrochloride in the system cyclohexanol–water was 3·2 at pH 7·2 and 28°C. Analysis of Cl: calculated 15·10%, found 14·93%. Infrared spectrum of the base (KBr): 760, 1170, 1304, 1376, 1430 + 1443, 1505, 1580, 1650, 2940, 3245, 3460 cm^{-1}. The melting points were measured on a Kofler block and are not corrected. The infrared spectrum was measured in Unicam SP 1000 Spectrophotometer. The dissociation constant of THA was determined spectrophotometrically.

Enzyme preparations. The purified preparation of butyrylcholinesterase was obtained from horse serum by the method of Strelitz[9]. The lyophilized preparation showed a specific activity of 4·8 μmol of hydrolyzed butyrylcholine. min^{-1} mg^{-1} (80 ncat) at 25°C and pH 8·0. As sources of acetylcholinesterase served lyophilized stromata of human erythrocytes, a purified soluble enzyme preparation from bovine erythrocytes, and a homogenate of whole rat brain. The stromata were prepared by hemolysis of human erythrocytes, obtained from fresh citrate blood, in a 20-fold volume of distilled water. The stromata were repeatedly washed, freed completely of hemoglobin, and lyophilized. This preparation showed a specific activity of 0·12 μmol of hydrolyzed acetylcholine . min^{-1} mg^{-1} (2 ncat) at 25°C and pH 8·0. The lyophilized preparation of soluble bovine erythrocyte acetylcholinesterase of specific activity of 0·28 μmol of hydrolyzed acetylcholine . min^{-1} mg^{-1} (4·6 ncat) at 25°C and pH 8·0 was prepared as described elsewhere[10]. The homogenate of whole rat brain was prepared from brains of Wistar rats of both sexes weighing 200—240 g. The animals were sacrificed by scission of the carotid arteries, the brains were excised rapidly, cut into several parts, washed with physiological saline, and stored at −18°C until treated further. Immediately before the experiment they were homogenized in Ultra-Turrax with deionized water (Elgastat, England) to a 10% (w/v) homogenate. The activity of the homogenate varied around 0·5 to 0·6 μmol of hydrolyzed acetylcholine . min^{-1} ml^{-1} at 25°C and pH 8·0.

Measurement of esterase activity. The activity of acetylcholinesterase and butyrylcholinesterase was measured in terms of the initial rate of substrate hydrolysis in a Radiometer pH-stat. All measurements were made at 25°C and pH 8·0. The acid released by the substrate during its hydrolysis was titrated by 0·05M-NaOH. Acetylcholine iodide and butyrylcholine iodide were used as substrates for the measurement of the activity of acetylcholinesterase and butyrylcholinesterase, respectively. The total volume of the reaction mixture was 20 ml. The measurements were allowed to proceed either in deionized water or in 75 mM- or 150 mM-NaCl. The measurement in deionized water was made in the absence of inorganic cations except for experiments where the effect of Ca^{2+}-ions on the kinetics of the enzymatic reaction was investigated; in such cases the pH of deionized water was adjusted to 8·0 by titration by 0·05M-NaOH in a Radiometer pH-stat. The incubation of the enzymes with THA was carried out at constant temperature (±0·1°C). The mathematical treatment of the results and the calculations of kinetic and thermodynamic constants were made in Minsk-22 computer. All values of constants are means ± confidence range for P 0·95.

RESULTS

Inhibition of Butyrylcholinesterase by 1,2,3,4-*Tetrahydro-9-aminoacridine*

When THA was incubated with horse plasma butyrylcholinesterase at 25°C, pH 8·0, in 150 mM-NaCl, a nonprogressive inhibition was observed, *i.e.* the degree of inhibition was dependent only on THA concentration and not on the time of incubation of the enzyme with the inhibitor. The magnitudes of I_{50} constants for 5-min incubation, 0·127 ± 0·009 μM, and for 30-min incubation, 0·135 ± 0·010 μM, are therefore the same. The character of the interaction of the enzyme with the inhibitor was expressed in the Hill plot[11]. The plot of $\log . \{v_i/(v_0 - v_i)\}$ *versus* $\log$ [THA] is linear in the range of THA concentrations used and the magnitude of Hill's coefficient (n) is 0·80 for both substrate concentrations used (Fig. 1). One inhibitor molecule

therefore interacts with one catalytic unit of the enzyme[11]. As obvious from the Lineweaver–Burk[12] plot (Fig. 2), THA is a mixed inhibitor of butyrylcholinesterase. The inhibition constant of the noncompetitive (K_i 0·07 μM) and competitive (K_i 0·17 μM) component of the inhibition was calculated from a secondary plot of slopes (K_m/V) and intersections ($1/V$) *versus* THA concentration. The magnitude of the inhibition

