Inhibition of Enzymatic Reactions. A Rapid Method to Determine the Index pI_{50}

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Z. Naturforsch. 57 c, 496–499 (2002); received December 5, 2001/February 25, 2002

Enzyme, Inhibition, Index pI_{50}

The activity of every substance I inhibiting an enzymatic reaction can be approximately evaluated by the index pI_{50}. This paper describes a simple and fast method of estimate and/or determination of this index. The method is based on the linearity of the dependence of the ratio of reaction rates of uninhibited and inhibited reaction vs. concentration of the inhibitor at constant initial substrate and enzyme concentrations for fully competitive, non-competitive, uncompetitive and mixed type of inhibition by the one inhibitor. The validity of the method is demonstrated by four inhibitors of hydrolysis of acetylthiocholine by butyrylcholine esterase.

Introduction

The index pI_{50} is an approximate but qualified quantitative parameter of the inhibition power of a given inhibitor I for a given reaction with the given substrate S and enzyme E (Hansch and Deutsch, 1966; Kaczmar et al., 1970; Metcalf, 1971; Kuchař and Rejholec, 1980; Čegan and Mindl, 1980), which is \(-\log [I]_{50}\), the decadic logarithm of the molar inhibitor concentration [I]_{50}, which reduces the (initial) rate of inhibited reaction \(v_i\) in comparison with the (initial) rate of uninhibited reaction \(v_o\) to one half, i.e. \(v_i = 0.5 \cdot v_o\), under given reaction conditions (temperature, pressure, concentration of S and E, pH, ionic strength etc.). The (initial) values of \(v_o\) or \(v_i\) are calculated usually from the experimental dependence on concentration of S or product (P) vs. time (t). The present determinations of pI_{50} (e.g. Pavlová, 1998) use the dependence of the ratio \(v_i/v_o\) vs. [I] or pI = \(-\log [I]\) measured at the chosen initial concentrations of S and E and other constant conditions mentioned above. From this dependence the value of pI for \(v_i/v_o = 0.5\) is determined by interpolation or extrapolation. Such method of pI_{50} determination of many inhibitors in short time appeared to us as too complicated, time-consuming and expensive. The dependence \(v_i/v_o\) vs. [I] or pI mentioned is not linear and therefore many points are needed to express exactly its interesting part. Therefore this paper deals with a faster and simpler method of pI_{50} determination.

Theoretical Part

The rate equation (1) describing the decrease of S or production of P in the form of the Michaelis-Menten relation can be derived from the general scheme of reaction of S with E inhibited by I (see e.g. Kotyk and Horáček, 1977) on condition that the steps S + E = ES [1], ES + I = ESI [2], E + I = EI [3] and EI + S = ESI [4] are reversible and in equilibrium during the whole course of the reaction, the steps ES \(\rightarrow\) E + P [5] and ESI \(\rightarrow\) E + I + P [6] are irreversible with the rate constants \(k_i\) and \(k_{ir}\) (i = 1 to 6, r = reverse step) and equilibrium constants \(K_1 = k_{1r}/k_1, K_2 = k_{2r}/k_2, K_3 = k_{3r}/k_3\):

\[
v_i = -d[S]/dt = -d[P]/dt = V_m \cdot [S]/(K_{M} + [S]) \quad (1)
\]

\[
V_m = V_m \cdot (K_2 + k_6 \cdot [I])/k_5/(K_2 + [I]) \quad (1.1)
\]

\[
K_M = K_M \cdot (1 + [I]/K_3)/(1 + [I]/K_2) \quad (1.2)
\]

\[
V_m = k_5 \cdot [E]_o \quad (1.3)
\]

\[
K_M = k_{1r}/k_1 = K_1 \quad (1.4)
\]

From the steps [1]–[6] four experimentally found types of full inhibition (\(k_6 = 0\)) can be derived: competitive (\(K_2 = \infty\)), noncompetitive (\(K_2 = K_3\)), uncompetitive (\(K_3 = \infty\)) and mixed inhibition. Application of these conditions in (1) gives different expressions of \(v_i\) for every of the four mentioned types of inhibition.

