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Acetylcholinesterase Kinetics

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Abstract. Three mechanisms have been suggested to describe the inhibition of acetylcholinesterase (EC. 3.1.1.7) by an excess of acetylcholine.

(i) Substrate inhibition occurs through the reaction of acetylcholine with acetylated enzyme. The deacetylation of this ternary complex is supposed to be completely inhibited.

(ii) A ternary complex is formed as in *(i)*. However, the deacetylation is not completely inhibited.

(iii) A two-site-mechanism is discussed. Acetylcholine binds either to the active site or to the modifier site. Binding to the latter changes the activity of the active site.

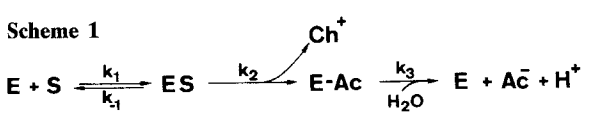
Steady state treatment was applied to *(i)*–*(iii)*. A least squares fit led to catalytic parameters. It is demonstrated that mechanism *(ii)* is the most simple one which can describe satisfactorily the experimental data. Limits for a set of rate constants are derived from the catalytic parameters. A numerical integration shows that the steady state approximation may be used even when the mechanisms are rather complex.

Key words: Acetylcholinesterase – Enzyme kinetics – Substrate inhibition – Steady state kinetics

1. Introduction

The hydrolysis of acetylcholine under the influence of acetylcholinesterase (EC. 3.1.1.7) can at low substrate concentrations be described by Scheme 1.

Scheme 1



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The substrate acetylcholine S is hydrolyzed to choline Ch^+ and acetic acid Ac^-H^+. E stands for the free and E-Ac for the acetylated enzyme. On steady-state assumption for all enzyme species the reaction rate is:

$$v = \frac{V}{1 + \frac{K_m}{[S]}} = \frac{k_{cat} \cdot [E]_t}{1 + \frac{K_m}{[S]}}. \tag{1}$$

The parameters in Eq. (1) are composed of rate constants and referred to as catalytic parameters.

V = maximum reaction rate at high substrate concentration = $k_{cat} \cdot [E]_t$,
$[E]_t$ = total concentration of enzyme monomers which is equal to the total concentration of active sites,

$$k_{cat} = \text{turnover number} = \frac{k_2 \cdot k_3}{k_2 + k_3},$$

$$K_m = \text{Michaelis constant} = \frac{k_3 \cdot (k_{-1} + k_2)}{k_1 \cdot (k_2 + k_3)}.$$

The reaction consists of at least three steps, the formation of the enzyme-substrate complex, the release of choline, which is paralleled by the acetylation of the enzyme, and the deacetylation of the acetyl enzyme. If an enzyme catalyzed reaction behaves according to Scheme 1 then the reaction rate approaches asymptotically the maximum value $V = k_{cat} \cdot [E]_t$ as the substrate concentration is enhanced. Acetylcholinesterase activity, however, does not reach such a limit but exhibits the phenomenon of substrate-inhibition. The activity of the enzyme decreases again when acetylcholine-concentration exceeds a certain optimum concentration. Therefore activity vs. pS[1] curves are "bell-shaped" as demonstrated, e.g., by Nachmansohn and Rothenberg (1945), Nachmansohn and Wilson (1951), Gentinetta and Brodbeck (1976), Bon and Massoulié (1976), Hopff (1976). Kinetic schemes more complex than Scheme 1 must be taken into account in order to obtain an adequate description of the experimental data. In this paper three possible mechanisms suggested earlier (Krupka and Laidler 1961; Rosenberry and Bernhard 1972) are examined as follows:

(i) An analytical expression for the reaction rate as a function of the substrate concentration is obtained by a steady state analysis.

(ii) A least squares fit carried out on previously published activity vs. pS curves (Gentinetta and Brodbeck 1976) has resulted in numerical values of the catalytic parameters with their standard deviations. The mean standard deviations of the fits were used to check adequacy of the mechanisms.

(iii) Additional bibliographical data were used to calculate a set of rate constants from averaged catalytic parameters.

(iv) Numerical integration of the set of differential equations related to a given scheme was performed in order to check wether these rate constants are

1 pS = Negative logarithm of the substrate concentration

consistent with experimental activity vs. pS curves and wether the steady state approximation was permissible.