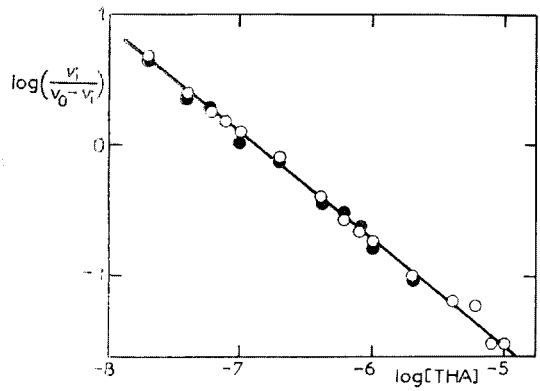


FIG. 1

Kinetics of Inhibition of Horse Plasma Butyrylcholinesterase by 1,2,3,4-Tetrahydro-9-aminoacridine in Hill Plot

Enzyme incubated with inhibitor 30 min. Acetylcholine concentration 2 (○) and 10 mM (●), respectively.

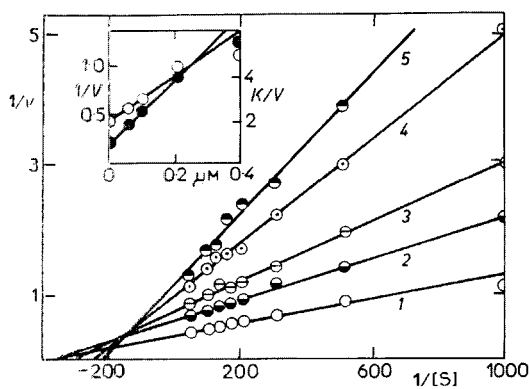


FIG. 2

Kinetics of Inhibition of Horse Plasma Butyrylcholinesterase by 1,2,3,4-Tetrahydro-9-aminoacridine in Lineweaver–Burk Plot

Final THA concentration: *1* 0; *2* 0·06; *3* 0·1; *4* 0·2; *5* 0·4 μM. The inserted diagram represents the dependence of intersections, $1/V$ (●) and slopes, K/V (○) on THA concentration in μM.

constant K_i calculated from the Dixon plot[13] of $1/v_i$ *versus* $[I]$ at two substrate concentrations (2 and 10 mM) was 0·14 μM.

Inhibition of Acetylcholinesterase by 1,2,3,4-*Tetrahydro-9-aminoacridine*

Acetylcholinesterase too is inhibited by THA in the nonprogressive manner. The affinity of THA for the three preparations used was the same. The values of I_{50} measured in 150 mM-NaCl (25°C, pH 8·0, 30-min incubation) were 2·38 ± 0·36 μM for rat brain acetylcholinesterase, 2·20 ± 0·42 μM for bovine erythrocyte acetylcholinesterase, and 2·10 ± 0·41 μM for human stromate acetylcholinesterase. Likewise, the values of Hill's coefficient were the same for all three enzyme preparations (from 0·98 to 1·02) and independent of the concentration of substrate used. The effect of sodium chloride concentration in the incubation mixture on the magnitude of I_{50} of THA was examined with human stromate acetylcholinesterase. The I_{50}-values measured were 1·55 ± 0·26 μM in deionized water, 1·80 ± 0·37 μM in 75 mM-NaCl, and 2·10 ± 0·41 μM in 150 mM-NaCl. The magnitudes of Hill's coefficient were not affected by sodium chloride concentration (0·98 – 1·03). The inhibition of acetylcholinesterase by THA is of noncompetitive character with respect to the substrate, as demonstrated with human stromate acetylcholinesterase in Fig. 3. K_i 2·22 ± 0·31 μM.

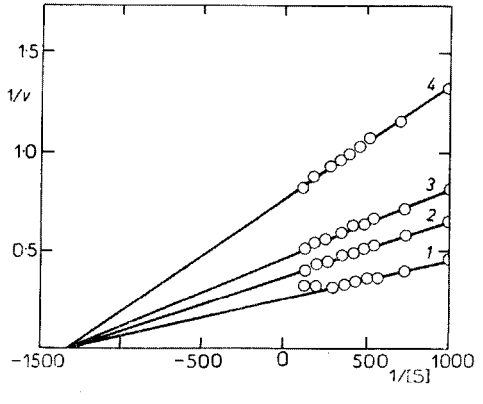


FIG. 3

Kinetics of Inhibition of Human Stromate Acetylcholinesterase by 1,2,3,4-Tetrahydro-9-aminoacridine in Lineweaver–Burk Plot

Final THA concentration: *1* 0; *2* 1; *3* 2; *4* 4 μM.

