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COMMENT ON COLORIMETRIC MONITORING OF ENZYMATIC HYDROLYSIS OF ACETYLTHIOCHOLINE

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The mechanism of the colorimetric reaction of thiocholine with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, RSSR) has been studied. It was found that its mechanism is more complex than acknowledged so far and conforms to two consecutive competitive steps (2) and (3) with the condition for the rate constants $k_2 \gg k_1$. *The applicability of this reaction to the very frequent on-line monitoring of the enzymatic hydrolysis of acetylthiocholine fortunately has not changed. The determined value of absorption coefficient of the measured yellow substance at pH = 8 (5-mercapto-2-nitrobenzoic acid, RSH) is* $\varepsilon(412) = 14950 \pm 840\ M^{-1}\ cm^{-1}$.

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Introduction

The enzymatic hydrolysis of acetylcholine by acetylcholinesterase and/or butyrylcholinesterase plays an important role in the timely transport of the nerve emotions in the human brain [1]. Their defect course is considered to be one of the possible reasons of Alzheimer disease. In the studies of this reaction *in vitro* acetylthiocholine ($XSCOCH_3$) is often used instead of acetylcholine, because the course of hydrolysis can easily be monitored continuously (on-line) by the Ellman colorimetric method [2]. It is based on the fact that thiocholine (XSH) produced in the reaction

$$(CH_3)_3N^+CH_2CH_2S.COCH_3 + H_2O + E \rightarrow (CH_3)_3.N^+CH_2CH_2SH + CH_3COOH + E \quad (1)$$

briefly: $XSCOCH_3 + H_2O + E \rightarrow XSH + CH_3COOH + E$

(E is acetylcholin- or butyrylcholinesterase) reacts immediately and irreversibly with the present surplus of 5,5´dithiobis-2-nitrobenzoic acid (RSSR), forming quantitatively a yellow product with the absorbance maximum at 412 nm. Since the time of publishing of the original Ellman´s paper [2] the following stoichiometry has been accepted

$$RSSR + XSH \rightarrow RSH + RSSX \quad (2)$$

where the measured yellow product is considered to be the 5-mercapto-2-nitrobenzoic acid (RSH) and its dissociated forms at pH = 8 and temperature 25 °C. We found in the literature the following values of the absorption coefficient of this yellow substance: ε(412) = 13600 [2], 14150 [3], ε(405) = 13300 [4], 13600 M^{-1} cm^{-1} [5]. Experimental verification of these values by means of the spectrophotometric curves did not give the same value of ε, when the RSSR was added to a surplus of XSH or *vice versa*. In the first case the calculated value of ε was practically twice as great as the literature values. This very well reproducible result indicates a more complicated course of the colour reaction between XSH and RSSR.

For several decades this reaction has provided the basis of a reputable and

frequently used method of continual monitoring of the enzymatic hydrolysis of acetylthiocholine and its inhibition. Therefore we have dealed in more detail with the course of this determination and we are offering the following interpretation of the quantitative experimental facts.

Materials and Methods

Chemicals

Acetylthiocholine iodide: *Sigma-Aldrich,* Praha, CZ, kept at 5 °C. From this substrate a fresh aqueous analytical solution ca 10^{-3} M was used for the preparation of thiocholine analytical solution.

5,5′-dithiobis-2-nitrobenzoic acid: *Sigma-Aldrich*, Praha, CZ, kept at laboratory temperature. From this substance the analytical aqueous solution ca 10^{-3} M was prepared, kept at 5 °C and used for the colorimetric titrations.

Butyrylcholinesterase (BCHE) preparation: hydrolysate from the horse plasma in pellets ca 6 g, kept at 5 °C. One pellet was dissolved in 100 ml of demi water.

Buffer: Sőrensen′s phosphate buffer pH 8.0, concentration 0.07 M, ionic strength I = 0.196 M (adjusted by NaCl 0.1 M) was used.

Thiocholine: A mixture of 11.9 ml of acetylthiocholine solution ca 10^{-5} M with 0.1 ml of BCHE preparation in the buffer (total volume 12 ml) gave, after 20 minutes, the solution with equivalent analytical concentration of XSH, which was used for the colorimetric titrations.

Methods and Apparatus

The colorimetric titration curves were measured at pH = 8 (phosphate buffer) and temperature 25 °C by means of a diode-array spectrograph HP 8452A, Hewlett-Packard, USA. A thermostated glass cuvette (25 °C) with the maximum volume of 30 ml and optical path 2 cm, equipped with a glass propeller, was used as the reactor. The cuvette was filled with the chosen volumes of buffer and solution of RSSR (or XSH). This mixture was titrated with the analytical solution of XSH (or RSSR) as the titrant. After every addition of the titrant the equilibrium absorbance A (412 nm) was measured.