2. Materials and Methods

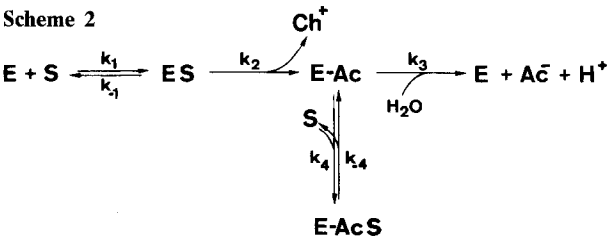
Kinetic experiments at low substrate concentrations ($< 10^{-3}$ M) require the use of the double-syringe technique described by Heilbronn (1958) in order to maintain constant substrate concentration. Our analysis is based on activity vs. pS curves reported by Gentinetta and Brodbeck (1976) making use of this technique. The reaction mixture consisted of 20 ml of 10 mM magnesium chloride in distilled water, and the activity was measured at pH 7.4 and at a temperature of 30° C. The enzyme activity at optimum substrate concentration amounted to 8.7 ± 0.1 IU (1 IU = 1 international unit = 1 μmole product produced per min). The enzyme used was the so called G form that means the 11 S form isolated from *Electrophorus electricus*. The molecular weight of this enzyme is 340,000 ± 20,000 g/mol (Dudai et al. 1973; Bon et al. 1976; Bon and Massoulié 1976; Anglister and Silman 1978). The spezific activity of the 11 S enzyme amounted to 10,000 ± 1,000 IU/mg (Rosenberry et al. 1972; Chen et al. 1974; Webb and Clark 1978). The enzyme consisted of four subunits each with one active site. The total concentration of monomers $[E]_t$ was obtained from the spezific activity of the enzyme and the measured activity at optimum substrate concentration and amounted to $[E]_t = (5.1 \pm 0.9) \cdot 10^{-10}$ M.

3. Results and Discussion

3.1. Mechanism 1

In Mechanism 1 the enzyme inhibition at high substrate concentration is described by the reversible binding of a second acetylcholine molecule either to the ES-complex or to the acetylated enzyme whereby the reaction flow is assumed to be completely blocked. Acetylcholine binding to the ES-complex was suggested, e.g., by Haldane (1930) and Murray (1930) and Nachmansohn and Wilson (1951). However, investigations by Krupka and Laidler (1961) as well as by Wilson and Alexander (1962) gave evidence that the inhibition is caused by acetylcholine binding to the acetylated enzyme as shown in reaction Scheme 2.

Scheme 2



Under steady state approximation the reaction rate is found to be

$$v = \frac{V}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}} = \frac{k_{cat} \cdot [E]_t}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}}, \tag{2}$$

where K_m and V are the same as defined with reaction Scheme 1 and Eq. (1) above. (V is the theoretical maximum reaction rate at a given enzyme concentration if no substrate inhibition would take place.)

