The enthalpimetric determination of inhibition constants for the inhibition of urease by acethydroxamic acid

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The effect of concentration of acethydroxamic acid (AHA) on inhibition of jack bean urease in phosphate buffer, pH 7.0, at 25°C, was studied. The measurements were performed at urease concentration of 2.5 mg/100 cm³ for concentrations of urease and AHA ranging in the range of 2–50 mmol dm⁻³ and 0.25–10 mmol dm⁻³, respectively. The reactions were monitored by two techniques: analytical and enthalpimetric. For the analytical technique the growth of ammonia concentration in the course of the reaction was determined. From the recorded progress curves the following parameters were calculated for each inhibitor concentration: the initial reaction rate, the steady-state rate and the inversion constant. From these parameters the inhibition constants of the initial and steady-state stages of the reaction, Kᵢ and Kᵢ*, were calculated. The former constant did not change whereas the latter one proved to decrease quickly with an increase in inhibitor concentration. This behaviour resulted from the fact that the inactive complex EI* was not a product of internal inversion but was formed in the reaction: \( \frac{2}{3} \text{I} + \text{EI} \rightarrow (\text{EI} \cdot \frac{2}{3} \text{I})^* \). The dissociation constant of this complex is equal to about \( 0.3 \times 10^{-3} \) (mol dm⁻³)²₃.

In the 1960s Kobashi et al. [1] found that acethydroxamic acid along with other hydroxamic acids R–CONH–OH ⇌ R–C(OH)=NOH and some of their derivatives are strong and specific inhibitors of plant and microbial ureases [1–3]. They proved that these inhibitors combine with urease stoichiometrically and block the active site with a –CONHOH group. They determined the inhibitory strength of numerous hydroxamates assuming as its measure the concentration of an inhibitor at which the urease activity falls to 50% of its original value.

Subsequent studies on urease inhibition by hydroxamic acids have been reviewed by Mobley & Hausinger [4]. Some of their results indicate that hydroxamic acids could be used in medical therapies.

The enzymatic kinetics of urea hydrolysis in the presence of acethydroxamic acid (AHA) was a subject of a number of papers [5–9]. Zerner et al. [5–8] studied a plant urease and proposed a mechanism which according to Morrison & Walsh [10] was classified as classical fast-binding competitive inhibition. Todd & Hausinger [9] investigated the microbial urease Klebsiella aerogenes and found AHA to be a slow-binding competitive inhibitor. This means that urea hydrolysis proceeds along scheme (1).

The aim of this study was to characterize the work of the system in the course of time.

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Abbreviation: AHA, acetylhydroxamic acid.
within the period sufficiently long for the steady-state rate \( v_0 \) to become visible on the progress curve, and within the inhibitor concentration range sufficiently broad for the relations \( 1/v_0, 1/v_{0s}, k \) vs \( \left[ I \right] \) to become clear. These relations were interpreted in terms of the aforementioned theory of slow-binding competitive inhibition. The progress of urea hydrolysis, i.e. the increase in \( \text{NH}_3 \) concentration with time, was recorded. Two techniques were applied: enthalpic and analytical. The former allowed to measure rates of the reaction at its initial fast stage with satisfactory accuracy. The latter was used for the reactions at high concentrations of the inhibitor; under these conditions a small amount of ammonia was produced and the amount of heat secreted was proportionally low.

### Basic equations and symbols

The general model of slow-binding inhibition introduced by Morrison & Walsh [10] covers relations shown in scheme (1):

\[
E + S \xrightleftharpoons[k_s]{k_1} ES \xrightarrow{k_y} E + P \\
E + I \xrightleftharpoons[k_s]{k_4} EI \xrightarrow{k_e} EI^*
\]

(1)

the equation of the progress curve, \( P \) vs \( t \), has the form (2):

\[
P = v_0 t + \frac{k_y - k_s}{K_1 k} \left( 1 - e^{-\frac{K_1 k t}{[I]}} \right)
\]