Experimental Facts

Two spectrophotometric titration curves were measured. The first one was obtained by the titration of the (constant) initial concentration of RSSR ($[RSSR]_0 = a$) by the volumetric solution of thiocholine. The second one was

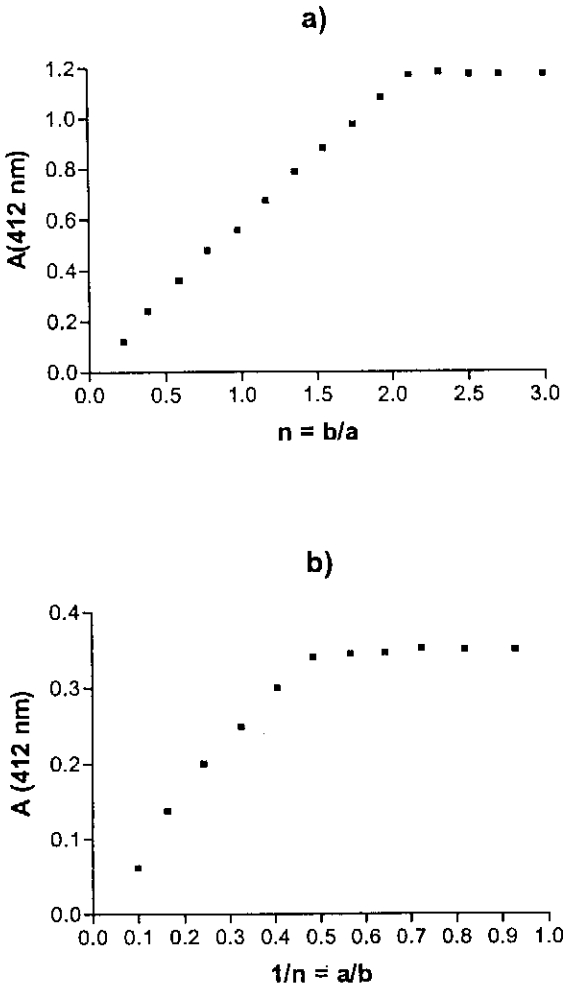

Fig. 1 a) Colorimetric titration curve absorbance A (412 nm) vs. ratio $n = b/a$. Initial concentration of Ellman's reagent $[RSSR]_0 = a = 2.126\times10^{-5}$ M, b = concentration of titrant thiocholine [XSH].
b) Colorimetric titration curve A (412 nm) vs. ratio $1/n = a/b$. Initial concentration of thiocholine $[XSH]_0 = b = 1.084\times10^{-5}$ M, a = concentration of titrant Ellman's reagent [RSSR]. Optical path $d = 2$ cm

obtained by the titration of the initial concentration of thiocholine ($[XSH]_0 = b$) by the volumetric solution of RSSR.

An example of the first curve is expressed graphically in Fig. 1a as the dependence of the absorbance ($A = \varepsilon d[RSH]$) of the equilibrium reaction mixture on the ratio of the initial concentrations of XSH and RSSR, $n = b/a$, i.e. A vs. n. The curve is formed by two straight lines crossing at $n = 2$.

The second curve is the dependence A vs. $1/n = a/b$ (For example see Fig. 1b) and consists also from two lines crossing at $1/n = 0.5$.

If the colour reaction between RSSR and XSH proceeded according to the scheme (2), i.e. based on the presumption presented in [2], both colorimetric curves would to be identical with the intercept at $n \equiv 1/n = 1$. This condition is not fulfilled and, therefore, the reaction of RSSR with XSH must be more complicated.

Interpretation

The obtained experimental facts can be explained by the system of two consecutive competitive irreversible reactions

$$RSSR + XSR \xrightarrow{k_1} RSH + RSSX \qquad (2)$$

$$RSSX + XSR \xrightarrow{k_2} RSH + XSSX \qquad (3)$$

where k_1, k_2 are the rate constants of the steps (2) and (3), respectively, and the supposed structure of XSSX is $(CH_3)_3N^+CH_2CH_2-S-S-CH_2CH_2N^+(CH_3)_3$. The existence of the this particle is supported by the paper [6]. The stoichiometry of the summary reaction

$$RSSR + 2XSH \rightarrow XSSX + 2RSH \qquad (4)$$

i.e. [RSSR] : [XSH] = 1 : 2 corresponds with both presented experimental curves

providing RSH as the only coloured (yellow) substance in the reaction mixture. Generally, the form of the equilibrium titration curves of a reaction is dictated also by its mechanism and kinetics [7]. Analysis of the differential kinetic equations describing the course of the reactions (2) and (3) gives the following common balance relations