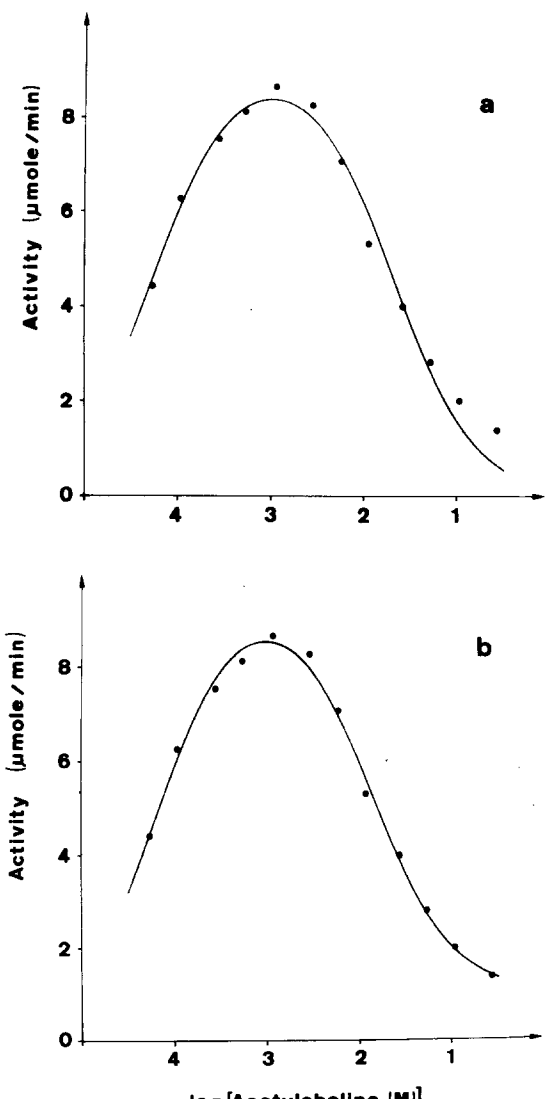


Fig. 1a and b. Least squares fit of acetylcholinesterase-activity vs. pS data (filled symbols) based on Mechanism 1 (**a**) [Scheme 2, Eq. (2)] and Mechanism 2 (**b**) [Scheme 3, Eq. (4)]. ($T = 30°$ C, pH 7.4, 10 mM $MgCl_2$)

K_i is the inhibition constant for substrate inhibition given by

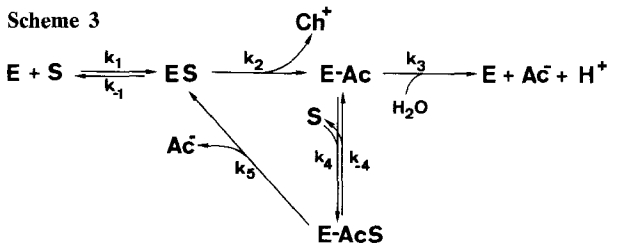
$$K_i = \frac{(k_2 + k_3) \cdot k_{-4}}{k_2 \cdot k_4}. \text{ For } k_2 \gg k_3 \text{ one obtains } K_i \approx \frac{k_{-4}}{k_4}.$$

A least squares fit (Fig. 1a) led to the following averaged constants: $K_m = (5.6 \pm 1.7) \cdot 10^{-5}$ M, $K_i = (2.0 \pm 0.5) \cdot 10^{-2}$ M, $V = 9.3 \pm 0.6$ IU. The errors correspond to the 90% confidence limits and the mean standard deviation of this fit is 0.41 IU. The turnover number k_{cat} was calculated from V and $[E]_t$ to be $k_{cat} = (1.5 \pm 0.4) \cdot 10^4\ s^{-1}$.

3.2. Mechanism 2

The above mentioned Mechanism 1 results in symmetrical activity vs. pS curves. However, recently published curves are distinctly asymmetric (Gentinetta and Brodbeck 1976; Bon and Massoulié 1976) in that way that inhibition at high substrate concentration is less efficient than expected from Mechanism 1. Kinetic mechanisms describing such a behaviour have been discussed by Krupka and Laidler (1961).

Scheme 3



In contrast to Mechanism 1 the deacetylation of the ternary complex (E-AcS) is not completely inhibited. The steady state treatment led to the following expression for the reaction rate:

$$v = \frac{V + V_i \cdot \dfrac{[S]}{K_i}}{1 + Q + \dfrac{K_m}{[S]} + \dfrac{[S]}{K_i}}, \tag{3}$$

where

$$V = \frac{k_2 \cdot k_3}{k_2 + k_3} \cdot [E]_t = k_{cat} \cdot [E]_t, \tag{3a}$$

$$V_i = \frac{k_2 \cdot k_5}{k_2 + k_5} \cdot [E]_t = k_{cat_i} \cdot [E]_t, \tag{3b}$$

$$K_m = \frac{k_3 \cdot (k_{-1} + k_2)}{k_1 \cdot (k_2 + k_3)}, \tag{3c}$$

$$K_i = \frac{(k_2 + k_3) \cdot (k_{-4} + k_5)}{(k_2 + k_5) \cdot k_4}, \tag{3d}$$

$$Q = \frac{k_{-1} \cdot k_4 \cdot k_5}{k_1 \cdot (k_2 + k_3) \cdot (k_{-4} + k_5)}. \tag{3e}$$