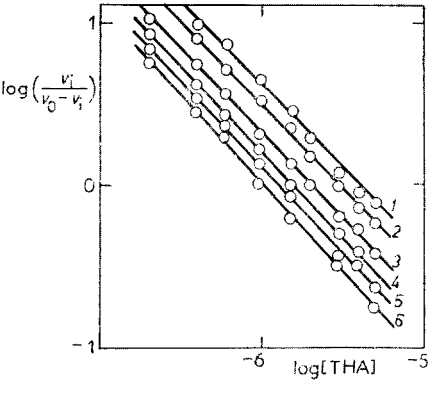


FIG. 4

Effect of Temperature on Kinetics of Inhibition of Human Stromate Acetylcholinesterase by 1,2,3,4-Tetrahydro-9-aminoacridine in Hill Plot

The incubation of the enzyme with the inhibitor proceeded 30 min at the following temperatures: *1* 5; *2* 11; *3* 16; *4* 22; *5* 27; *6* 34°C.

Test of Inhibition Reversibility

The test of the reversibility of the inhibition of horse plasma butyrylcholinesterase, rat brain acetylcholinesterase, and human stromate acetylcholinesterase by THA was carried out by the dilution experiment and by removal of the inhibitor by dialysis. Butyrylcholinesterase was incubated 30 min with 1 μM THA in 150 mM-NaCl at 25°C and pH 8·0 (84% inhibition) and then diluted by 150 mM-NaCl at a ratio of 1 : 1 to 1 : 20 in ten steps altogether. The enzyme activity was measured with 2 mM butyrylcholine as substrate. The per cent of inhibition was independent of the dilution degree and varied between 83 and 85%. A 24-h dialysis against 3 changes of a 500-fold volume of 150 mM-NaCl at 20°C decreased the inhibition to 42–45%; a prolongation of the dialysis time to 48 h decreased the inhibition to 8–12% (values obtained with five samples). Analogous experiments with acetylcholinesterase incubated 30 min in 150 mM-NaCl at pH 8·0 and 25°C with 10 μM THA showed that a dilution of the reaction mixture to a 20-fold of the original volume is without effect on the degree of inhibition (76 to 82%). A decrease of inhibition to 72- to 79% was observed after dialysis against a 500-fold volume of 150 mM-NaCl at room temperature, yet a prolongation of the dialysis time to 48-h did not influence the per cent of inhibition any considerably (70 to 78%, values obtained with ten samples).

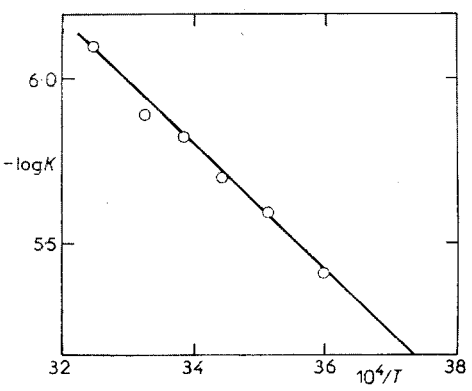


FIG. 5

Effect of Temperature on Dissociation Constant of Complex Acetylcholinesterase-1,2,3,4-Tetrahydro-9-aminoacridine

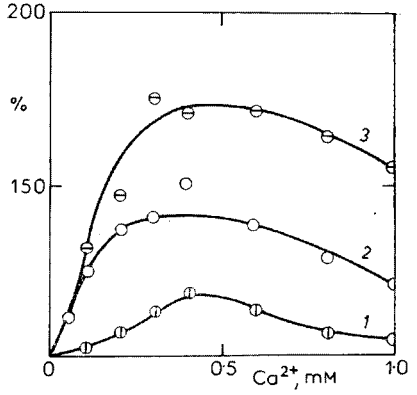


FIG. 6

Effect of Concentration of Ca^{2+}-Ions on Activity of Human Stromate Acetylcholinesterase in Presence and Absence of 1,2,3,4-Tetrahydro-9-aminoacridine

Final THA concentration: *1* 0; *2* 10; *3* 6 μM.

