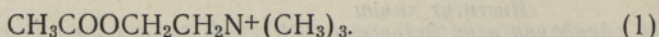УДК 577.153

A. SEPP, A. LANGEL, Y. ROZENGART, J. JÄRV

PRODUCT INHIBITION IN BUTYRYLCHOLINESTERASE REACTION WITH 1,2-DIMETHYL-1-(2'-ACETOXYETHYL)PIPERIDINIUM IODIDE
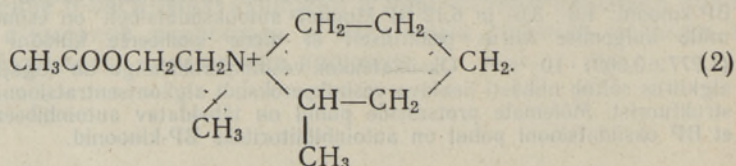
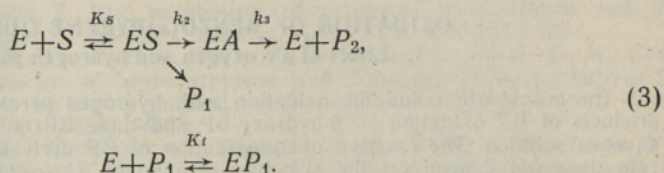*(Presented by O. Eisen)*

Introduction

The hydrophobic binding site for the substrate leaving group in the active center of butyrylcholinesterase (3.1.1.8) can accommodate substituents not longer than the choline residue of the natural substrate acetylcholine (1):

$$CH_3COOCH_2CH_2N^+(CH_3)_3. \tag{1}$$

If the substrate leaving group is longer, the rest of it does not participate in the hydrophobic interaction with the binding site and, therefore, has no effect on the overall binding effectiveness of the substrate molecule in the enzyme active center [1, 2]. At the same time all ammonium compounds which act as reversible inhibitors of cholinesterases seem to locate more freely in the active center as there are no strict spatial limitations for the interaction of their hydrophobic groups with the enzyme. This may complicate experiments with substrates possessing extra bulky substituents in their leaving groups as the product inhibition effect may interfere with the kinetic analysis. In the present study such a strong product inhibition effect has been found in the case of the butyrylcholinesterase-catalyzed hydrolysis of 1,2-dimethyl-1-(2'-acetoxyethyl)piperidinium iodide (2):

$$
CH_3COOCH_2CH_2N^+
\begin{array}{c}
\diagup CH_2{-}CH_2 \diagdown \\
\diagdown CH{-}CH_2 \diagup \\
 | \\
 CH_3
\end{array}
CH_2.
\qquad
\begin{array}{c}
\diagup \\
CH_3
\end{array}
\tag{2}
$$

This substrate has a particularly bulky ammonium group and the alcohol product P_1 of its hydrolysis inhibits the enzyme according to the following scheme:

$$
E+S \overset{K_s}{\rightleftharpoons} ES \overset{k_2}{\rightarrow} EA \overset{k_3}{\rightarrow} E+P_2,
$$
$$
 \searrow
$$
$$
 P_1
\tag{3}
$$
$$
E+P_1 \overset{K_t}{\rightleftharpoons} EP_1.
$$

The competitive mechanism of product inhibition was established by means of the integrated form of the Michaelis-Menten equation [3]:

$$\frac{[P]}{t[E]_0} = \beta + \frac{\alpha}{t[E]_0} \ln \frac{[S]_0}{[S]_0-[P]}, \tag{4}$$

where $[S]_0$ and $[E]_0$ denote the initial concentrations of the substrate and the enzyme, $[P]$ is the product concentration at time t. In this analysis a more complicated calculation procedure was used instead of the linear plot according to Eq. (4). This approach involved adjustment of $[S]_0$ by a non-linear least-squares method and allowed us to obtain a better fit of Eq. (4) with the experimental data than the simple linear plot would have afforded.

Experimental

Horse serum butyrylcholinesterase was obtained from the Mechnikov Institute of Sera and Vaccine, Moscow. The enzyme stock solutions were made in 0.15 M KCl and they can be kept at +4 °C for several days without any change in activity. The operational normality of the solutions was determined by titration with O,O-diethyl-p-nitrophenylphosphate as described in [4]. Acetylcholine iodide (from «Chemapol», Czechoslovakia) was recrystallized from the absolute alcohol-ether mixture. 1,2-dimethyl-1-(2′-acetoxyethyl)piperidinium iodide was obtained from the Institute of Bioorganic Chemistry of the Uzbek SSR Academy of Sciences and is described in [5].

