New Findings about Ellman's Method to Determine Cholinesterase Activity

Alena Komersová[a,*], Karel Komers[a], and Alexandr Čegan[b]

[a] Department of Physical Chemistry, Faculty of Chemical Technology, University of Pardubice, Nám. Legií 565, 532 10 Pardubice, Czech Republic. Fax: +420466037068. E-mail: alena.komersova@upce.cz

[b] Department of Biological and Biochemical Sciences, University of Pardubice, Štrossova 239, 53002 Pardubice, Czech Republic

* Author for correspondence and reprint requests

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The original Ellman's spectrophotometrical method for cholinesterase activity determination uses 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) as a chromogen and records the level of cholinesterase activity as an increase of absorbance at 412 nm. Although this procedure usually poses no problem, exceptions arise when the concentration of DTNB is far higher than the concentration of acetylthiocholine (ATCH). It was found that the ratio of concentrations of DTNB/ATCH is an important parameter for the ATCH hydrolysis course: high excess of DTNB decreases the hydrolysis rate resulting in a lower measured enzyme activity. Our experiments indicate that this influence of DTNB concentration can be explained by the inhibition of ATCH hydrolysis by DTNB.

Key words: Ellman's Method, Acetylthiocholine, Hydrolysis

Introduction

Enzymatic hydrolysis of acetylcholine (ACH) is an important step in the nerve transport (Giacobini, 2000), and its defect course is considered to be one of the possible reasons of Alzheimer disease. Ellman's procedure (Ellman *et al.*, 1961) is commonly used for the determination of cholinesterase activity and also for monitoring of the ACH hydrolysis by acetylcholinesterase (ACHE) or butyrylcholinesterase (BCHE) *in vitro*. In these experiments acetylthiocholine (ATCH) is disposed instead of ACH because its similar qualitative kinetic behaviour provides on-line monitoring of the hydrolysis by the Ellman's method. Ellman's procedure can be applicated also for the determination of cholinesterase activity of tissue extracts, homogenates, cell suspensions etc. but the procedure is disturbed by high hemoglobin absorption at 412 nm. Therefore modified methods were developed (Worek *et al.*, 1999; Willig *et al.*, 1996).

The original Ellman's colorimetric procedure is based on the reaction of thiocholine (one of the products of enzymatic hydrolysis of ATCH) with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) forming a yellow product (5-mercapto-2-nitrobenzoic acid and its dissociated forms) at pH 8, see reaction (1). The maximum absorption coefficient was found at 412 nm.

As the substrate is hydrolyzed the increase of absorbance at 412 nm is recorded, and the activity of the enzyme calculated as the reaction rate from the slope of the linear part of the time-dependence of the absorbance. Reaction (1) is sufficiently rapid and thus not rate-limiting in the enzymatic hydrolysis.

Ellman's method presents a frequently used method for the determination of the ACHE and BCHE activity and the continuous monitoring of the enzymatic hydrolysis of ATCH. We have dealed in detail with the influence of the DTNB concentration on the reaction course.

In the original Ellman's paper the following stoichiometry has been accepted:

$$\mathrm{RSSR} + \mathrm{XSH} \xrightarrow{k_a} \mathrm{RSH} + \mathrm{RSSX}, \qquad (1)$$

where RSSR is Ellman's reagent, XSH is thiocholine, RSH is the measured yellow product (5-mercapto-2-nitrobenzoic acid) and RSSX is the compound consisting of one half of RSSR and thiocholine.

The mechanism of the colorimetric reaction of thiocholine with Ellman's reagent has been studied in detail (Komers *et al.,* 2003) and it was found that this mechanism is more complex than acknowledged so far. Experimental facts, *i.e.* the

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stoichiometric ratio [XSH]/[RSSR] = 2 (determined by spectrophotometric titration curves of XSH and RSSR at 412 nm), can be rationally explained by the consecutive reactions (1) and

$$\mathrm{RSSX + XSH} \xrightarrow{k_b} \mathrm{RSH + XSSX} \qquad (2)$$

with the condition $k_a \ll k_b$. For similar (the same order) values of rate constants, $k_a \approx k_b$, the absorbance of the reaction mixture would not be proportional to the thiocholine concentration.

The hydrolysis of ATCH (ACH) by cholinesterases can be described (Zdražilová *et al.,* 2006) by the reaction course

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \xrightarrow{k_2} \mathrm{P + HA + E}, \qquad (3)$$

where E is the enzyme (BCHE or ACHE), S the substrate (ATCH or ACH), ES the complex substate-enzyme which decomposes into the product of hydrolysis P [thiocholine (TCH) or choline (CH)] and acetic acid (HA).