(2)

The slope of the tangent at \( t = 0 \) is equal to \( v_0 \), whereas the slope of the asymptote is equal to \( v_y \). The half-time, \( t_{1/2} \), is the abscissa of the point of intersection of the progress curve with the straight line which divides the intercept of the asymptote into halves and is parallel to the asymptote. The time \( t_{1/2} \) is connected with the inversion constant by eqn. (3):

\[
k = \frac{\ln 2 \left[ I \right]}{t_{1/2} K_1}
\]

(3)

At high inhibitor concentrations, 4,...,10 mmol dm \(^{-3} \) eq. (4) was used:

\[
k = \frac{2v_0}{n\text{NH}_3 K_1}
\]

(4)

where \( n\text{NH}_3 \) denotes final ammonia contents in moles (cf. Table 2).

The following linear relations between variables \( 1/v_0, 1/v_{0s} \) and inhibitor concentration appear in the theory of the slow-binding competitive inhibition:

\[
\frac{1}{v_0} = \frac{K_m}{K_v \left[ S \right]_0} [I] + \frac{1}{v_{\text{max}}} \left( 1 + \frac{K_m}{[S]_0} \right)
\]

(5)

\[
\frac{1}{v_s} = \frac{K_m}{K_i \left[ S \right]_0} [I] + \frac{1}{v_{\text{max}}} \left( 1 + \frac{K_m}{[S]_0} \right)
\]

(6)

\( K_m \) is the Michaelis constant, \( K_i \) and \( K_i^* \) are inhibition constants of initial and steady-state stages of the reaction, respectively. The former can also be identified with the dissociation constant of EI complex (eqn. 7), as justified by the fact that the reaction \( E + I \xrightleftharpoons{} EI \) reaches equilibrium quickly. On the contrary, the latter one cannot be related to any equilibrium, therefore the term "overall dissociation constant" for \( K_i^* \) will not be used in this study. In equations (8) the term \( K_i^* \) appears only as a substitute.

\[
K_i = [E] \left[ I \right] / [E] = k_4 / k_3
\]

(7)

\[
K_i^* = [E] \left[ I \right] / ([E] + [E^*]) = k_4 k_e / (k_5 + k_6)
\]

(8)

Symbols:

\( * \) \( k \) is the inversion constant for the establishment of the equilibrium between EI and EI*,

\[
k = k_6 \left( 1 + \frac{[S]_0 \left[ I \right]}{K_m K_i^*} \right)
\]

(9)

\( * \) \( k_j \) is the rate constant related to an elementary reaction \( j \) in scheme (1); \( k_1 \) and \( k_2 \) are in mol\(^{-1}\) dm\(^{-3}\) s\(^{-1}\), the others in s\(^{-1}\),

\( * \) \( v_{\text{max}}, v_0 \) and \( v_s \), in mol s\(^{-1}\), are the maximum reaction rate defined by the Michaelis-Menten equation, initial reaction rate and steady-state reaction rate of
the hydrolysis of urea, respectively, all related to the known constant mass of the enzyme used in the experiment. Other symbols have generally accepted meanings and are expressed in SI units.

Eqn. (5) and (6) allow to verify the hypothesis that acetohydroxamic acid is a competitive slow-binding inhibitor of plant urease.

EXPERIMENTAL

Materials

Jack bean urease was Sigma type III. Its specific activity was 32 units per mg of active protein. One unit is the amount of enzyme that liberates 1.0 μmol of NH₃ from urea per minute at pH 7 and 25°C. Acetohydroxamic acid (Analar grade) was purchased from Sigma. Urea and all other chemicals (Analar grade) were obtained from POCh (Gliwice, Poland).