The uninhibited reaction (i.e. \([I]_o = 0\) relates to classical Michaelis-Menten equation \(v_o = V_m \cdot [S]/(K_M + [S])\), where \(V_m\) is the maximum rate of the given enzyme reaction under given conditions and at
the saturation of E by S, $K_M$ is the Michaelis constant.

The ratio of $v_i/v_i$ gives then following dependences on S, E and I for fully competitive (2), noncompetitive (3), uncompetitive (4) and mixed (5) inhibition type

$$v_i/v_i = [K_M/(K_3 \cdot (K_M + [S]))] \cdot [I] + 1 \quad (2)$$

$$v_i/v_i = [I]/K_2 + 1 = [I]/K_3 + 1 \quad (3)$$

$$v_i/v_i = ([S]/(K_2 \cdot (K_M + [S])) \cdot [I] + 1 \quad (4)$$

$$v_i/v_i = ([K_M/K_3 + [S]/K_2]/(K_M + [S])) \cdot [I] + 1 \quad (5)$$

It can be seen from Eqns (2) to (5) that all these dependences $v_i/v_i$ vs. $[I]$ have a linear character at constant $[S]_o$ and $[E]_o$. The Eqns (2) and (4) for fully competitive and uncompetitive inhibition are valid also in the case of a steady state for the concentrations of E, ES, EI and ESI instead of equilibrium of the steps $[1] \rightarrow [2]$, but instead of Eqn. (1.4) it holds $K_M = (K_{1r} + K_2)/(K_1)$. The linearity of the dependence $v_i/v_i$ vs. $[I]$ is very advantageous for the simple and fast determination of the pI$_{50}$ of a given inhibitor. The value of $[I]$ at the ratio $v_i/v_i = 2$ corresponds with the concentration $[I]_{50}$ reducing $v_i$ to $v_i/2$ under given conditions (see Fig. 1). The straight lines $v_i/v_i$ vs. $[I]$ have always the intercept 1 on the $v_i/v_i$-axis. So, an approximate estimate of pI$_{50}$ can be theoretically made by this intercept and only one determined ratio $v_i/v_i$ for one concentration of I. The exact pI$_{50}$ determination requires, of course, more measurements of $v_i$ for more $[I]$ at constant $[S]_o$, $[E]_o$, but always much less than for the construction of the generally nonlinear dependence $v_i/v_i$ vs. $[I]$ mentioned in the Introduction.

**Remark**

Besides the determination of pI$_{50}$ the type of inhibition described above can be detected according to the dependence of the value of the slope of $v_i/v_i$ vs. $[I]$ on the used concentration of S. With increasing $[S]_o$ the value of the slope decreases in the case of fully competitive inhibition (2), increases by fully uncompetitive inhibition (4) and by fully mixed inhibition (5) both alternatives are possible ($K_2 < K_1$ increasing, $K_2 > K_1$ decreasing). The slope of $v_i/v_i$ vs. $[I]$ for fully noncompetitive inhibition (3) does not depend on $[S]_o$. On condition of knowledge of the type of inhibition, the values of corresponding constants $K_i$ and $K_M$ can be calculated from the corresponding slopes according to Eqns (2) – (5).

For all other types of the described inhibition, i.e. fully uncompetitive and mixed inhibition in steady state and all partial inhibitions ($K_6 \neq 0$) in equilibrium or steady state, the Dixon relation $1/v_i$ vs. $[I]$ are not linear but hyperbolic (Kotyk and Horák, 1977). In these cases the value of pI$_{50}$ must be determined by nonlinear regression.

The described method of pI$_{50}$ determination was checked on the enzymatic hydrolysis of acetyltihochole (ATCH) with butyrylcholine esterase (BCHE), inhibited by four inhibitors: three carbamate derivatives (A, B, C) and 7-methoxytaxacin (D). The inhibition type of these inhibitors in this reaction is not yet known.