Michaelis-Menten equation describing the decrease of S or production of P and HA for this enzymatic hydrolysis is

$$v = \frac{\mathrm{d[P]}}{\mathrm{d}t} = \frac{\mathrm{d[HA]}}{\mathrm{d}t} = -\frac{\mathrm{d[S]}}{\mathrm{d}t} = \frac{V_m\,[\mathrm{S}]}{K_M + [\mathrm{S}]}, \qquad (4)$$

where v is the actual and V_m the maximum reaction rate under given conditions and K_M the Michaelis constant of the given reaction.

Irreversibly inhibited hydrolysis of the substrate with a reversible binding step and irreversible catalytic step follows reaction scheme (5), where I is the inhibitor and EI the enzyme-inhibitor complex:

$$\begin{array}{l} \mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{ES} \xrightarrow{k_2} \mathrm{P + HA + E} \\ +\mathrm{I} \downarrow k_3 \\ \mathrm{EI} \end{array} \qquad (5)$$

Material and Methods

Chemicals

Acetylcholinesterase (EC. 3.1.1.7, ACHE), lyophilizate from electric eel, kept at −20 °C, acetylthiocholine iodide (ATCH), kept at 5 °C, and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), kept at laboratory temperature, were all from Sigma-Aldrich. Phosphate buffer, pH 8.

Analytical solutions

ACHE preparation

The original lyophilizate was dissolved in the phosphate buffer, pH 8, and this stock solution was kept at −20 °C. A suitable amount of the stock solution was defrosted each working day and ACHE activity was measured.

0.01 M DTNB and 0.01 M acetylthiocholine iodide stock solutions

Appropriate amounts of the substances were dissolved in phosphate buffer, pH 8, and these solutions were kept at 5 °C not longer than 2–3 d. The suitable working concentrations of DTNB and acetylthiocholine iodide were prepared immediately before use by dilution with buffer solution.

Methods and apparatus

The ACHE stock solution activity and kinetics of ATCH hydrolysis were measured by the Ellman's method at pH 8, ionic strength I = 0.11 M (ensured by concentration of buffer) and 25 °C using a Hewlett-Packard spectrometer 8453 A with a diode array detector. A thermostated glass cuvette with the maximum volume of 30 ml and optical path of 2 cm, equipped with a glass propeller, was used as the reactor. Initial concentration of ATCH was in all cases $[\mathrm{ATCH}]_0$ = 0.16 mM, and $[\mathrm{DTNB}]_0$ = 0.2 mM in the ACHE activity determination and various in the kinetics of ATCH hydrolysis determination (see Results and Discussion). Every working day at first the actual value of ACHE preparation activity (in U units) was determined and based on this value, such volume of the ACHE preparation was used (for all ATCH hydrolyses) which ensured the chosen initial ACHE activity of 3.5 U in the reaction mixture. The activity of 1 U (unit) includes such amount of ACHE preparation which converts 1 μmol of the substrate in 1 min.

Suitable volumes of buffer, pH 8, ACHE and DTNB stock solutions were mixed in the cuvette and thermostated 5 min at 25 °C. Then a suitable volume of ATCH stock solution was added under vigorous mixing. This moment was taken as the zero time of the hydrolysis (t = 0). The total volume of the reaction mixture was always 25 ml; initial concentrations of ATCH and DTNB are given in Tables I and II. During the whole reaction course the dependence of absorbance A (412 nm) vs. time t was continually measured.

Results and Discussion

The dependences A (412 nm) *vs.* t were continually measured at pH 8 and 25 °C in the reaction mixtures including constant initial concentrations of buffer, ATCH (0.16 mM), ACHE (3.5 U) and various initial concentrations of DTNB (0.2 to 0.717 mM). The A (412 nm) values were overcalculated to actual TCH concentrations based on Lambert-Beer's law: A (412 nm) = ε (412 nm) · [TCH] · d, using ε (412 nm) = 14150 (M cm)$^{-1}$ and d = 2 cm. All so obtained [TCH] *vs.* t dependences were tested for validity of the Michaelis-Menten equation (4) in Briggs-Haldane modification (V_m = k_2 · [ACHE]$_0$; K_M = $(k_{-1} + k_2)/k_1$) by the PC program GEPASI (Zdražilová *et al.,* 2006). In positive cases the optimal values of k_1, k_{-1}, k_2, [ACHE]$_0$ and the corresponding standard deviation (SD) were obtained.