Enzymatic reaction

The hydrolysis of urea catalyzed by jack bean urease \( \text{CO}(\text{NH}_2)_2 + \text{H}_2\text{O} \rightarrow 2\text{NH}_3 + \text{CO}_2 \) was studied. All measurements were carried out at enzyme concentration of 2.5 mg/100 cm\(^3\) in phosphate buffer, pH 7.0 (50 mmol dm\(^{-3}\), 1 mmol dm\(^{-3}\) EDTA) at 25°C. Jack bean urease is known to reach the highest activity at neutral pH [11].

Reaction without inhibitor. In the first series of buffer solutions, in which the initial urea concentrations were: 2, 3, 5...50 mmol dm\(^{-3}\), the concentrations of ammonia were measured after 2, 3, 4 and 5 minutes of the reaction (analytical method). From these data the initial reaction rates were calculated. The reaction was initiated by addition of 0.25 cm\(^3\) of the urease solution (2.5 mg cm\(^{-3}\)) into 25 cm\(^3\) of the assay mixture.

Reaction with inhibitor. In the second series of buffer solutions, urea concentration was kept constant at 50 mmol dm\(^{-3}\), while those of AHA were: 0, 0.5, 1, 1.5 and 3 mmol dm\(^{-3}\). The reactions were carried out in a calorimeter, and temperature increments were recorded as a function of time, \( \Delta T \text{ vs } t \).

In the third series of solutions of higher AHA concentration: 4, 5, 6, 8 and 10 mmol dm\(^{-3}\), the reaction was observed in the same conditions by the analytical method. The reaction of both series were initiated by addition of 1 cm\(^3\) of the urease solution (2.5 mg cm\(^{-3}\)) into 100 cm\(^3\) of the assay mixture. The obtained two families of progress curves: calorimetric and analytical, were used to calculate the initial reaction rate \( v_0 \), steady-state reaction rate \( v_s \) and constant \( k \) as a function of inhibitor concentration.

Methods

In the calorimetric method the reaction was carried out in the isoperibol set and the rate of temperature change was recorded at 2 s intervals with an accuracy of 0.001°C. The observed differential increments \( \Delta T \) were corrected for heat exchange into \( \Delta T^* \). The detailed description of the calorimetric apparatus was presented elsewhere [12].

In the analytical method the concentration of ammonia was determined by the phenol-hypochlorite colorimetric method; the absorbance was measured at 625 nm [13].

RESULTS

Michaelis constant \( K_m \) and maximum reaction rate \( v_{\text{max}} \)

The measured initial rates of urea hydrolysis (in the absence of the inhibitor) as a function of urea concentration form a hyperbola: \( v_0 = v_{\text{max}} \left( [S]_0 + [S]_0 \right) / K_m + [S]_0 \) (Fig. 1A). In the Hanes coordinate system [14] this relationship becomes linear (10),

\[
\frac{[S]_0}{v_0} = \frac{1}{v_{\text{max}}} \frac{[S]_0 + K_m}{[S]_0}
\]

as presented in Fig. 1B. According to the statistical analysis performed by Wilkinson, this linearization procedure is the most reliable of the methods commonly used [15].

From the parameters of the straight line the kinetic constants of the reaction were calculated:


\[ K_{\text{eq}} = (10.2 \pm 0.5) \times 10^{-3} \text{ mol dm}^{-3} \]

\[ v_{\text{max}} = (1.075 \pm 0.02) \times 10^{-6} \text{ mol s}^{-1} \]

The value of \( K_{\text{eq}} \) obtained here is higher than that reported previously [12], \( 6.5 \pm 0.2 \times 10^{-3} \), because it has been determined at

monia \( n_{\text{NH}_3} \) (Fig. 3) are correlated by the following equation:

\[ \Delta T = \frac{1}{2} \frac{\Delta H}{R_{\text{cal}}} n_{\text{NH}_3} = 74.85 n_{\text{NH}_3} \]  

(11)

where \( \Delta H = -59580 \text{ J mol}^{-1} \) is the enthalpy of urea hydrolysis [12] and \( R_{\text{cal}} = 398.00 \text{ J K}^{-1} \) is the heat capacity of the calorimeter.