The parameters K_m and V are composed of the same rate constants as in Mechanism 1. V_i is the theoretical maximum reaction rate at a given enzyme concentration for the case that formation of product only would occur by deacetylation of the ternary complex E-AcS. K_i is again the inhibition constant for substrate inhibition and Q is a parameter which may be neglected because its value is much smaller than one as demonstrated below. As the reaction rate according to the structure of Eq. (3) is determined by only four parameters, one parameter has to be eliminated. This can be done by dividing numerator and denominator of Eq. (3) by the value $(1 + Q)$ resulting in

$$v = \frac{V' + V_i \cdot \frac{[S]}{K_i'}}{1 + \frac{K_m'}{[S]} + \frac{[S]}{K_i'}} \tag{4}$$

with $V' = V/(1 + Q)$, $K_m' = K_m/(1 + Q)$, $K_i' = K_i \cdot (1 + Q)$. A least squares fit (Fig. 1b) based on Eq. (4) led to numerical values for the four parameters V', V_i, K_m', and K_i', and the mean standard deviation of the fit. The latter amounts to 0.22 IU and is considerably smaller than that observed with Mechanism 1. Corresponding results are obtained by the evaluation of activity vs. pS curves published by Bon and Massoulié (1976). Therefore Mechanism 2 should be considered to be more reasonable than Mechanism 1. Finally an estimate of the maximum value of Q is possible by means of Eqs. (3a–e).

$$Q = \frac{V_i}{K_i' \cdot V'} \cdot K_D \cdot \frac{k_3}{k_2 + k_3} \text{ or } Q < \frac{V_i}{K_i' \cdot V'} \cdot K_D,$$

where $K_D = k_{-1}/k_1$ is the dissociation constant of the enzyme substrate complex.

By using the fitted values for V_i, V', and K_i' (see below) the maximum value of Q amounts to $8 \cdot K_D$. The value of K_D is unknown, however, the corresponding values for the cationic ligands 1-Methyl-7-hydroxyquinolinium and N-Methylacridinium have been estimated to be about $2 \cdot 10^{-7}$ M (Mooser et

al. 1972; Neumann et al. 1978). If K_D of the acetylcholine-enzyme complex is of similar magnitude, Q may be neglected and the fitted parameters K'_m, K'_i, V' are identical with K_m, K_i, and V.

They amount to

$$K_m = (6.3 \pm 1.1) \cdot 10^{-5}\,\mathrm{M},$$

$$K_i = (1.3 \pm 0.3) \cdot 10^{-2}\,\mathrm{M},$$

$$V = 9.7 \pm 0.4\,\mathrm{IU},$$

$$V_i = 1.0 \pm 0.4\,\mathrm{IU}.$$

Indicated errors correspond to the 90% confidence limits. If equivalent parameters obtained for Mechanism 1 and 2 are compared with each other, one should take into account that the introduction of an additional parameter (V_i in the case of Mechanism 2) may influence the remaining parameters of the least squares fit, i.e., K_m, K_i, and V, respectively. The turnover numbers k_{cat} and k_{cat_i} were calculated from $[\mathrm{E}]_t$ and the maximum reaction rates V and V_i:

$$k_{\mathrm{cat}} = (1.6 \pm 0.3) \cdot 10^4\,\mathrm{s}^{-1},\quad k_{\mathrm{cat}_i} = (1.7 \pm 0.9) \cdot 10^3\,\mathrm{s}^{-1}.$$

Rate constants of Mechanism 2 are determined on two assumptions as described below.

First, deacetylation of the enzyme is expected to be the rate-limiting step in the course of acetylcholine hydrolysis (Wilson and Cabib 1956; Krupka 1964). Therefore it is possible to indicate lower and upper limits of the corresponding rate constant k_3 using Eq. (3a) with $k_2 = k_3$ and $k_2 \gg k_3$, respectively.

$$k_{\mathrm{cat}} < k_3 < 2\,k_{\mathrm{cat}} \tag{5a}$$

k_1, k_2, and k_5 are obtained as functions of k_3 from Eqs. (3a), (3b), and (3c)

$$k_1 = \frac{k_{\mathrm{cat}}}{K_m - K_D \cdot \dfrac{k_3 - k_{\mathrm{cat}}}{k_3}}, \tag{5b}$$

$$k_2 = \frac{k_3 \cdot k_{\mathrm{cat}}}{k_3 - k_{\mathrm{cat}}}, \tag{5c}$$

$$k_5 = \frac{k_{\mathrm{cat}_i}}{1 - k_{\mathrm{cat}_i} \cdot \left(\dfrac{1}{k_{\mathrm{cat}}} - \dfrac{1}{k_3}\right)}. \tag{5d}$$

Second, assuming K_D to be of similar magnitude for both, acetylcholine and the quinolinium- and acridinium-ligands, i.e., $\leq 10^{-5}$ M, one obtains $k_1 = (2.6 \pm 0.7) \cdot 10^8\ (\mathrm{sM})^{-1}$.