**Results and Discussion**

Table I includes the dependences of the ratio $v_i/v_i$ vs. $[I]_o$ at constant [ATCH]$_o$ and [BCHE]$_o$ for all studied inhibitors A, B, C, D and the estimates of the values of pI$_{50}$ for each experimental point. The graphical proof of the linearity (see correlation coefficients R$^2$ in Table II) on the dependences for all inhibitors studied is shown in the Fig. 1.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$[I]_o$, [µM]</th>
<th>0</th>
<th>8</th>
<th>32</th>
<th>56</th>
<th>80</th>
<th>104</th>
</tr>
</thead>
<tbody>
<tr>
<td>v$_i$/v$_i$</td>
<td>pI$_{50}$</td>
<td>4.13</td>
<td>4.33</td>
<td>4.31</td>
<td>4.34</td>
<td>4.35</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<th>Inhibitor</th>
<th>$[I]_o$, [µM]</th>
<th>0</th>
<th>9.6</th>
<th>38.4</th>
<th>67.2</th>
<th>96</th>
<th>125</th>
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<tr>
<td>v$_i$/v$_i$</td>
<td>pI$_{50}$</td>
<td>3.92</td>
<td>4.16</td>
<td>4.16</td>
<td>4.16</td>
<td>4.15</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<table>
<thead>
<tr>
<th>Inhibitor</th>
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<th>10.3</th>
<th>41.3</th>
<th>72.2</th>
<th>103</th>
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<tr>
<td>v$_i$/v$_i$</td>
<td>pI$_{50}$</td>
<td>4.23</td>
<td>4.30</td>
<td>4.29</td>
<td>4.31</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$[I]_o$, [µM]</th>
<th>0</th>
<th>2.80</th>
<th>11.2</th>
<th>19.6</th>
<th>28</th>
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<tbody>
<tr>
<td>v$_i$/v$_i$</td>
<td>pI$_{50}$</td>
<td>5.88</td>
<td>5.77</td>
<td>5.77</td>
<td>5.77</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Fig. 1. The dependences $v_o/v_i$ vs. $[I]$ from Table I for the reaction of acetylthiocholine with butyrylcholine esterase at 25 °C and pH 7.6 inhibited by four inhibitors A, B, C, D (see Table I). Their linearity is demonstrated by the deviation bars of 95% confidence of linear regression at the experimental data points. At the line D the size of the points is equal or greater than the corresponding bars. Intersections of the dashed line $v_o/v_i = 2$ with the calculated lines determine the values of $[I]_{50}$ and therefore also of $pI_{50}$.

In Table II an overview is included of the estimated and determined $pI_{50}$ values of the measured inhibitors for the hydrolysis at given conditions. The determined value $pI_{50}$ of Sevin (A) = 4.33 could be compared with two data from Pavlova (1998) and Patocka (1999), $pI_{50} = 4.08$ and 4.11, measured at about the same conditions. The exact values of $pI_{50}$ calculated from the straight lines correspond well with the average estimated values (calculated without the first experimental points measured for too small concentrations of inhibitors).

Beside this, the linearity of the dependence $v_o/v_i$ vs. $[I]$ at constant [BCHE] and [ATCH] for all inhibitors implies, that for the reaction between ATCH and BCHE probably these inhibitors fulfil some of the four described fully inhibition types (see Remark in Theoretical Part).

**Experimental**

Butyrylcholine esterase (BCHE) preparation: lyophylizate from the horse plasma, pressed in pellets ca 6 g. Kept in refrigerator at 5 °C.

Acetylthiocholine (ATCH) iodide: substrate, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman’s reagent): both from Sigma/Aldrich, Prague, CZ. Kept at 5 °C.

1-Naphthyl N-methylcarbamate (Sevin), Fluka, Prague, CZ. 4-Nitrophenyl N-isobutylcarbamate was prepared according to Nesymov and Pelkis (1964). 4-Nitrophenyl N-butylcarbamate was synthesized according to Linh Gialih et al. (1999).