The kinetics of the ester hydrolysis was followed titrimetrically on a «Radiometer» (Denmark) titrigraph RTS in 5—250 ml thermostated vessels at pH 7.5 and 25 °C in 0.15 M KCl solution under argon atmosphere. A 6 mM solution of KOH was used for titration of the acetic acid liberated in the hydrolysis reaction.

The initial rate data for the substrate hydrolysis were analyzed according to the Eadie-Hofstee transformation of the Michaelis-Menten equation [3]:

$$v = k_{cat}[E]_0 - K_m \frac{v}{[S]_0}, \tag{5}$$

where v is the initial rate, $[E]_0$ and $[S]_0$ denote the initial concentrations of the enzyme and the substrate.

Under the pseudo first-order conditions where $[S]_0 \ll K_m$ substrate hydrolysis is described by the kinetics of the first-order process:

$$v = -\frac{d[S]}{dt} = K_I[S], \tag{6}$$

where $k_I = k_{II}[E]_0$. The k_I values were calculated from the first-order kinetic curves by means of the differential method of E. Rudakov [6]. The second-order rate constants of the substrate hydrolysis were obtained from the k_I vs $[E]_0$ plot. At least five different enzyme concentrations were used.

The progress curves of substrate hydrolysis obtained at substrate concentrations around the K_m values were analyzed by Eq. 4. The approach used involved calculation of the approximate α and β values from the $[P]/[E]_0 t$ vs $\frac{1}{t[E]_0} \ln \frac{[S]_0}{[S]_0 - [P]}$ plot. Further the parameter $[S]_0$ was adjusted by the increment $\Delta[S]_0$, which was calculated by means of a non-linear least-squares programme. The new $[S]_0$ value was then used to obtain new values for α and β by the linear regression analysis according to Eq. (4). This procedure for adjustment of the $[S]_0$ value was repeated until the following increment $\Delta[S]_0$ was less than $10^{-3}\%$ of the preceding $[S]_0$ value. The refinement technique for $[S]_0$ was based on taking the partial first derivatives of Eq. (4) as described by G. N. Wilkinson [7] and by W. W. Cleland [8]. Calculation of the α and β values together with adjustment of the $[S]_0$ value was important to obtain the closest fit of the rate

equation with the experimental data, because random errors in the arguments of Eq. (4) would introduce systematic errors in the α and β values [9].

All calculations and the statistical treatment of the kinetic data were performed on an «Искра 1256» personal computer.

Results

Under the conditions commonly used for monitoring the initial rates of the cholinesterase-catalyzed hydrolysis of ester substrates the reaction of butyrylcholinesterase with 1,2-dimethyl-1-(2′-acetoxyethyl)piperidinium iodide deviated remarkably from the zero-order reaction course. These deviations were more noticeable at lower substrate concentrations, where the relative degree of conversion of the substrate into reaction products was high. It has been found that this phenomenon can be avoided by remarkably increasing the volume of the reaction mixture. The latter fact points to the inhibition of the reaction by one of the reaction products. Application of large volumes allows titrimetric monitoring of the acid liberated at a very low conversion degree of the substrate, where the concentration of products is small. In this particular case the zero-order kinetics of the hydrolysis could be followed if 250 ml of the reaction mixture were titrated instead of 5 ml samples. Such an increase in the reaction volume leads to a 50-fold increase in the consumption of the enzyme and the substrate. Moreover, only relatively low reaction rates can be measured titrimetrically in such large volumes owing to low effectiveness of mixing of the sample and the relatively slow equilibration of a large amount of the reaction mixture.

Under the conditions described above the initial rates of the 1,2-dimethyl-1-(2′-acetoxyethyl)piperidinium iodide hydrolysis were measured at 0.1—2.0 mM concentrations and the values of K_m and k_{cat} were estimated from the v vs $v/[s]$ plot shown in Fig. 1. However, for a more complex kinetic analysis of this reaction together with a characterization of the product inhibition effect the progress kinetic curves of the reaction were measured at substrate concentrations around the K_m value. In these experi-
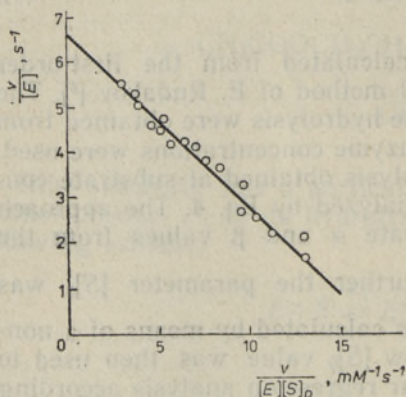