Effect of Temperature on Inhibition of Acetylcholinesterase

Human stromate acetylcholinesterase was incubated with 0·2 to 5 μM THA 30 min at six different temperatures varying in the range 5 – 34°C, in 75 mM-NaCl at pH 8·0. The residual enzyme activity was then measured and the dependence on the inhibition degree on THA concentration at the different temperatures was expressed by using Hill's equation[11]. A series of parallel lines (Fig. 4) was obtained from which the values of constants I_{50}, n, and pK were calculated; their numerical values are given in Table I. The values of standard free energy of the binding of the inhibitor to the enzyme for the individual temperatures were calculated according to the formula $\Delta G^\circ = -2{\cdot}303\boldsymbol{R}T\mathrm{p}K$. The standard free enthalpy of the binding of THA to acetylcholinesterase, ΔH°, was calculated from the slope of the linear dependence of pK on $1/T$ (Fig. 5) which numerically equals the value of $\Delta H^\circ/2{\cdot}303\boldsymbol{R}$. The value of $\Delta H^\circ = 8{\cdot}75 \pm 0{\cdot}12$ kcal mol^{-1} was calculated by the method of regression analysis. The values of standard entropy change were calculated according to the formula $\Delta S^\circ = (\Delta H^0 - \Delta G^0)/T$. The numerical values of all thermodynamic constants are given in Table I.

Activation of Acetylcholinesterase by Ca^{2+}-Ions

The activation effect of Ca^{2+}-ions on human stromate acetylcholinesterase was examined at pH 8·0 and 25°C in a medium not containing any inorganic cations with the exception of Ca^{2+}. The concentration of Ca^{2+}-ions varied between 0 and 1 mM. It was found that a slight activation effect of the Ca^{2+}-ions is increased in the presence of THA as obvious from Fig. 6.

The effect of Ca^{2+}-ions alone and the combined effect of Ca^{2+}-ions and THA on the kinetic parameters of the hydrolysis of acetylcholine by human stromate ace-

TABLE I

Effect of Temperature on Kinetic and Thermodynamic Parameters of Interaction of 1,2,3,4-Tetrahydro-9-aminoacridine with Human Stromate Acetylcholinesterase

t °C	pI_{50}	n	pK	ΔG^0 kcal mol^{-1}	ΔS^0 (e.u.)
5	5·41 ± 0·04	1·00 ± 0·02	5·41	−6·85	+5·60
11	5·52 ± 0,03	1·01 ± 0,01	5·60	−7·20	+5·60
16	5·71 ± 0·02	0·99 ± 0·01	5·70	−7·52	+5·62
22	5·82 ± 0·04	1·00 ± 0·02	5·82	−7·88	+5·61
27	5·88 ± 0·02	1·01 ± 0·01	5·90	−8·08	+5·61
34	5·98 ± 0·02	1·02 ± 0·01	6·10	−8·50	+5·60

tylcholinesterase were examined in a medium not containing any inorganic cations at pH 8·0 and 25°C. The results of these measurements were treated graphically by dual reciprocal plot according to Lineweaver and Burk[12] and the kinetic parameters obtained are given in Table II.

DISCUSSION

1,2,3,4-Tetrahydro-9-aminoacridine inhibits *in vitro* the activity of acetylcholinesterase and butyrylcholinesterase. The affinity of THA for horse plasma butyrylcholinesterase (pI_{50} 6·90, recorded data[4] for human plasma butyrylcholinesterase, pI_{50} 7·6) is higher than for acetylcholinesterase (pI_{50} 5·70 for all three kinds of enzymes, recorded data[4], pI_{50} 6·2). The degree of inhibition of both enzymes is independent of the time of incubation of the enzyme with THA, as described elsewhere[4].

The interaction of THA with acetylcholinesterase has a character of noncompetitive inhibition with K_i 2·22 μM and Hill's coefficient of 1·0. That means that one molecule of THA binds to one catalytic unit of the enzyme. The interaction is slightly affected by the concentration of sodium chloride in the incubation medium: an increase of salt concentration decreases the affinity of THA for the enzyme. On the contrary an affinity increase was observed after an increase of the temperature. The standard free energy of of the binding of THA to the active surface of acetylcholinesterase, ΔG^0 varies in the temperature range 5–34°C between −5·41 and −6·10 kcal mol^{-1}. The standard free enthalpy of the binding, ΔH^0, is constant over the given temperature range and its value is 8·75 kcal mol^{-1}. The standard entropy change, ΔS^0, is also constant and numerically equals 5·61 *e.u.*

