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Michaelis Constants and Substrate Inhibition Constants for the Reaction of Acetylthiocholine with Acetylcholinesterase and Cholinesterase

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The activity of erythrocyte acetylcholinesterase at 5 °C and 25 °C and serum cholinesterase between 10 °C and 40 °C was measured with acetylthiocholine as a substrate. Michaelis constants (K_m) and substrate inhibition constants (K_{ss}) were calculated. K_m constants were calculated according to the Michaelis and Hill equations, while the K_{ss} constants were assesed graphically from pS curves and also calculated according to the equation which takes into account the hydrolysis of the enzyme-substrate inhibited complex. K_{ss} constants and K_m constants from the Hill equation were obtained at minimum χ^2 value. K_m and K_{ss} constants are almost independent of temperature while the maximal activities increase with temperature. Energies of activation were calculated from these data.

Acetylthiocholine is a substrate of cholinesterases. This substrate has been widely used for measuring cholinesterase activities[1] ever since a very simple and fast method was developed by Ellman *et al.*[2] There are many published values of the Michaelis constant (K_m) for acetylcholinesterase and acetylthiocholine but the value of the substrate inhibition constant (K_{ss}) is not well established for this system which also applies to K_m for the serum cholinesterase.

The aim of this work was to determine these constants, which are required to characterize the reaction, and to interprete the mechanisms of reversible inhibition whereby acetylthiocholine is often used as a substrate [3,4].

MATERIALS AND METHODS

Enzyme preparation: Purified bovine erythrocyte acetylcholinesterase (E. C. 3.1.1.7) (Winthrop Lab Inc. New York, USA), purified horse serum (Sigma Chemical Co., St. Louis, Mo., USA), and purified human serum cholinesterase (E. C. 3.1.1.8) AB Kabi, Stockholm, Sweden) were used. Stock solutions were made up in water and kept no longer than 2—3 days. Dilutions were prepared immediately before use. During the assay the concentrations of purified bovine erythrocyte acetylcholinesterase were 10 μg/ml and 20 μg/ml at 25 and 5 °C, respectively, those of purified horse serum ranged from 5 μg/ml to 20 μg/ml and of purified human plasma from 30 to 160 μg/ml.

The enzyme activity was measured using the method of Ellman *et al.*[2]. All experiments were done in 0.1 M phosphate buffer, pH = 7.4. Acetylthiocholine (0.5 and 0.1 M) was dissolved in distilled water of the same temperature at which

the activity was measured immediately before the experiment. The thiol reagent (5,5'-dithiobis-2-nitrobenzoate), the buffer and the enzyme, previously stored at the assay temperature, were put into the test tube, the substrate added and the activity measured by reading the absorbance at 15 s intervals for 1.0 to 2.0 min. The spontaneous hydrolysis of acetylthiocholine was substracted from the measured enzyme activity. The total volume of the reaction mixture was 3.0 ml. More details of the procedure for measuring the enzyme activity were described earlier[5].

RESULTS AND DISCUSSION

Acetylcholinesterase

The activities of bovine erythrocyte acetylcholinesterase were measured at 5 °C and 25 °C in the range of acetylthiocholine concentration from 0.1 to 50 mM. Bell-shaped pS curves were obtained at both temperatures (Fig. 1). From a narrow range of substrate concentrations (0.1 to 0.5 mM) K_m was evaluated using the Wilkinson plot[6] (*i. e.* [S]/v against [S]) and was found to be 0.11 mM for both themperatures, the maximum velocities being 6.0 and 13.2 ΔA min^{-1} mg^{-1} at 5 °C and 25 °C, respectively.

Acetylthiocholine at concentrations higher than 1 mM inhibits acetylcholinesterase. When reciprocal activities ($1/v$) were plotted against substrate concentrations ($1.0 \leqq [S] \leqq 50$ mM) a curve was obtained (Fig. 2). The mechanism of substrate inhibition is not known. However, it is generally considered that inhibition of enzyme by higher substrate concentrations is due to formation of an ESS complex. Regardless of the nature of this complex (*i. e.* the binding sites for the substrate and/or possible existence of various enzyme-substrate species), it was attempted in this paper to fit a curve to the experimental data using the most simple equation which takes into account the enzyme inhibition with excess substrate[7]:

$$v = V \frac{\beta [S] + K_{ss}}{[S] + K_{ss}} \cdot \frac{[S]}{[S] + K_m} \tag{1}$$

V is the maximal activity, K_m is the Michaelis constant, K_{ss} is the dissociation constant of the enzyme-substrate inhibited complex ESS and β is the ratio of the rate constants of decomposition of the ESS and ES complexes.