Furthermore Eqs. (5a) and (5d) result in $k_5 = (1.7 \pm 0.9) \cdot 10^3\ \mathrm{s}^{-1}$. Concerning k_4 and k_{-4} it is not possible to get numerical values because the only information about these two constants is the ratio $(k_{-4} + k_5)/k_4$ which is part of the parameter K_i. Thus only a relation between k_{-4} and k_4 can be derived from Eq. (3d).

$$k_{-4} = \frac{k_{\mathrm{cat}}}{k_3} \cdot K_i \cdot \frac{k_5}{k_{\mathrm{cat}_i}} \cdot k_4 - k_5 \approx \frac{k_{\mathrm{cat}}}{k_3} \cdot K_i \cdot k_4 - k_{\mathrm{cat}_i}\,. \tag{5e}$$

The approximation of Eq. (5e) makes use of the fact that in our case k_5 and k_{cat_i} are equal within the limits of experimental error (see above). Because $k_{-4} > 0$ and $k_3 > k_{\mathrm{cat}}$, a minimal value for k_4 may be estimated from Eq. (5e): $k_4 > k_{\mathrm{cat}_i}/K_i$, resulting in $k_4 > 1.3 \cdot 10^5\ (\mathrm{sM})^{-1}$.

Figure 2 shows the constants k_1, k_2, k_3, and k_5 as depending on the fraction k_2/k_3. Two attempts have been made to determine the value of this fraction. Wilson and Cabib (1956) have estimated k_2/k_3 to be about six. Based on this value one would obtain $k_2 = (1.1 \pm 0.2) \cdot 10^5\ \mathrm{s}^{-1}$ and $k_3 = (1.8 \pm 0.4) \cdot 10^4\ \mathrm{s}^{-1}$. A least squares analysis of the experimental data from Wilson and Cabib (1956) based on the Arrhenius equation led to energies of activation for acetylation (Ea_3) and deacetylation (Ea_4) of $Ea_3 = 57 \pm 11$ kJ/mol, and $Ea_4 = -4 \pm 8$ kJ/mol, respectively. These values are in good agreement with those published by Wilson and Cabib (1956) who found $Ea_3 = 63-84$ kJ/mol, and $Ea_4 = 5.0$ kJ/mol, respectively. However, the frequency factors including the entropies of activation, could not be fitted with

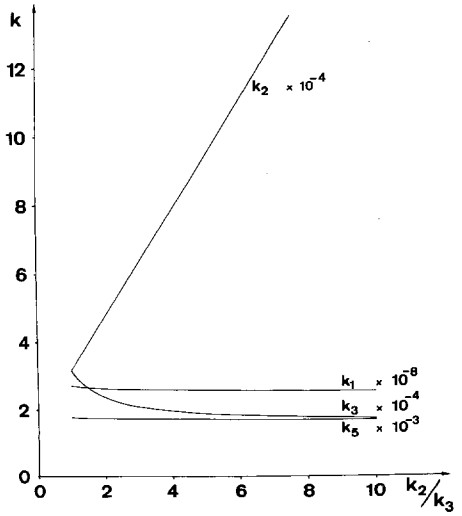


Fig. 2. Rate constants of Mechanism 2 (Scheme 3) as depending on the ratio k_2/k_3. ($T = 30°$ C, pH 7.4, 10 mM $MgCl_2$)

significance. Furthermore, the calculated energy of activation for the hydrolysis of the acetyl-enzyme is rather small compared with the corresponding value for the hydrolysis of acetylchymotrypsin which has been estimated by Bender et al. (1964) to be about 43 kJ/mol. Finally, it should be noted that Beauregard and Roufogalis (1979) observed a non-linear Arrhenius plot with cardiolipin-associated erythrocyte acetylcholinesterase which was similar to that reported by Wilson and Cabib (1956). They found that the deflexion at 20° C depended on the presence of Ca^{2+}-ions and of cardiolipin. In the view of this finding and of the unlikely small energy of activation for acetyl-enzyme hydrolysis ($Ea_4 = -4 \pm 8$ kJ/mol) one should ask, wether the interpretation of the non-linear Arrhenius plot by Wilson and Cabib (1956) leading to $k_2/k_3 \cong 6$ was realistic. Froede and Wilson (1980) have made another approach to determine this ratio. Corresponding results will be discussed below.