$$a = [\mathrm{RSSR}] + [\mathrm{RSSX}] + [\mathrm{XSSX}] \tag{5}$$

$$b = [\mathrm{XSH}] + [\mathrm{RSH}] \tag{6}$$

$$[\mathrm{RSH}] = [\mathrm{RSSX}] + 2[\mathrm{XSSX}] \tag{7}$$

where the terms in brackets are the equilibrium concentrations of the reaction components that are determined also by the values of rate constants k_1 and k_2. At their comparable values ($k_1 \approx k_2$) the dependence [RSH] vs. n and therefore also the corresponding colorimetric curve A vs. n need not be expressed by straight lines. But if $k_2 \gg k_1$, then for $n < 2$ the following more simple balances are valid

$$a = [\mathrm{RSSR}] + [\mathrm{XSSX}] \tag{8}$$

$$b = [\mathrm{RSH}] + 2[\mathrm{XSSX}] \tag{9}$$

because thiocholine and the „mixed" substance RSSX cannot be present in the subequivalent equilibrium reaction mixture due to the very fast step (3).

The spectrophotometric titration curve A vs. n (i.e. titration of a constant initial concentration $[\mathrm{RSSR}]_0 = a$ by $[\mathrm{XSH}] = b$) fulfils for n (0; 2), according to (9), the equation of the increasing line

$$A = \varepsilon d[\mathrm{RSH}] = \varepsilon d b = \varepsilon d a \frac{b}{a} = \text{const.}\, n \tag{10}$$

The overequivalent part of the spectrophotometric curve ($n > 2$) is formed by the horizontal line given by the expression $A = \text{const.}\, 2$.

Unfortunately, the opposite unequality of the rate constants $k_1 \gg k_2$ gives, after similar analysis, the same form of the spectrophotometric curve A vs. n. Hence, the cases $k_2 \gg k_1$ and $k_1 \gg k_2$ are in this way undistinguishable.

The difference between both mechanisms is revealed by means of the spectrophotometric titration curve A vs. $1/n$, i.e. titration of $[XS]_0 = b$ by [RSSR] = a.

On condition $k_2 \gg k_1$ it holds for subequivalent ($1/n \leq 0.5$) equilibrium states

$$[RSH] = 2a = 2b\frac{a}{b} = 2b\frac{1}{n} \tag{11}$$

Then, for $1/n$ (0; 0.5) the theoretical spectrophotometric titration curve A vs. $1/n$ has the form

$$A = \varepsilon d[RSH] = 2\varepsilon da = 2\varepsilon db\frac{a}{b} = \text{const}'.\left(\frac{1}{n}\right) \tag{12}$$

For $1/n > 0.5$ the A value remains constant and it is $A = \text{const}'.0.5$.

On the condition $k_1 \gg k_2$ the step (3) can run only after the step (2) is completed. Therefore, the dependence [RSH] vs. $1/n$ increases linearly in the interval $1/n$ (0; 1). In this interval the theoretical spectrophotometric titration curve A vs. $1/n$ has the form

$$A = \varepsilon d[RSH] = \varepsilon da = \varepsilon db\frac{a}{b} = \varepsilon db\frac{1}{n} = \text{const}''.\frac{1}{n} \tag{13}$$

For $1/n > 1$ the A value remains constant and it holds $A = \text{const}''$.

Results and Discussion

The experimental curves A vs. n and A vs. $1/n$, presented in Figs 1a and 1b, conform to the reaction scheme of two consecutive competitive reactions (2), (3) with the condition $k_2 \gg k_1$. Thanks to validity of this condition the continuous monitoring of the hydrolysis of acetylthiocholine by means of determination of the product thiocholine with Ellman´s reagent is possible. For comparable values of both rate constants the equilibrium absorption A of the reaction mixture would not be proportional to the thiocholine concentration.

The average value (from three measurements for every type of curve) of the mixed absorption coefficient ε of RSH and its dissociated forms at pH = 8 and temperature 25 °C, calculated from the determined values of const., const', used values of optical path of the cuvette (*d*) and *a* or *b*, is ε(412 nm) = 14950 ± 840 (±5.6%) $M^{-1} cm^{-1}$ (14110 $M^{-1} cm^{-1}$ from the dependence *A* vs. *n* , 15789 $M^{-1} cm^{-1}$ from the dependence *A* vs. $1/n$).

Acknowledgements

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