It follows from Fig. 1 and 2 that the numerical value of β must be $0 \leqq \beta \leqq 1$. If $\beta = 0$, a plot of $1/v$ *vs.* [S] is a straight line; if $\beta = 1$, no substrate inhibition occurs. To fit a curve onto the experimental values v, pairs of β and K_{ss} values were systematically varied (in the ranges $0 \leqq \beta \leqq 1$, $1 \leqq K_{ss} \leqq 50$) and χ^2 values calculated according to the equation

$$\chi^2 = \frac{(v_{exp} - v_{calc})^2}{v_{calc}} \tag{2}$$

where v_{exp} are the activities from Fig. 1 and v_{calc} were obtained using eqn. 1.

The minimum χ^2 was obtained for $0 \leqq \beta \leqq 0.10$ and $5 \leqq K_{ss}/mM \leqq 7$ at 5 °C while at 25 °C the respective ranges were $0.05 \leqq \beta \leqq 0.15$ and $8 \leqq K_{ss}/mM \leqq 12$ (Fig. 3). Any other pair of β and K_{ss} gave a worse fit to the experimentally obtained activities. The solid lines in Fig. 2 were calculated using β and K_{ss} values giving a minimum in χ^2.

The K_{ss} constants obtained in this manner agree well with the K_{ss} constants assessed graphically from Fig. 1 at the concentration of substrate for

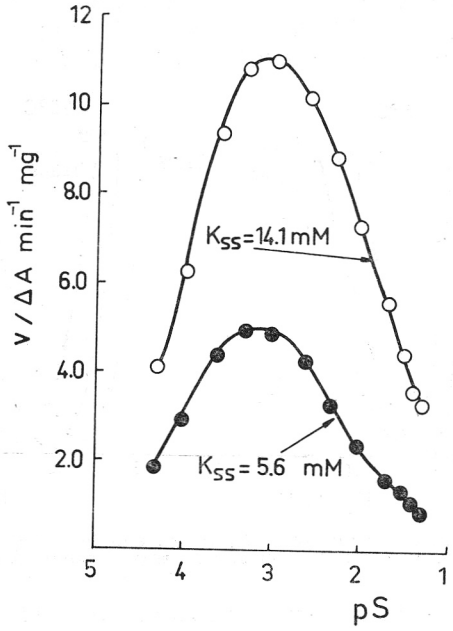

Fig. 1 — Activity $v/\Delta A$ min^{-1} mg^{-1} of bovine erythrocyte acetylcholinesterase as function of the acetylthiocholine concentration at 25 °C (○) and and 5 °C (●). Each point is the mean value of three separate experiments, each of which is made in duplicate. The K_{ss} constants marked on the figure are estimated graphycally as that substrate concentration for which the activity is 50% of its maximal value.

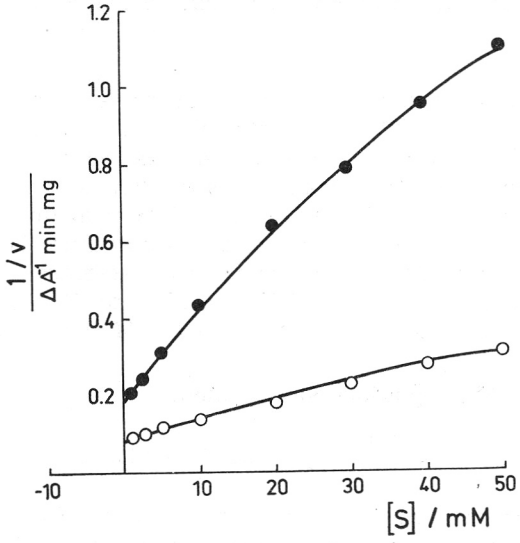

Fig. 2 — Results from Fig. 1 plotted as $1/v$ against substrate concentration. The solid lines were calculated from Eqn. (1) using β and K_{ss} at the minimum χ^2 value (Fig. 3): $K_{ss} = 10$ mM and $\beta = 0.10$ at 25 °C (○) and $K_{ss} = 6$ mM and $\beta = 0.05$ for 5 °C (●).

which the activity is inhibited 50%. K_{ss} values obtained in such a way are 5.6 mM and 14.1 mM at 5 and 25 °C, respectively (Fig. 1). Such agreement is expected because of the small numerical value of β (*cf.* Fig. 3).