Numerical Integration

One purpose of the numerical integration was to check wether the estimated rate constants were consistent with the measured activity vs. pS curve, i.e., whether the use of the steady state assumption was adequate. The set of differential equations describing the mechanism belonged to the class of stiff differential equations. The method which has been used for the integration is a Gear method, i.e., an implicit linear multi-step method, which is suitable to solve equations with stiff terms. For further information concerning this methods the reader is referred to Gear (1971). The subroutine we used has been developed by D. K. Kahaner, National Bureau of Standards and C. D. Sutherland, Los Alamos Scientific Laboratory. Any set of positive rate constants which satisfies Eqs. (5a)–(5e) led when numerical integration was performed to an activity vs. pS curve which was identical to the curve described by the analytical expression given by Eq. (4). The two curves were identical within a rms-deviation of $\Delta v_{rms} = 8 \cdot 10^{-5}$ IU. Thus the use of the steady state assumption turned out to be adequate in this case, and therefore a further refining of the rate constants by "curve-fitting" was not possible. By numerical integration one obtains not only the concentrations of the substrate and the products but also those of the different enzyme intermediates.

Figure 3 shows the degree of acetylation, i.e., the concentration of E-Ac and E-AcS compared to the total enzyme concentration. This degree of acetylation is dependent on the ratio k_2/k_3 and the acetylcholine concentration, however, it is not influenced by the enzyme concentration as long as the latter is much smaller than the substrate concentration (Fig. 3 is applicable to enzyme concentrations smaller than 10^{-7} M). Froede and Wilson (1980) have found experimentally that 60–70% of the enzyme are acetylated at a substrate concentration of 0.1–0.5 mM. Unfortunately, these authors did not indicate accurate substrate concentrations (10^{-4} to $5 \cdot 10^{-4}$ M) so that a reliable determination of the ratio k_2/k_3 is not possible. From Fig. 3 one only can conclude that $k_2/k_3 \geq 2$ that means $k_2 \geq 5 \cdot 10^4\ s^{-1}$ and $k_3 = (2.1 \pm 0.8) \cdot 10^4\ s^{-1}$.

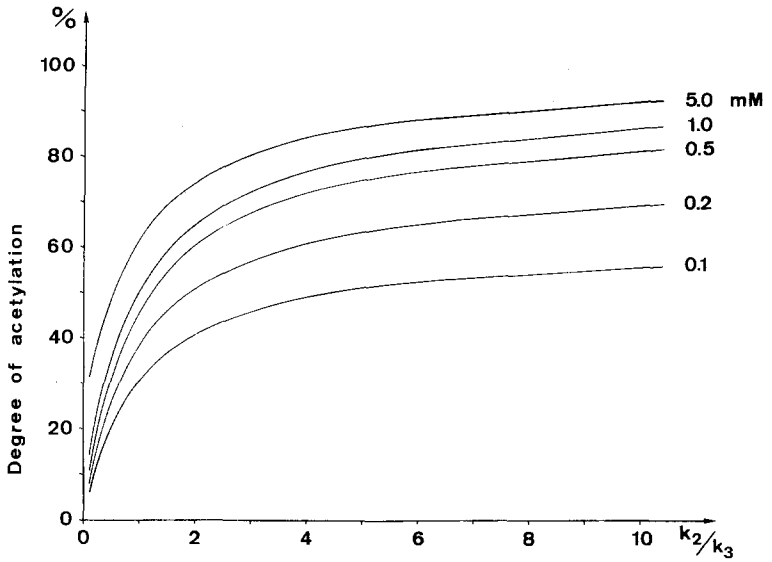
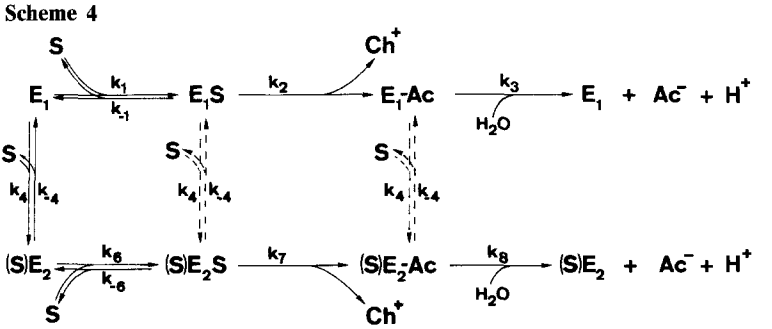