The kinetic experiments mentioned above have shown the influence of the DTNB concentration on the reaction course of ATCH hydrolysis. From Table I it follows, that for [DTNB]$_0$ = 0.2 to 0.598 mM (ratio [DTNB]$_0$/[ATCH]$_0$ = 1.25 to 3.74) the ATCH hydrolysis fulfils equation (4) and the hydrolysis rate is only slightly affected by [DTNB]$_0$. Any concentration of DTNB from this range can be used for enzyme activity determinations and the same resultes are obtained. But initial DTNB concentrations higher than 1.6 mM ([DTNB]$_0$/[ATCH]$_0$ > 10, see Table II) markedly decrease the rate of hydrolysis, the TCH production (as shown in Fig. 1), and thereby also the measured enzyme activity. In this DTNB concentration range, the ATCH hydrolysis does not follow equation (4). It appears that decreasing of the reaction rate with increasing concentration of DTNB is the consequence of ACHE inhibition by DTNB. Probably DTNB can attach the enzyme molecules and therefore, at higher concentrations of DTNB, hydrolyses of ATCH by ACHE do not follow the Michaelis-Menten reaction mechanism. We studied the kinetic model of enzyme-catalyzed reaction in the presence of DTNB as irreversible inhibitor [see (5)], and it was found that all measured hydrolyses with ratio [DTNB]$_0$/[ATCH]$_0$ = 10 to 44.8 follow this kinetic model. As can be seen from Table II, if the ratio [DTNB]$_0$/[ATCH]$_0$ = 10 to 27.4 then the binding step (formation of ES complex) is irreversible, but if [DTNB]$_0$/[ATCH]$_0$ > 29.9 the rate constant k_{-1} increases and the binding step is again reversible.

Table I. Mean optimal values and standard deviations of rate constants k_i and initial enzyme concentration [E]$_0$ according to (3) for ATCH hydrolyses with soft excess of DTNB.

[DTNB]$_0$ × 10^{-4} [M]	[DTNB]$_0$/[ATCH]$_0$	$k_1 \times 10^{-5}$ [M^{-1} s^{-1}]	k_{-1} [s^{-1}]	k_2 [s^{-1}]	[E]$_0$ × 10^8 [M]
2.00	1.25	1.316 ± 0.19	4.46 ± 1.81	17.95 ± 3.82	8.27 ± 0.83
2.79	1.74	1.288 ± 0.24	5.073 ± 2.65	17.21 ± 3.63	8.12 ± 0.76
3.60	2.25	1.201 ± 0.15	6.286 ± 2.96	16.75 ± 3.51	7.94 ± 0.78
4.38	2.74	1.326 ± 0.23	4.186 ± 2.14	17.43 ± 3.24	8.23 ± 0.87
5.18	3.24	1.246 ± 0.19	6.116 ± 2.81	17.26 ± 3.46	8.02 ± 0.86
5.98	3.74	1.251 ± 0.13	5.436 ± 2.77	16.96 ± 3.37	8.05 ± 0.79

Table II. Mean optimal values and standard deviations of rate constants k_i and initial enzyme concentration [E]$_0$ according to (5) for ATCH hydrolyses with high excess of DTNB.

[DTNB]$_0$ × 10^{-3} [M]	[DTNB]$_0$/[ATCH]$_0$	$k_1 \times 10^{-5}$ [M^{-1} s^{-1}]	k_{-1} [s^{-1}]	k_2 [s^{-1}]	k_3 [M^{-1} s^{-1}]	[E]$_0$ × 10^8 [M]
1.6	10.0	1.607 ± 0.33	–	13.36 ± 4.23	5.66 ± 1.70	8.39 ± 0.83
2.39	14.9	1.618 ± 0.37	–	13.31 ± 3.86	5.76 ± 1.62	8.28 ± 0.91
3.19	19.9	1.688 ± 0.39	–	12.82 ± 3.91	5.56 ± 1.68	8.16 ± 0.84
4.0	25.0	1.86 ± 0.48	–	10.68 ± 3.44	6.63 ± 1.83	7.81 ± 0.97
4.78	29.9	1.707 ± 0.46	0.034 ± 0.011	9.955 ± 3.12	7.48 ± 1.94	7.86 ± 0.84
5.58	34.9	1.720 ± 0.41	1.27 ± 0.36	9.149 ± 3.67	8.02 ± 2.11	7.68 ± 0.79
6.38	39.9	1.258 ± 0.45	4.81 ± 1.41	10.79 ± 3.93	7.07 ± 1.84	7.52 ± 0.92
7.17	44.8	1.307 ± 0.44	4.50 ± 1.53	8.58 ± 3.62	7.33 ± 1.88	7.39 ± 0.90