Fig. 1. Initial rate plot of the butyrylcholinesterase-catalyzed hydrolysis of 1,2-dimethyl-1-(2′-acetoxyethyl)piperidinium iodide, 25 °C, 0.15 M KCl, pH 7.5.
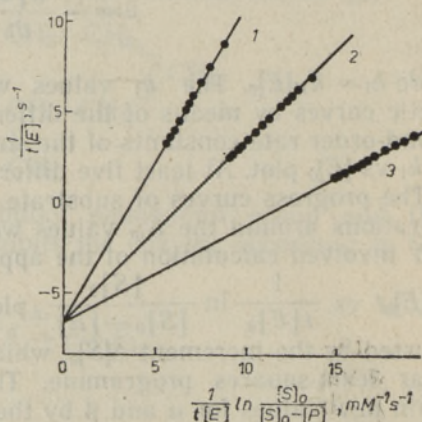
Fig. 2. Integrated rate plot of the butyrylcholinesterase-catalyzed hydrolysis of 1,2-dimethyl-1-(2′-acetoxyethyl)piperidinium iodide, 25 °C, 0.15 M KCl, pH 7.5.
Substrate concentrations:
1 — 0.98 mM; 2 — 0.55 mM; 3 — 0.21 mM.

ments the common 5—15 ml vessels designed for the «Radiometer» pH-stat equipment can be used.

Fig. 2 shows that according to Eq. (4) the plots of the kinetic data in the coordinates $[P]/t[E]_0$ vs $\dfrac{1}{t[E]_0} \ln \dfrac{[S]_0}{[S]_0-[P]}$ provide straight lines with slopes α and intercepts β. The latter value remains constant at different substrate concentrations. This means that the variation of the product concentration during the hydrolysis reaction has no effect on the catalytic rate constant. On the other hand, the α values depend upon $[S]_0$ showing that the effective K_m changes with the increase in the product concentration during the hydrolysis reaction. This interrelationship is a linear one, as shown in Fig. 3.

In summary, the results described above show that the product of the hydrolysis of 1,2-dimethyl-1-(2′-acetoxyethyl)piperidinium iodide has a remarkable inhibitory effect upon the butyrylcholinesterase-catalyzed hydrolysis of this substrate. This inhibition can be regarded as belonging to the competitive type, in the case of which the constants α and β of Eq. (4) have the following meaning:

$$\alpha = -\frac{K_m}{1-K_m/K_i}\left(1+\frac{[S]_0}{K_i}\right),$$

$$\beta = k_{cat}/(1-K_m/K_i).$$

The plot of α vs $[S]_0$ allows the estimation of both constants K_m and K_i. These results, together with k_{cat} obtained from the β value, are listed in the Table.

It should be noted that all the plots shown in Fig. 2 were obtained after computer fitting of the kinetic data to obtain the best values of parameters α, β and $[S]_0$ in Eq. (4), which minimize the sum of the squares of the deviations of the experimental points from this linear equation. Adjustment of the $[S]_0$ values was found to be important according to the F-test and it gave minimal standard deviations, for even small random deviations in this parameter result in systematic changes in both α and β. This aspect of data processing by means of Eq. (4) is discussed more thoroughly in [9]. The plots of α vs $[S]_0$ obtained with and without adjustment of the latter parameter are compared in Fig. 3. It can be seen that the intercept obtained for the latter case has a negative sign and is in conflict with the positive sign of the slope of this plot. The random distribution of the differences between the initial and adjusted values of $[S]_0$ is illustrated in Fig. 4. It can be seen that the maximal values of these differences do not exceed 10—15% and thus remain within the error

Butyrylcholinesterase-catalyzed hydrolysis of 1,2-dimethyl-1-(2′-acetoxyethyl)-piperidinium iodide in 0.15 M KCl at pH 7.5 and 25 °C