Fig. 3. Degree of acetylation of acetylcholinesterase as depending on the ratio k_2/k_3 and the acetylcholine concentration ($T = 30°$ C, pH 7.4, 10 mM $MgCl_2$)

3.3. Mechanism 3

Several authors (Meunier and Changeux 1969; Rosenberry and Bernhard 1971; Wermuth and Brodbeck 1973; Mooser and Sigman 1974) have suggested the existence of a peripheral binding site, a so called modifier site which when occupied has an influence on the catalytic activity of the active site. Rosenberry and Bernhard (1972) described the substrate inhibition with such a two-site model. According to Scheme 4 acetylcholine should react not only with the active but also with the peripheral site and thereby reduce the activity of the catalytic centre.

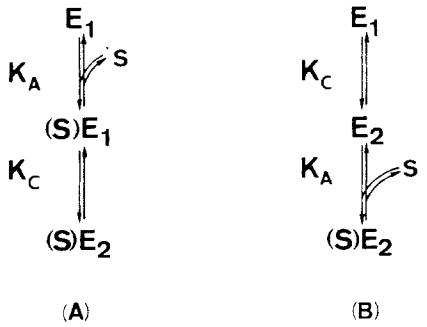
Scheme 4



The reason for the change of enzymatic activity might be either a conformational change of the protein as a consequence of the association of a substrate molecule with the peripheral site [cf. Scheme 4.1 (A)], or the existence of a

conformational equilibrium between a high and a low active state where only the peripheral site of the latter would be able to bind substrate [cf. Scheme 4.1 (B)].

Scheme 4.1



Index 1 stands for the enzyme conformation with the high, and index 2 for that with the low activity, respectively. K_A is the association constant related to acetylcholine binding by the peripheral site, and K_c is the equilibrium constant of the conformational change of the enzyme. K_E, the equilibrium constant between the high active conformation E_1 and the low active conformation (S)E_2 is equal to the product of the equilibrium constant of the two steps of acetylcholine association and conformational change, i.e., $K_E = K_A \cdot K_c$. Mechanisms as described in Scheme 4.1 have been discussed by Bolger and Taylor (1979) for the reaction of bisquaternary ammonium ligands with acetylcholinesterase. For our analysis it does not matter whether Mechanism 4.1 (A) or 4.1 (B) takes place. In order to simplify steady state treatment of Mechanism 3 the dashed reaction paths in Scheme 4 have not been considered, i.e., only the peripheral site of the free enzyme is expected to react with the substrate. However, it should be noted that all reaction paths of Scheme 4 have been taken into account on numerical integration (see below). The steady state reaction rate as a function of the substrate concentration is found to be:

$$v = \frac{V_1 + V_2 \cdot \frac{K_{m_1}}{K_{m_2}} \cdot K_E \cdot [\mathrm{S}]}{1 + K_{m_1} \cdot K_E + \frac{K_{m_1}}{[\mathrm{S}]} + \frac{K_{m_1}}{K_{m_1}} \cdot K_E \cdot [\mathrm{S}]}, \tag{6}$$

where K_{m1}, K_{m2}, V_1, and V_2 denote the Michaelis constants and the maximum reaction rates of the two species E_1 and (S)E_2. They are given by

$$K_{m_1} = \frac{k_3(k_{-1} + k_2)}{k_1(k_2 + k_3)}, \tag{6a}$$

$$K_{m_2} = \frac{k_8(k_{-6} + k_7)}{k_6(k_7 + k_8)}, \tag{6b}$$

$$V_1 = \frac{k_2 \cdot k_3}{k_2 + k_3} \cdot [\mathrm{E}]_t \,, \tag{6c}$$

$$V_2 = \frac{k_7 \cdot k_8}{k_7 + k_8} \cdot [\mathrm{E}]_t \,, \tag{6d}$$

$$K_E = \frac{k_4}{k_{-4}} \,. \tag{6e}$$

As the reaction rate according to Eq. (6) is determined by only four independent parameters, the number of actual parameters has to be reduced by one. This may be done by dividing numerator and denominator of Eq. (6) by the factor $(1 + K_{m1} \cdot K_E)$ leading to:

$$v = \frac{C_1 + C_2 \cdot [\mathrm{S}]}{1 + \dfrac{C_3}{[\mathrm{S}]} + C_4 \cdot [\mathrm{S}]} \,, \tag{7}$$

