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

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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, noncompetitive, 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 unhibited reaction v_o to one half, i.e. $v_i = 0.5\ v_o$, 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 Horák, 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 → E + P [5] and ESI → 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 $K_1 = k_{1r}/k_1$, $K_2 = k_{2r}/k_2$, $K_3 = k_{3r}/k_3$:

$$v_i = -\mathrm{d}[S]/\mathrm{d}t = \mathrm{d}[P]/\mathrm{d}t = V'_m \cdot [S]/(K'_M + [S]) \quad (1)$$

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

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

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

$$K_M = k_{1r}/k_1 \equiv K_1 \quad (1.4)$$

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

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the saturation of E by S , K_M is the Michaelis constant.

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

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

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

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

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

It can be seen from Eqns (2) to (5) that all these dependences v_o/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]–[4], but instead of Eqn. (1.4) it holds $K_M = (k_{1r} + k_2)/k_1$. The linearity of the dependence v_o/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] for the ratio $v_o/v_i = 2$ corresponds with the concentration $[I]_{50}$ reducing v_i to $v_o/2$ under given conditions (see Fig. 1). The straight lines v_o/v_i vs. [I] have always the intercept 1 on the v_o/v_i-axis. So, an approximate estimate of pI_{50} can be theoretically made by this intercept and only one determined ratio v_o/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_o 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_o/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 ($K_2 < K_3$ increasing, $K_2 > K_3$ decreasing). The slope of v_o/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 acetylthiocholine (ATCH) with butyrylcholine esterase (BCHE), inhibited by four inhibitors: three carbamate derivates (A, B, C) and 7-methoxytacrin (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_o/v_i vs. [I] 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 I. Dependences of the ratio v_o/v_i vs. $[I]_o$ at $[ATCH]_o$ = 4 mM, $[DTNB]_o$ = 1.3 mM, $[BCHE]_o$ (0.5 ml of solution of BCHE preparation in 12.5 ml of reaction mixture), 25 °C and pH 7.6 (phosphate buffer, J ≈ 0.262 M) and estimates of indices pI_{50} for used inhibitors A (Naphthyl N-methylcarbamate, Sevin), B (4-Nitrophenyl N-isobutylcarbamate), C (4-Nitrophenyl N-butylcarbamate), D (7-methoxytacrin). ATCH = acetylthiocholine, BCHE = butyrylcholine esterase, I = inhibitor, DTNB = Ellman's reagent.

Inhibitor A						
$[I]_o$ [μM]	0	8	32	56	80	104
v_o/v_i	1	1.11	1.68	2.16	2.77	33.42
pI_{50}		4.13	4.33	4.31	4.34	4.35
Inhibitor B						
$[I]_o$ [μM]	0	9.6	38.4	67.2	96	125
v_o/v_i	1	1.08	1.55	1.97	2.39	2.78
pI_{50}		3.92	4.16	4.16	4.16	4.15
Inhibitor C						
$[I]_o$ [μM]	0	10.3	41.3	72.2	103	
v_o/v_i	1	1.17	1.82	2.42	3.09	
pI_{50}		4.23	4.30	4.29	4.31	
Inhibitor D						
$[I]_o$ [μM]	0	2.80	11.2	19.6	28	
v_o/v_i	1	1.21	1.66	2.16	2.64	
pI_{50}		5.88	5.77	5.77	5.77	

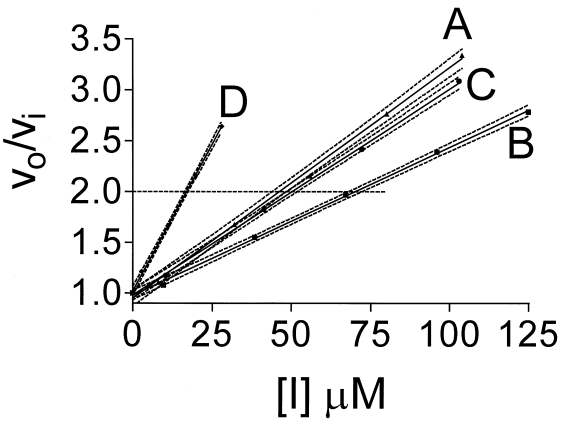

Fig. 1. The dependences v_o/v_i vs. $[I]_o$ from Table I for the reaction of acetylthiocholine with butyrylcholine esterase at 25 °C and pH 7.6 inhibited by four inhibitors A, B, C, D (see Table I). Their linearity is demonstrated by the deviation bars of 95% confidence of linear regression at the experimental 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 Pavlová, (1998) and Patočka (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 studied 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).

Table II. Average estimated and determined values pI_{50} and correlation coefficients R^2 of linearity of the dependences v_o/v_i vs. $[I]_o$ from Table I for inhibition of hydrolysis of acetylthiocholine by butyrylcholine esterase by means of four inhibitors A, B, C and D (see Table I).

Inhibitor	Estimate pI_{50}	Determination pI_{50}	R^2	Reference
A	4.33	4.33	0.9981	4.08*, 4.11**
B	4.16	4.16	0.9990	
C	4.30	4.28	0.9995	
D	5.77	5.78	0.9993	

* (Pavlová, 1998); **(Patočka, 1999).

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 synthetized 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 Bielavský (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 mM 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 thiocholine (TCH), which gives a yellow product with excess of DTNB; maximum of absorbance at 412 nm (A). 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

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 × 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 cm^3. Thus, the initial concentrations were $[ATCH]_o$ = 4 × 10^{-3} M and $[DTNB]_o$ = 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 $[I]_o$ are given in Table I.

All dependences *A* vs. *t* were measured for small coversion of ATCH (Δ[ATCH] < 2 molar% of $[ATCH]_o$), 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 $v = \Delta A/\Delta t$, or $v_o/v_i = \Delta A_o/\Delta A_i$ (for an equal period Δt). The estimate of pI_{50} was calculated from each experimental point of the dependence v_o/v_i vs. [I] for every inhibitor used. The exact determination of the pI_{50} was calculated by means of linear regression of all dependences mentioned including a minimum of five points.

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