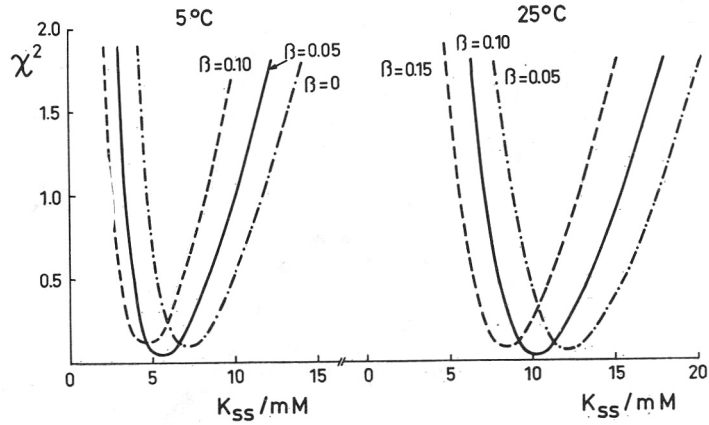

Fig. 3 — χ^2 values calculated (for different K_{ss} values and indicated β) from Eqns (1) and (2) and from activities determined experimentally (Fig. 1).

The K_m determined in our experiments are very similar to the published data obtained with the same enzyme preparation[2,8–10]. The K_{ss} constants obtained by the methods described above are smaller than the value of 30 mM reported by Ellman *et al.*[2]. Aldridge and Reiner[11] recalculating the values obtained by Ellman *et al.*[2] found the K_{ss} constant to be about 3.3 mM assuming that the ESS complex hydrolyzes with a β value of 0.09 to 0.1. A similar K_{ss} constant was obtained by these authors from the kinetics of competition between acetylthiocholine and some coumarin derivatives.

Cholinesterase

The cholinesterase activity of human and horse serum was measured at 10 °C, 20 °C, 30 °C, and 40 °C at acetylthiocholine concentrations ranging from 0.1 to 10 mM. The activities of both enzyme preparations increase with increasing substrate concentration and do not reach a maximal activity even at 10 mM acetylthiocholine (Fig. 4). A plot of [S]/v against [S] is curved for both enzymes and at all temperatures, but this curvature is more pronounced for human serum than for horse serum cholinesterase. Consequently, the Michaelis eqn. is not obeyed and different K_m and V are obtained for the substrate concentration range from 0.1 to 1.0 mM than from 1.0 to 10 mM (Tables I and II).

Therefore, the Hill equation was applied:

$$\log \frac{v}{V-v} = -\log K_m + n \log [S] \qquad (3)$$

and a χ^2 analysis performed in order to determine the best set of numbers n, K_m and V. For the human enzyme preparation the best fit was obtained assuming $n \approx 0.6$ while for horse serum $n \approx 0.8$. The smaller n for human serum agrees with the more pronounced deviation from the Michaelis kinetics

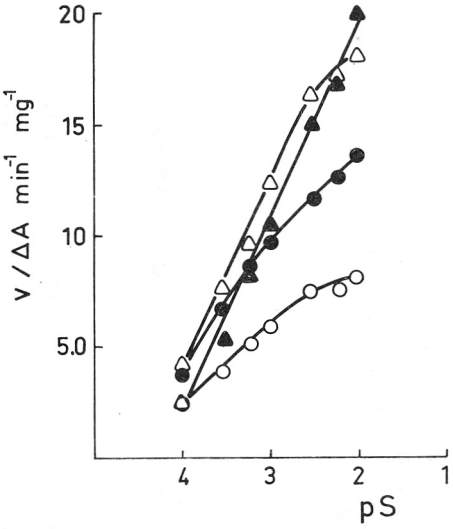

Fig. 4 — Activity $v/\Delta A$ min^{-1} mg^{-1} of horse serum cholinesterase as a function of acetylthiocholine concentration at 10 °C (○), 20 °C (●), 30 ° (△) and 40 °C (▲). Each point is the mean of three separate experiments, each of which is made in duplicate.

(*cf.* Table I). In Tables I and II are shown the K_m, V, and n values giving a minimum χ^2.

Some authors also found that the reactions of human and horse serum cholinesterases with acetylthiocholine deviate from the Michaelis kinetics[9,12] considering it to be due to the impurity from enzyme preparation[12]. K_m values published by these authors (from the Michaelis eqn.) agree with our results obtained in the corresponding ranges of substrate concentrations (Tables I and II). For the enzyme preparation used in our study, the Hill equation describes the reaction between enzyme and substrate better than the Michaelis kinetics would do.

Influence of temperature on K_m and V

The K_m values obtained for erythrocyte acetylcholinesterase and acetylthiocholine are the same at 5 °C and 25 °C. The K_m constants for human serum cholinesterase slightly decrease with increasing temperature (Table I) while for horse serum cholinesterase the K_m does not change appreciably between 10 °C and 30 °C but an obvious increase does occur between 30 °C and 40 °C (Table II and Fig. 4).