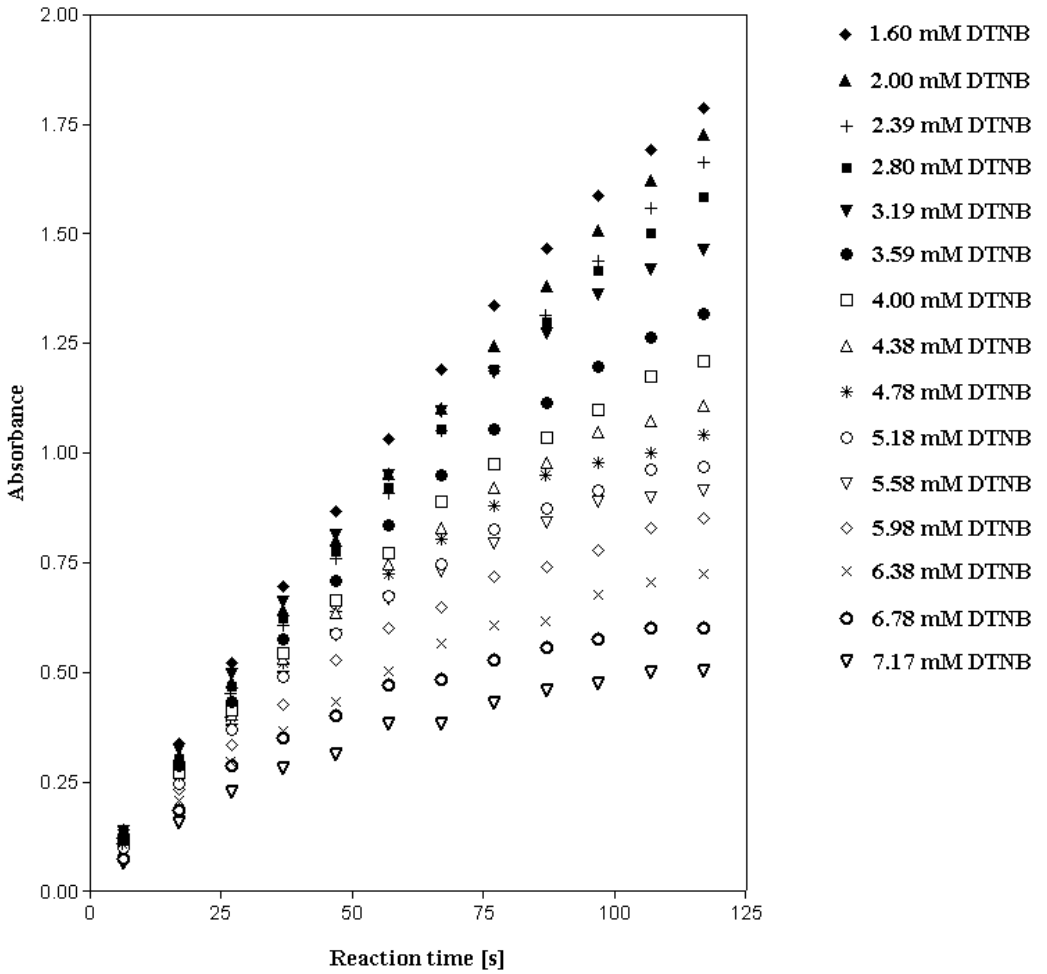

Fig. 1. Hydrolysis of ATCH by ACHE. Initial concentrations: $[ATCH]_0$ = 0.16 mM, $[DTNB]_0$ = 1.6 to 7.17 mM ($[DTNB]_0/[ATCH]_0$ = 10.0 to 44.8); initial ACHE activity: 3.5 U, phosphate buffer, pH 8; ionic strength: I = 0.11 M; 25 °C.

From the performed experiments it cannot be definitely decided, whether the high excess of DTNB affects the ACHE activity (as an inhibitor) or decreases the concentrations of ATCH or TCH, or operates in a complex way. But it is sure that high excess of DTNB in the reaction mixture markedly falsifies the true reaction course of the studied hydrolyses.

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