![Figure 1. Initial rate of urea hydrolysis by urease as a function of the substrate concentration.](image1)

System without inhibitor; \( u_0 \) (mol s\(^{-1}\)), [urea] (mol dm\(^{-3}\)). A, the solid line was calculated by fitting experimental data to Michaelis-Menten equation; B, replotted in the Hanes coordinate system.

a higher concentration of phosphate buffer. It is known that phosphates are urease inhibitors of medium strength [16] independently of the presence of other inhibitors.

![Figure 2. Enthalpimetric progress curves \( \Delta T vs t \), for urease-catalyzed urea hydrolysis in the presence of acetohydroxyacid.](image2)

Numbers denote \([\text{AHA}]\) (mmol dm\(^{-3}\)). A, the whole course of the reaction, experimental points are not shown in the plots; B, \( \Delta T vs \) time for the initial stage of reaction.

The initial reaction rate, \( u_0 \), at low and moderate inhibitor concentrations was determined from the first (0 \( \leq t \leq 60 \) s) linear segment of the calorimetric progress curve (Fig. 2B). Linear least squares analysis yielded the slope (K s\(^{-1}\)), which multiplied by the factor: \( R_{\text{cal}}/\Delta H = 398/59580 = 0.006680 \) mol K\(^{-1}\) yielded the rate \( u_0 \) (mol s\(^{-1}\)) related to a given inhibitor concentration (Table 1). At high concentrations only the analytical method could be used (Table 2).
Inversion constant \( (k) \)

The experimental values of inversion constant \( k \) obtained from the progress curves corresponding to all the inhibitor concentrations studied between 0.25 and 10 mmol dm\(^{-3}\), are given in the second column of Table 3.

**DISCUSSION**

The values of \( K_m \) and \( v_{\text{max}} \) determined in this study for the system without the inhibitor and the experimental data \( v_0, v_9, k \) obtained for the systems with the inhibitor allow to calculate the inhibition constants \( K_i \) and \( K_i^* \). The functional dependence of the constants on inhibitor concentration, \( K_i, K_i^* \), \( k \) vs [I], described in this section, is essential for kinetic analysis of the system.

**Inhibition constant of the initial state \( K_i \)**

The experimental points ([I], \( v_0 \)) listed in Tables 1 and 2, plotted in the coordinate system \( 10^5 v_0 \) vs \( 10^5 [I] \) produce a straight line (Fig. 4A) described with eqn. (12):

\[
\frac{10^5}{v_0} = 0.1460 \times 10^5 [I] + 1.1200
\]

(12)

From eqn. (5) for \([S]_0 = 0.05 \text{ mol dm}^{-3}\) the value \( K_i \) was calculated:

\[
K_i = 10^{-6} \times 10.2(0.146 \times 1.075 \times 0.05) = 1.30 \times 10^{-3} \text{ mol dm}^{-3}
\]

Both techniques were applied to determine the steady-state rate, \( v_9 \), in the range of lower concentrations (Table 1). At higher concentrations the slope of the asymptotes and consequently the values of \( v_9 \) become lower than the experimental error.

**Table 1. The initial \( v_0 \) and steady-state reaction rate \( v_9 \) at low and medium inhibitor concentrations**

<table>
<thead>
<tr>
<th>( 10^5 [I] ) (mol dm(^{-3}))</th>
<th>( 10^6 v_0 ) (mol s(^{-1}))</th>
<th>( 10^5 v_9 ) (mol s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>0.50</td>
<td>0.635</td>
<td>–</td>
</tr>
<tr>
<td>1.0</td>
<td>0.782</td>
<td>0.257</td>
</tr>
<tr>
<td>1.5</td>
<td>0.755</td>
<td>0.088</td>
</tr>
<tr>
<td>2.0</td>
<td>–</td>
<td>0.055</td>
</tr>
<tr>
<td>3.0</td>
<td>0.646</td>
<td>0.026</td>
</tr>
<tr>
<td>4.0</td>
<td>–</td>
<td>0.059</td>
</tr>
<tr>
<td>0.25</td>
<td>–</td>
<td>0.012</td>
</tr>
<tr>
<td>0.06</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>0.025</td>
<td>0.006</td>
<td></td>
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<tr>
<td>0.025</td>
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<tr>
<td>0.006</td>
<td>0.006</td>
<td></td>
</tr>
</tbody>
</table>
This value is valid for the whole studied range of inhibitor concentration up to the concentration of 0.010 mol dm\(^{-3}\).