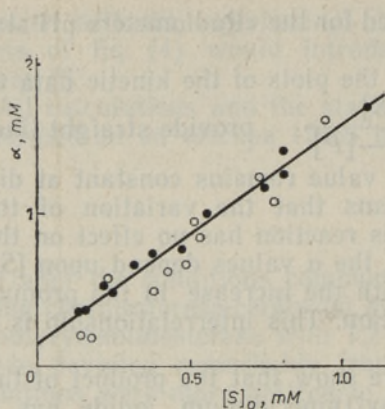| Equation	Conditions	Constants	Constants' values
Integrated rate equation (4)	$[S]_0 \sim K_m$	K_m, mM k_{cat}, s^{-1} K_i, mM	0.41 ± 0.02 6.1 ± 0.3 0.099 ± 0.022
Differential rate equation (5)	$[P] < K_i$	K_m, mM k_{cat}, s^{-1}	0.41 ± 0.04 6.4 ± 0.5
Pseudo first-order conditions	$[S]_0 \ll K_m$ $[P] < K_i$	k_{II}, $M^{-1}s^{-1}$	$(1.0 \pm 0.1) \, 10^4$

Fig. 3. Plot of α vs $[S]_0$ for the integrated form of the rate equation (4), calculated with ($\bullet$) and without ($\bigcirc$) computer adjustment of $[S]_0$.
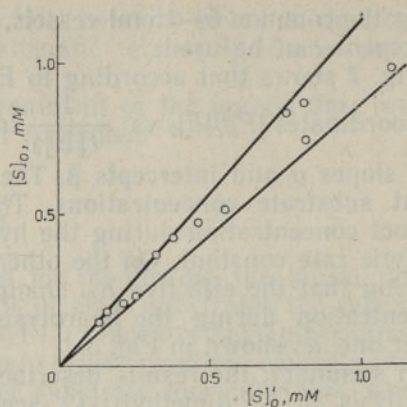
Fig. 4. Comparison of the initial ($[S]_0$) and adjusted ($[S]_0'$) values of the substrate concentrations.

limits of the experiment. These data clearly show that the application of the integrated rate equation requires very high exactness in determining the ligand concentrations [9].

Kinetic experiments of a third type were carried out under the pseudo first-order conditions where $[S]_0 \ll K_m$ and $[P] \ll K_i$. In these kinetic runs also 250 ml of the reaction mixture was titrated to fulfil both the conditions given above. The reactions carried out at different enzyme concentrations followed the first-order reaction course and allowed the application of Eq. (6) for the calculation of the second-order rate constants listed in the Table.

Discussion

Constants K_m and k_{cat} for 1,2-dimethyl-1-(2'-acetoxyethyl)piperidinium iodide can be compared with constants K_s and k_2 of scheme (3) by making use of the deacetylation rate constant $k_3 = 840$ s^{-1} for butyrylcholinesterase reaction with acetic esters [1]. Comparison of the latter value with the kinetic parameters in the Table shows that $K_m = K_s$ and $k_{cat} = k_2$ for the piperidinium derivative of acetylcholine, while for the parent compound constants $k_2 = 1.5 \cdot 10^3$ s^{-1} and $K_s = 2.5$ nM can be calculated from data in [1]. It can be seen that the binding effectiveness of substrates (1) and (2) is close in spite of the remarkable difference in their hydrophobicity and agrees with the idea of a limited range of the appropriate binding site. At the same time, however, the alcohol product of the enzymatic hydrolysis of the piperidinium derivative binds with the enzyme approx. four times more effectively than the substrate itself. It can be noted that in the case of non-productive binding of substrates in the enzyme active center the experimental values of K_m (and K_s) are lower than the actual substrate constant. As a result of this the difference between the binding effectiveness of the substrate and that of the product will become still greater.

In summary, the present results confirm that reliable kinetic parameters for extra bulky substrates can be obtained by taking into account the product inhibition effect, preferably by means of the progress curve analysis. The importance of the product inhibition effect arises from the peculiarities of the structure-activity relationships in the case of butyryl-cholinesterase substrates and reversible inhibitors.
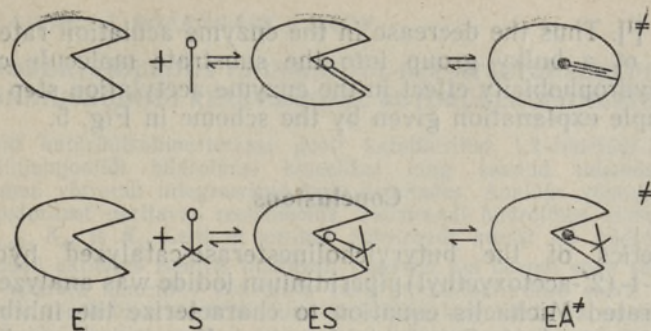
Fig. 5. Schematic representation of the non-covalent binding step and the following enzyme acylation step for «normal» (upper scheme) and «bulky» (lower scheme) substrates. Closing of the hydrophobic slit stabilizes additionally the activated complex of the bond-breaking step.