7-Methoxy-1,2,3,4-tetrahydro-acridin-9-ylamine (7-methoxytacrin, 7-MEOTA) was obtained according to Bielavsky (1977). The melting points of all inhibitors agree with those of the above literature. Kept in refrigerator at 5 °C.

A well homogenized aqueous solution of BCHE was prepared by suspending several lyophylizate pellets in demineralized water. The total volume of the solution was divided into aliquots necessary for the assays of a day. These were separately kept frozen to prevent the loss of the BCHE activity. A 0.1 m fresh aqueous solution of ATCH iodide was prepared for the measurement every day. The aqueous solution of 0.04 m DTNB was prepared and kept in darkness at 5 °C. 1 nm solutions of inhibitors were used: Analytical solutions of all carbamates were prepared by solving them in acetone, 7-methoxytacrin was dissolved in water. Solutions with lower concentrations were prepared by dilution with water. All solutions were kept at 5 °C.

The initial rates $v_o$ and $v_i$ of the given uninhibited and inhibited reaction were determined according to Ellman et al. (1961). The substrate ATCH and enzyme BCHE produce thiocoline (TCH), which gives a yellow product with excess of DTNB; maximum of absorbance at 412 nm ($A_t$). The ratio $v_o/v_i$ is then equal to the ratio $(dA_o/dA_i)$ at the given time $t$. The standard solution consisted of 5 ml buffer, 7 ml water and 0.5 ml 0.04 m DTNB to eliminate the weak absorption of excess of DTNB in the reaction mixture at 412 nm. The dependences $A$ vs. $t$ were

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Estimated $pI_{50}$</th>
<th>Determined $pI_{50}$</th>
<th>$R^2$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.33</td>
<td>4.33</td>
<td>0.9981</td>
<td>4.08*, 4.11**</td>
</tr>
<tr>
<td>B</td>
<td>4.16</td>
<td>4.16</td>
<td>0.9990</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.30</td>
<td>4.28</td>
<td>0.9995</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5.77</td>
<td>5.78</td>
<td>0.9993</td>
<td></td>
</tr>
</tbody>
</table>

*(Pavlová, 1998); **(Patocka, 1999).
measured by means of a spectrograph 8452A diode array (Hewlett-Packard, USA). A glass thermostated (25 ± 0.1 °C) and mixed cuvette of 30 ml volume with an optical path of 2 cm served as the reactor. The initial reaction mixture was obtained by fast (<1 s) mixing of the aqueous buffer solution and BCHE with the aqueous solution of ATCH, DTNB and I: 5 ml buffer, 0.5 ml BCHE and x ml water with 0.5 ml 0.1 M ATCH , 0.5 ml 0.04 M DTNB, y ml I, ca 10⁻³ M, total volume 12.5 cm³. Thus, the initial concentrations were [ATCH]₀ = 4 × 10⁻³ M and [DTNB]₀ = 1.3 × 10⁻³ M and the mixture had the ionic strength (from the buffer) J ≈ 0.262 M. The used initial concentrations of inhibitors [I]₀ are given in Table I.

All dependences A vs. t were measured for small coversion of ATCH (Δ[ATCH] < 2 molar% of [ATCH]₀), i.e. at the beginning of the reaction. In this reaction state the dependences A vs. t were strictly linear. Thus, the initial reaction rates, or their ratios, could be calculated as v = ΔA/Δt, or vₒ/vᵢ = ΔAₒ/ΔAᵢ (for an equal period Δt). The estimate of pI₅₀ was calculated from each experimental point of the dependence vₒ/vᵢ vs. [I] for every inhibitor used. The exact determination of the pI₅₀ was calculated by means of linear regression of all dependences mentioned including a minimum of five points.

Acknowledgment

This work was financially supported by the Czech Ministry of Education, Health and Sport (Research project CZ 310008/2010/3340) and by the grant of FRVŠ 1615/2001.