TABLE II

Effect of Ca^{2+}-Ions and 1,2,3,4-Tetrahydro-9-aminoacridine on Kinetic Parameters of Hydrolysis of Acetylcholine by Human Stromate Acetylcholinesterase

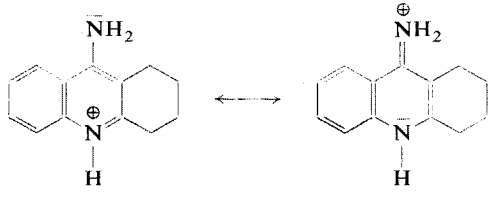
The incubation of the enzyme with both effectors and the measurement of enzymatic activity proceeded in a medium free of inorganic anions at pH 8·0 and 25°C. The kinetic constants were calculated from the $1/v$ *versus* $1/[S]$ plot. K_m, Michaelis constant; V_m, maximum rate of hydrolysis of acetylcholine in the absence of effectors; V_p, maximum rate of hydrolysis of acetylcholine in the presence of effectors; K_i, inhibition constant.

$[Ca^{2+}]$ mM	THA μM	K_m mM	V_m	V_p	K_i μM
0	0	0·455 ± 0·022	3·70 ± 0·12	—	—
0·5	0	0·448 ± 0·031	—	3·91 ± 0·15	—
0	6·0	0·436 ± 0·036	—	1·32 ± 0·08	3·34
0·5	6·0	0·475 ± 0·041	—	1·82 ± 0·12	5·80

THA behaves toward butyrylcholinesterase as a mixed competitive-noncompetitive inhibitor characterized by $K_{i\text{-compet.}}$ 0·17 μM and $K_{i\text{-noncompet.}}$ 0·17 μM. Likewise, the inhibition of butyrylcholinesterase involves the interaction of one molecule of THA with one catalytic unit of the enzyme. Hill's coefficient is 0·8.

The bond between THA and the active surface of both enzymes is very rigid. A mere dilution of the reaction mixture to a 20-fold of its original volume is without effect on the degree of inhibition. Only after dialysis for several days the bond between THA and the enzyme is disconnected and the activity of the enzyme is restored; the inhibition may therefore be regarded as being irreversible rather than reversible as reported by Heilbronn[4], even more because the restoration of the enzymatic activity of acetylcholinesterase is incomplete.

The rigidity of the bond between THA and the active surface of both enzymes indicates that the bond between the enzyme and the inhibitor is most likely not effected by electrostatic forces but predominantly by hydrophobic interactions. The magnitude of pK_a shows that the molecule of THA is fully ionized at pH 8·0 used in our experiments. The protonization of the molecule of THA is similarly to the molecules of aminopyridines[14] of such a type that the structure of the ionized molecule is expressed by mesomeric structures. These structures indicate that the positive



charge is not allocated to a defined site of the THA molecule. It is probable that this displacement of the charge makes the hydrophobic character of the THA molecule more explicit. This could explain the higher affinity of THA for butyrylcholinesterase which bears more "hydrophobic domains" in the neighborhood of the active center than acetylcholinesterase[15].

The noncompetitive character of the inhibition of acetylcholinesterase by THA excludes the possibility that THA binds to the α-anionic (catalytic) site which binds the substrate[16]. Since an antagonismus in the effect of Ca^{2+}-ions and THA on acetylcholinesterase has not been demonstrated we can also exclude the possibility of binding of THA to the β-anionic (allosteric) site of the active center of the enzyme. The activating effect of Ca^{2+}-ions on acetylcholinesterase is explained by binding of this cation to the β-anionic site also called the β-accelerating site[16]. We may thus assume that the bond between THA and acetylcholinesterase is effected at the site of the so-called "hydrophobic region" which lies approximately 10 Å apart from the esteratic site[17] and is also referred to as the γ-anionic site[16]. This γ-anionic site is also allosteric and if occupied by the effector it will affect the activity of both the catalytic and the β-accelerating site[16]. The activating effect of Ca^{2+}-ions is therefore enhanced

by the presence of THA. A similar situation has been observed also with gallamine, another inhibitor likewise binding to the γ-anionic site of acetylcholinesterase[16]. The results obtained provide evidence showing that THA binds to the hydrophobic domain of acetylcholinesterase in the neighborhood of its active center, alters the conformation of its molecule by its allosteric effect and thus decreases the rate of deacylation of the enzyme without changing its affinity for the substrate. Similarly, the binding of Ca^{2+}-ions to the β-anionic site does not change the affinity of the substrate for the enzyme yet the rate of deacylation is increased as a result of this binding.

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