Table 2. The initial reaction rate \(v_0\) and the final ammonia contents \(n_{NH_3}\) at high inhibitor concentrations

<table>
<thead>
<tr>
<th>(10^3[1]) (mol dm(^{-3}))</th>
<th>(10^6v_0) (mol s(^{-1}))</th>
<th>(10^3n_{NH_3}) (mol)</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.59</td>
<td>0.152</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.54</td>
<td>0.128</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0.50</td>
<td>0.112</td>
<td>0.006</td>
</tr>
<tr>
<td>8</td>
<td>0.44</td>
<td>0.076</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0.39</td>
<td>0.067</td>
<td>1</td>
</tr>
</tbody>
</table>

Linear dependence of \(1/v_0\) on \([I]\) predicted by eqn. (5), observed over the whole range of inhibitor concentration, means that in the initial stage of the reaction (1–2 min) AHA is a classical competitive inhibitor.

Inhibition constant of the steady-state \(K_i^*\)

In the range 0<\([I]\)<1 mmol dm\(^{-3}\) the relation, \(10^{-6}v_0\) vs \(10^3[I]\), is linear (Fig. 4B) and given by eqn. (13):

\[
10^{-6}v_0 = 5.5814 \times 10^3[I] + 1.1200
\]

(13)

In this range \(K_i^*\) has a constant value. It was determined by comparing the slopes of straight lines (6) and (13) and was found to be:

\[
K_i^* = \frac{10^{-6} \times 10.2}{(5.5814 \times 10^3 \times 0.05)} = 0.0340 \times 10^{-3} \text{ mol dm}^{-3}
\]

The values of \(k_5\) and \(k_8\) constants in this inhibitor concentration range were calculated from the transformed eqns. (8) and (9). Using the values:

\[
K_i = 1.30 \times 10^{-3} \quad K_i^* = 0.034 \times 10^{-3} \quad K_n = 10.2 \times 10^{-3} \quad [S]_0 = 0.05
\]

the following was obtained:

<table>
<thead>
<tr>
<th>(10^3[I])</th>
<th>(10^3k_5)</th>
<th>(10^3k_6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.435 (uncorr)</td>
<td>1.22</td>
</tr>
<tr>
<td>0.5</td>
<td>0.919 (uncorr)</td>
<td>1.66</td>
</tr>
<tr>
<td>1.0</td>
<td>2.04 (corr)</td>
<td>2.15</td>
</tr>
</tbody>
</table>

For higher inhibitor concentrations, \([I] > 1 \text{ mmol dm}^{-3}\), it was assumed that \(K_i\) has a constant value and that \(k_6 = 0.0578 \times 10^{-3} \text{ s}^{-1}\). From the same eqns. (8) and (9) taking \(k\)-corrected, \(K_i^*\) and \(k_5\) were calculated.

Figure 4. The reciprocal rate of hydrolysis of urea vs concentration of acetohydroxamic acid; \([I]\) (mol dm\(^{-3}\)).

A. The initial reaction rate \(v_0\) (mol s\(^{-1}\)); the ordinate of the first point was calculated from the slope of the line 0 in Fig. 2B; the remaining values were taken from Table 1 and 2. B. The steady-state reaction rate \(v_0\) (mol s\(^{-1}\)); values \(\bigcirc\), \(\bigcirc\) taken from Table 1; points in the range \(0 < 10^3[I] < 1\) determine the line (13). Solid lines are drawn through experimental points. Dotted lines represent eqn. (25).