The V values increase with temperature for both acetylcholinesterase and cholinesterase preparations. The activation energy (E_a) calculated for acetylcholinesterase and acetylthiocholine was found to be 27.1 kJ mol^{-1}; this is only a rough estimate because V was determined only at two temperatures. The Arrhenius plot for V (Hill eqn., Table I) of human serum cholinesterase is a straight line, the E_a being 23.8 kJ mol^{-1}. The Arrhenius plot for V (Hill eqn., Table II) of horse serum cholinesterase is curved; taking the results between 20 °C and 40 °C the E_a is similar to that obtained for human serum cholinesterase.

TABLE I

K_m and V values for purified human serum cholinesterase and acetylthiocholine calculated according to the Michaelis and Hill equations. K_m and V from the Michaelis equation was obtained by a linear regression analysis of the Wilkinson plot; S. E. is the standard error of the intercept of the abscissa. K_m, V and Hill coefficient n from the Hill equation were obtained by a χ^2 analysis (the K_m and V given in the Table correspond to the minimum χ^2 value).

t/°C	Michaelis equation				Hill equation		
	$0.1 \leq$ [S]/mM ≤ 1.0		$1.0 \leq$ [S]/mM ≤ 10		$0.1 \leq$ [S]/mM ≤ 10		
	$K_m \pm$ S. E. / mM	V / ΔA min^{-1} mg^{-1}	$K_m \pm$ S. E. / mM	V / ΔA min^{-1} mg^{-1}	n	K_m / mM	V / ΔA min^{-1} mg^{-1}
10	0.12 ± 0.03	0.40	1.03 ± 0.26	0.68	0.5	1.00	0.80
20	0.19 ± 0.05	0.73	0.76 ± 0.13	1.02	0.6	0.70	1.05
30	0.14 ± 0.05	1.06	0.73 ± 0.20	1.51	0.6	0.60	1.50
40	0.16 ± 0.05	1.44	0.82 ± 0.31	2.04	0.6	0.60	2.10

TABLE II

K_m and V values for purified horse serum cholinesterase and acetylthiocholine calculated according to the Michaelis and Hill equations. K_m and V from the Michaelis equation was obtained by a linear regression analysis of the Wilkinson plot; S.E. is the standard error of the intercept of the abscissa. K_m, V and the Hill coefficient n from the Hill equation were obtained by a χ^2 analysis (the K_m and V given in the Table correspond to the minimum χ^2 value).

t/°C	Michaelis equation				Hill equation		
	$0.1 \leq$ [S]/mM ≤ 1.0		$0.1 \leq$ [S]/mM ≤ 10		$0.1 \leq$ [S]/mM ≤ 10		
	$K_m \pm$ S.E. / mM	V / ΔA min^{-1} mg^{-1}	$K_m \pm$ S.E. / mM	V / ΔA min^{-1} mg^{-1}	n	K_m / mM	V / ΔA min^{-1} mg^{-1}
10	0.26 ± 0.03	7.39	0.49 ± 0.20	8.49	0.8	0.40	8.0
20	0.23 ± 0.02	11.9	0.66 ± 0.28	14.3	0.7	0.60	15.0
30	0.32 ± 0.08	15.5	0.59 ± 0.14	18.9	0.8	0.60	20.0
40	0.72 ± 0.03	17.8	1.44 ± 0.56	22.2	0.8	1.40	24.0

The activation energies obtained in this work for acetylcholinesterase and cholinesterase fit well into the published data of the effect of temperature on V for cholinesterases and substrates other than acetylthiocholine (*cf.* ref. 13).

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SAŽETAK

Michaelisove konstante i konstante inhibicije supstratom za reakciju acetiltiokolina s acetilkolinesterazom i kolinesterazom

Vera Simeon

Mjerena je aktivnost eritrocitne acetilkolinesteraze pri 5 °C i 25 °C, te serumske kolinesteraze pri 10 °C i 40 °C s acetiltiokolinom kao supstratom. Izračunane su Michaelisove konstante (K_m) i konstante kompleksa enzima inhibiranog sa supstratom (K_{ss}). Konstante K_m računane su po Michaelisovoj i Hillovoj jednadžbi, dok su konstante K_{ss} ocijenjene grafički iz pS-krivulje i računane po jednadžbi u kojoj se uzima u obzir hidroliza enzima inhibiranog supstratom. Da bi se odredilo minimalno odstupanje od eksperimentalnih podataka korištena je χ^2-analiza. Konstante K_m i K_{ss} gotovo su nezavisne o temperaturi, dok maksimalne brzine reakcije rastu s temperaturom, pa su iz te veličine izračunane energije aktivacije.

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