The results obtained are also important for improving our previous results on structure-activity relationships [1]. It appears that introduction of an extra bulky ammonium group into the substrate molecule remarkably reduces the rate of the enzyme acylation step in comparison with similar data for acetylcholine. At the same time the electrophilicity of the carbonyl carbon in esters (1) and (2) remains practically unchanged. As the binding effectiveness of these substrates is similar, the different rate constants k_2 must refer to some special stereochemical factor governing the bond-breaking step. This factor should be sensitive to the branching of a substituent at a long distance from the reaction center and thus differ essentially from the steric hindrance at the reaction center quantified by the Taft steric constants E_s (E_s^0) [10].

A more thorough structure-activity analysis performed earlier [1] has shown that hydrophobicity of the substrate leaving group is also revealed in the acylation step of the butyrylcholinesterase-catalyzed reaction. This phenomenon can be described by the correlation equation [1]:

$$\log k_2 = \log k_2^0 + \varrho^* \sigma^* + \varphi_a \pi, \qquad (7)$$

where the terms $\varrho^* \sigma^*$ and $\varphi_a \pi$ describe the contributions of the inductive and hydrophobic effects of the substrate leaving group. Formally this equation means that the «second» hydrophobic sorption of the substrate molecule occurs after the non-covalent ES-complex formation (see the scheme (3)). This double effect of hydrophobicity has been explained by a conformational change accompanying the bond-breaking step and consisting in the shutting of a hydrophobic «slit» in which the substrate is located (Fig.5) [11]. According to this hypothesis it can be expected that very bulky substituents of substrate molecule will not fit into the «closed slit» of the hydrophobic binding site, and that will destabilize the activated complex of the acylation step (Fig. 5).

If this is the case for the piperidinium derivative (2), the difference between the $\log k_2$ values for this substrate and acetylcholine should be equal to the hydrophobicity increment $\varphi_a \pi$ in Eq. (7). Making use of the acetylation rate constants k_2 given above for substrates (1) and (2), $\Delta \log k_2 = 2.3$ can be found. The hydrophobicity of the choline residue can be characterized by the relative hydrophobicity constant $\pi^* = 2.5$, calculated additively [10] without taking into account the ammonium atom, as this is a common structural element in both substrates. These $\Delta \log k_2$ and π^* values give $\varphi_a = 0.9$, which is close to $\varphi_a = 0.7 \pm 0.2$, earlier found for the acylation step of the butyrylcholinesterase-catalyzed hydrolysis of

acetic esters [1]. Thus the decrease in the enzyme aculation rate due to the introduction of a bulky group into the substrate molecule corresponds well to the hydrophobicity effect in the enzyme acetylation step and agrees with the simple explanation given by the scheme in Fig. 5.

Conclusions

The kinetics of the butyrylcholinesterase-catalyzed hydrolysis of 1,2-dimethyl-1-(2′-acetoxyethyl)piperidinium iodide was analyzed by means of the integrated Michaelis equation to characterize the inhibitory effect of the reaction product. Constants k_{cat} and K_m as well as the enzyme-product complex dissociation constant K_i were determined. It was shown that the hydrolysis of the substrate was strongly interfered with by-product inhibition, as the binding effectiveness of the substrate molecule with the enzyme active center was considerably lower than that of the alcohol produced by the ester hydrolysis. The results obtained by means of the integrated Michaelis equation are in agreement with the second-order rate constants determined under the pseudo first-order conditions. It was found that the introduction of the bulky piperidinium substituent into the ammonium group of the substrate reduced more than 200-fold the enzyme acylation rate constant as compared with acetylcholine. The substrate constant K_s was close to that for acetylcholine. The results are analyzed with reference to the structure-activity relationships for the substrates and inhibitors of butyrylcholinesterase and some implications of the structure and action mechanism of the enzyme active center discussed.