The values presented in Table 3 show that \(K_i^*\) and \(k_5\) do not remain constant. The rate constant \(k_5\) increases with an increase in inhibitor concentration followed by a decrease in \(K_i^*\) (eqn. 8) and by an increase in \(1/v_0\) faster than predicted by the linear equation (6) (Fig. 4B). Supposedly, the inhibitor
Table 3. The values of the inversion constant \( k \), experimental and calculated from equation

\[ 10^3 \, k = 2.04 \times 10^3 \, [I]^{-1.37} \]

(see text), of the rate constant \( k_0 \), and of the inhibition constant of steady-state \( K_i^* \). The last digit of \( K_i^* \) values are given with the accuracy of ±2.

<table>
<thead>
<tr>
<th>( [I] ) (mol dm(^{-3}))</th>
<th>( k ) (s(^{-1}))</th>
<th>( k ) corrected</th>
<th>( K_i^* ) (mol dm(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.435</td>
<td>1.22</td>
<td>0.034</td>
</tr>
<tr>
<td>0.50</td>
<td>0.919</td>
<td>1.86</td>
<td>0.034</td>
</tr>
<tr>
<td>1.0</td>
<td>2.21</td>
<td>2.15</td>
<td>0.034</td>
</tr>
<tr>
<td>1.5</td>
<td>4.40</td>
<td>3.13</td>
<td>0.024</td>
</tr>
<tr>
<td>2.0</td>
<td>6.49</td>
<td>3.94</td>
<td>0.019</td>
</tr>
<tr>
<td>3.0</td>
<td>11.9</td>
<td>5.34</td>
<td>0.014</td>
</tr>
<tr>
<td>4.0</td>
<td>23.7</td>
<td>6.56</td>
<td>0.011</td>
</tr>
<tr>
<td>6.0</td>
<td>41.3</td>
<td>8.69</td>
<td>0.0086</td>
</tr>
<tr>
<td>8.0</td>
<td>70.8</td>
<td>10.6</td>
<td>0.0071</td>
</tr>
<tr>
<td>10.0</td>
<td>88.2</td>
<td>12.3</td>
<td>0.0061</td>
</tr>
</tbody>
</table>

has an effect on the equilibrium \( \frac{k}{k_1} \). The inhibitor shifts the equilibrium to the right, this was neither predicted nor excluded by scheme (1). This question will be further discussed in next sections of this study.

**Inversion constant \( (k) \)**

Having accepted the scheme and assumptions of the theory of slow-binding inhibition and introducing no further assumptions, eqn. (2) describing the reaction progress curve was obtained, which is different from the original version of Morrison & Walsh [10]. Consequently, Morrison’s constant \( k \), denoted here as \( k_{app} \), is different from constant \( k \) introduced in this study:

\[ k_{app} = k \, K_i / [I] \].

Progress equation (2) is an alternative version of the equation of Morrison & Walsh [10].

The constant \( k \) is not connected with any elementary reaction, that is why here it is not called a rate constant. It describes the kinetics of a number of simultaneous reactions proceeding from the initial values \([E], [EA], [E1] \) to the final values \([E]_s, [EA]_s, [E1]_s, [E1]^* \). This is why estimation of the inversion constant seems appropriate.

The theory of slow-binding competitive inhibition (10) does not predict any functional dependence of \( k \) on \([I] \). This dependence was found empirically.

The values of \( \frac{K_i^*}{[I]} \times 10^5 \) \( k \) and \( 10^3 [I] \) were found linearly related according to eqn. (14):

\[ \frac{K_i}{[I]} \times 10^5 k = m \times 10^3 + n \]  

(14)

where \( m = 1.16 \) mol\(^{-1} \) dm\(^3\) s\(^{-1}\), and \( n = 1.90 \) s\(^{-1}\); correlation coefficient 0.981 (Fig. 5). This means that the points corresponding to the same value of \( 10^3 k \) and \( 10^3 [I] \), form a parabola (15):

\[ 10^3 k = \frac{m}{10^3 K_i} \times (10^3 [I])^2 + \frac{n}{10^3 K_i} \times 10^3 [I] \]  

(15)

where \( \frac{m}{10^3 K_i} = \frac{1.16}{1.30} = 0.89 \) and \( \frac{n}{10^3 K_i} = \frac{1.90}{1.30} = 1.46 \). The parabola is represented by a solid line in Fig. 6 (the big graph).