where the constants C_1, C_2, C_3, and C_4 are composed of K_{m1}, K_{m2}, V_1, V_2, and K_E. Comparison of Eqs. (3) and (7) reveals that the steady state solutions related to Mechanisms 2 and 3 have identical structure. Therefore it is not possible to distinguish Mechanism 2 from Mechanism 3 by means of a steady state analysis of activity vs. pS curves. Numeric values of C_1, C_2, C_3, and C_4 have been obtained by a least squares fit. The relation between these constants and the rate constants indicated in Scheme 4 is given by Eqs. (6a)–(6e) and (7), however, no attempt was made to get more information about rate constants because of the considerably higher complexity of Mechanism 3 with respect to Mechanism 2.

Numerical Integration

By numerical integration it could be demonstrated that any set of kinetic constants which satisfies Eqs. (6a)–(6e) and (7) leads to an activity vs. pS curve which is identical with the curve defined by Eq. (7). If the simplification of Scheme 4 is removed, i.e., if the reaction paths described by dashed lines were also taken into account the activities obtained by numerical integration would turn out to be too small at high substrate concentrations. Obviously, because there were now three paths leading from the high-active to the low-active state of the enzyme, whereas the rate constans k_4 and k_{-4}, have been derived from conditions ($C_1 \ldots C_4$) which are valid for a system with one path only. However, perfect agreement of the two curves could be obtained again by decreasing K_E.

4. Conclusions

The substrate inhibition of acetylcholinesterase might be explained either by sterical hinderance of the deacetylation as a result of the formation of a ternary

complex with an additional substrate molecule (Mechanism 1 and 2), or by a regulatory model (Mechanism 3). To describe the activity vs. pS curves published by Gentinetta and Brodbeck (1976) with sufficient accuracy, one has to use an analytical expression of the reaction rate with four parameters. Therefore, Mechanism 1 in which only three parameters are involved is unlikely.

Mechanisms 2 and 3 lead both to equivalent expressions for the reaction rate, i.e., four parameters and the same dependence with respect to the substrate concentration. Therefore, these two mechanisms can not be distinguished by means of least squares fits of activity vs. pS curves based on steady state analysis. Thus, Mechanism 2 (see Scheme 3) is the most simple one which leads to asymmetric bell-shaped activity vs. pS curves. The four corresponding parameters (K_m, K_i, k_{cat}, k_{cat_i}) are found to be

$$K_m = (6.3 \pm 1.1) \cdot 10^{-5}\,\mathrm{M},$$

$$K_i = (1.3 \pm 0.3) \cdot 10^{-2}\,\mathrm{M},$$

$$k_{cat} = (1.6 \pm 0.3) \cdot 10^{4}\,\mathrm{s}^{-1},$$

$$k_{cat_i} = (1.7 \pm 0.9) \cdot 10^{3}\,\mathrm{s}^{-1}.$$

They represent four relations among the seven rate constants involved in this mechanism. Any set of positive rate constants which satisfies these relations leads upon numerical integration to an activity vs. pS curve which is identical with the corresponding curve obtained by insertion of the fitted parameters K_m, K_i, k_{cat} and k_{cat_i} into the solution of the steady state approximation. This fact indicates that steady state analysis is adequate in this case.

Using the experimental finding that deacetylation is the rate limiting step (Krupka 1964; Froede and Wilson 1980) and the assumption that the dissociation constant of the enzyme-substrate complex is smaller than 10^{-5} M, it is possible to evaluate values or limits for the rate constants involved in mechanism 2:

$$k_1 = (2.6 \pm 0.6) \cdot 10^{8}\,(\mathrm{sM})^{-1},$$

$$k_2 \geq 5.0 \cdot 10^{4}\,\mathrm{s}^{-1},$$

$$k_3 = (2.1 \pm 0.8) \cdot 10^{4}\,\mathrm{s}^{-1},$$

$$k_4 > 1.3 \cdot 10^{5}\,(\mathrm{sM})^{-1},$$

$$k_5 = (1.7 \pm 0.9) \cdot 10^{3}\,\mathrm{s}^{-1}.$$

Thus under the assumptions already mentioned above, it has been demonstrated that rate constants of fast (k_1) and not directly accessible reaction steps (k_5) can

be evaluated by means of least squares analysis of experimental data obtained under steady state conditions.

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