REFERENCES

1. *Ярв Я. Л., Лангел Ю. Л.* Эффекты отщепляемой группы субстратов в реакции с бутирилхолинэстеразой. — Биоорг. хим., 1979, 5, № 5, 746—755.
2. *Järv, J.* Stereochemical aspects of cholinesterase catalysis. — Bioorganic Chemistry, 1984,12, 259—278.
3. *Cornish-Bowden, A.* Principles of Enzyme Kinetics. London, 1976, 142—152.
4. *Ярв Я. Л., Аавиксаар А. А., Годовиков Н. Н., Лангел Ю. Л., Паст Н. Б.* Ацетил-холинэстераза яда кобры. Определение концентрации активных центров. — Биохимия, 1976, 41, № 5, 827—835.
5. *Жоров Б. С., Розенгарт Е. В., Говырин В. А., Хромов-Борисов Н. В., Бровцына Н. Б.* Теоретический конформационный анализ некоторых субстратов холинэстеразы с циклическим строением аммониевой группировки. — Докл. АН СССР, 1976, 231, 215—218.
6. *Рудаков Е. С.* Дифференциальные методы расчета констант скорости несложных химических процессов. — Кинетика и катализ, 1960, 1, № 1, 177—187.
7. *Wilkinson, G. N.* Statistical estimations in enzyme catalysis. — Biochem. J., 1961, 80, 324—332.
8. *Clealand, W. W.* The statistical analysis of enzyme kinetic data. — Adv. Enzymol., 1967, 29, 1—32.
9. *Atkins, G. L.* The reliability of Michaelis constants and maximum velocities estimated by using the integrated Michaelis-Menten equation. — Biochem. J., 1973, 135, 779—784.
10. *Hansch, C., Leo, A. J.* Substituent Constants for Correlation Analysis in Chemistry and Biology. London—Toronto—New York, 1979, 9—17.
11. *Aaviksaar, A., Paris, J., Palm, V.* The relationship between catalysis and binding in chymotrypsin reactions. — Organic Reactivity, 1971, 8, 817—832.

Tartu State University

I. M. Sechenov Institute
of Evolutionary Physiology and Biochemistry

A. SEPP, A. LANGEL, E. ROZENGART, J. JÄRV

PRODUKTPIDURDUS 1,2-DIMETÜÜL-1-(2′-ATSETOKSÜETÜÜL)-PIPERIDIINIUMJODIIDI REAKTSIOONIL BUTÜRÜÜLKOLIINESTERAASIGA

On uuritud butürüülkoliinesteraasi poolt katalüüsitud 1,2-dimetüül-1-(2′-atsetoksü-etüül)-piperidiiniumjodiidi hüdrolüüsi kineetikat ning saadud tulemusi analüüsitud Michaelis-Menteni võrrandi integreeritud kuju kasutades. Analüüs võimaldas iseloomustada produktpidurdust uuritavas reaktsioonis. Substraadi hüdrolüüsi kineetikast määrati konstandid k_{cat}, K_m ja K_i. Leiti, et uuritud substraadi korral on hüdrolüüsi produkti sidumine ensüümi aktiivses tsentris tunduvalt tugevam kui ühend enda sidumine. Seoses sellega saab määrata kineetilisi konstante koos produktpidurduse efekti arvestamisega. Saadud andmete võrdlemine atsetüülkoliini hüdrolüüsi kineetiliste parameetritega võimaldab arutada butürüülkoliinesteraasi aktiivtsentri funktsioneerimise mõningaid iseärasusi.

А. СЕПП, А. ЛАНГЕЛ, Е. РОЗЕНГАРТ, Я. ЯРВ

ТОРМОЖЕНИЕ ПРОДУКТОМ В РЕАКЦИИ ГИДРОЛИЗА 1,2-ДИМЕТИЛ-1-(2′-АЦЕТОКСИЭТИЛ)-ПИПЕРИДИНИЙ ИОДИДА БУТИРИЛХОЛИНЭСТЕРАЗОЙ

Измерена кинетика катализируемого бутирилхолинэстеразой гидролиза 1,2-диметил-1-(2′-ацетоксиэтил)-пиперидиний иодида и определен на основе полученных данных эффект торможения реакции продуктом по интегральному уравнению Михаэлиса—Ментена. Определены константы k_{cat}, K_m и K_i гидролиза этого субстрата. Показано, что эффективность связывания продукта с активным центром фермента превышает эффективность связывания субстрата, и определение значимых кинетических параметров возможно только при одновременном учете торможения продуктом. Полученные данные сопоставлены с кинетическими параметрами гидролиза ацетилхолина и с представлениями о механизме функционирования активного центра бутирилхолинэстеразы.