The double logarithmic plot of \( 10^3 k \) vs \( 10^3 [I] \) was also linear (insert in Fig. 6):

\[ \log(10^3 k) = \bar{m} \log(10^3 [I]) + \bar{n} \]  

(16)
where $m = 1.67$ and $n \log 2.04 = 0.31$; correlation coefficient $0.996$.

Equation (16) is an equivalent of function (17):

$$10^3k = 2.04 \ (10^3[I])^{1.67}$$  \hspace{1cm} (17)

Because of the high value of the correlation coefficient of the line or function (16) eqn. (17) could be used to "smooth" the values $k$. The obtained $k$-corrected values were used to calculate $K_3^{a+1}$ and $k_5$ constants listed in Table 3. Equation (17) was included into the discussion presented below.

![Graph](image)

**Figure 5. Linearization of the relation: inversion constant $k$ (s$^{-1}$) vs concentration of acetohydroxamic acid: [I] (mol dm$^{-3}$).**

$k$ values taken from Table 3; $K_3 = 1.30 \times 10^{-9}$ mol dm$^{-3}$.

The presented analysis provides evidence that the inhibitor must produce an effect on the inversion constant $k$, similar to that mentioned in preceding section. The strong dependence of the constant $k$ on its concentration (Table 3) indicates that the inversion $EI \rightarrow EI^*$ does not occur spontaneously but with participation of inhibitor molecules according to kinetics of an order of magnitude higher than one.

Supposedly, there occurs reaction (18), in which 'a' is a value to be determined and $EI_{a+1}$ can be identified with $EI^*$:

$$aI + EI = EI_{a+1}$$  \hspace{1cm} (18)

Eqn. (18) can be interpreted in two ways:

1) Eqn. (18) represents the reaction proceeding at a rate given by eqn. (19):

$$-\frac{d[EI]}{dt} = \text{const} \ [I]^{n}[EI]$$  \hspace{1cm} (19)

or by eqn. (20) simplified to eqn. (21):

$$-\frac{d[EI]}{dt} = \frac{d[EI]}{dt} = k_{app}[EI] - [EI_n]$$  \hspace{1cm} (20)

$$\frac{d[EI]}{dt} = k_{app}[EI]$$  \hspace{1cm} (21)

Comparing eqn. (19) with (21) and taking into account that $k_{app} \rightarrow \frac{kK_i}{[I]}$ the following relationship was found: $k = \frac{\text{const} \ [I]^{a+1}}{K_i}$ substitution of eqn. (17) with the latter function allowed to calculate: $a+1 = 1.67$, i.e. $a = 2/3$.

Since the constant $k$ was determined with a satisfactory accuracy over the whole range of inhibitor concentrations 0.25–10 mmol dm$^{-3}$, and since the experimental points in this range are well approximated by eq. (17), the value $a = 2/3$ was considered to be well-founded and will be used in further discussion.

2) Equation (18) represents the equilibrium: $aI + EI \leftrightarrow EI_{a+1}$, with the equilibrium constant:

$$\frac{[I]^{a}[EI]}{[EI_{a+1}]} = K$$  \hspace{1cm} (22)

The following data allow to calculate $K$: for $[I] = 10^{-3}$, $k_5 = 2.15 \times 10^{-3}$ and $k_6 = 0.0578 \times 10^{-3}$; $a = 2/3$. Hence:

$$K = \frac{k_5 [I]^{a+1}}{k_6} = \frac{0.0578 \times 10^{-2}}{2.15 \times 10^{-3}} = 0.269 \times 10^3 \text{mol dm}^{-3}$$

Outside the region of linearity the following relations hold:

$$\frac{k_5}{k_6} = \frac{[I]^{a+1}}{K}$$  \hspace{1cm} (23)

$$\frac{1}{K_i} = \frac{1}{K} \left(1 - \frac{[I]^{a+1}}{K} \right)$$  \hspace{1cm} (24)

$$\frac{1}{i_n} = \frac{1}{i_{nmax}} \left[ \frac{K_n}{K} \left(1 - \frac{[I]^{a+1}}{K} \right) \left([I]/[I_n] \right) \left(1 + \frac{K_n}{[I_n]} \right) \right]$$  \hspace{1cm} (25)
A new nonlinear function $1/\nu_{5}$ vs $[I]$ was obtained. All the parameters in eqn. (25) have constant values over the whole range of inhibitor concentrations from 0.25 to 10 mmol dm$^{-3}$. After introducing the numerical data eqn. (25) changes into eqn. (26):

$$10^{-6}\nu_{5} = 0.146 [37.235(I) \times 10^{3}]_{2.67} + ([I] \times 10^{3}) + 1.12$$

(26)

![Figure 6. Plot of inversion constant $k$ (s$^{-1}$) vs concentration of acetohydroxamic acid; [I] (mol dm$^{-3}$).](image)

$k$ Values taken from Table 3. The solid line in the main plot is a representation of eqn. (15): $Y = 0.89 X^2 + 1.46 X$. In the insert a double logarithmic plot of the same values ($k; 10^6 [I]$) is the line (16): $Y = 1.67 X + 0.31$.

Equation (26) is presented as a dotted line in Fig. 4B. The experimental points, with satisfactory accuracy, are well approximated by eqn. (26).

CONCLUDING REMARKS

An enzymatic system with a competitive slow-binding inhibitor, not disturbed, is characterized by seven constants: $[S]$ $k_1$, $k_2$, $[I]k_3$, $k_4$, $k_5$, $k_6$, $k_7$ (s$^{-1}$). In such a system four secondary parameters: $K_M$, $v_{\text{MAX}}$, $K_I$ and $K_{I^*}$ are independent of inhibitor concentration, whereas three: $1/\nu_0$, $1/\nu_{5}$ and $k$ show a linear dependence. Symptoms of disturbance are connected with the constant $k_{5*}$. It remains invariable at $[I] \leq 1$ mmol dm$^{-3}$. At higher concentrations of the inhibitor, $k_{5}$, as the only parameter of the seven given above, does not remain constant. The disturbance is transferred onto the constants $K_{I^*}$ and $k$ which are directly dependent on $k_{5*}$. The disturbance of the system can be followed in scheme (1); it occurs in the inversion $EI \rightleftharpoons EI^*$. The observed effect of inhibitor concentration on the value of both inversion constant $k$ and rate constant $k_{5}$ provides sound evidence that the inactive complex $EI^*$ is a product of the reaction: $aI + EI = EI_{a+1}$. The composition of the complex seems to be independent of the concentration of [I], and the stoichiometric coefficient 'a' is close to 2/3. This leads to the formula (3EI $\cdot$ 2I). Therefore, to inactivate 3 active sites of urease 5 inhibitor molecules are needed. It is probable that the ratio 3:5 corresponds to the scheme (4) presented by Todd & Hausinger in [9]. As the urease active site contains two nickel atoms (confirmed in our laboratory by Olesch [17] with the use of EPR), the following coordination systems corresponding to the ratio 3E:5I are possible:

$$\left\{ \begin{array}{c} \text{Ni-AHA} \text{Ni-AHA} \text{Ni-AHA} \text{Ni-AHA} \\ \text{Ni-AHA} \text{Ni-AHA} \text{Ni-AHA} \text{Ni-AHA} \end{array} \right\}$$

REFERENCES


