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Calorimetric study of inhibition of urease by 2-mercaptoethanol
Procedures based upon integrated rate equations

Adam Juszkiewicz, Mirosława Kot*, Wiesława Zaborska

Faculty of Chemistry, Jagiellonian University, 30-060 Kraków, Ingardena 3, Poland

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Abstract

Urease-catalyzed hydrolysis of urea was studied in the absence, and presence, of 0.3 and 0.8 mmol dm^{-3} 2-mercaptoethanol in phosphate buffer at pH 7.0 at 25°C with the use of an isoperibol calorimeter. The extent of reaction with time, ΔT vs. t, was interpreted with the help of the integrated Michaelis–Menten equation, and the inhibition constant K_i was obtained from linear transformations of the equation (Jennings–Niemann, Yun–Suelter and Booman–Niemann). The obtained value of K_i was equal to 0.87 ± 0.10 mmol dm^{-3}. © 1998 Elsevier Science B.V.

Keywords: Enzymatic calorimetry; 2-Mercaptoethanol; Urease; Urease inhibition

1. Introduction

The study of urease inhibition has a medical and agronomic significance, as well as providing insight into the urease catalytic mechanism [1–4].

Inhibition constants are commonly determined by differential methods from initial reaction rates, and require the observation of the system at short initial-reaction periods. The reaction has to be carried out in a series of solutions of growing substrate concentration at a fixed inhibitor concentration. Less commonly used methods involve applied integration methods based on the reaction progress curves, monitoring the whole duration of the reaction up to the thorough exhaustion of the substrate [5]. Integration methods require observation of two solutions: one without, and the other with an inhibitor. The buffer of the reaction mixture should be selected in such a way that its pH does not change throughout the whole period of the observation.

The enzyme urease catalyzes the hydrolysis of urea to yield ammonia and carbon dioxide: $CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2$. The enzymatic hydrolysis of urea is an exothermic process: for which $\Delta H = -59\,580$ J mol^{-1} in phosphate buffer and $\Delta H = -16\,580$ J mol^{-1} in TRIS buffer [6]. The measurement of temperature increment, ΔT, for the reaction system produces a record of the total reaction progress curve ΔT vs. t. The calorimetric technique monitors the reaction continuously and is, therefore, independent of the limitations of other methods. The progress curves of enzymatic urea hydrolysis are frequently recorded by determination of ammonia concentration by the phenol-hypochlorite method over the time intervals of the reaction. This technique requires numerous sampling of the reaction mixture;

*Corresponding author.

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besides it coincides with some inhibitors, e.g. those containing thiol groups –SH [7]. In another continuous method, which uses glutamate dehydrogenase, a lag in recording occurs as compared to the reaction progress. The interpretation of the progress curves is, therefore, difficult; besides, the coupled enzymatic reaction may be inhibited by urease inhibitors [8]. Other methods of measurement of urea hydrolysis rates, such as ammonium ion electrodes [9], pH-stat [10] or pH indicator assay [11], cannot be applied for recording of the total reaction progress curves, because of high buffer concentrations required for the assay of thorough decomposition of urea.

Calorimetric technique has been used for the determination of K_M, v_{max} and K_i values for urea–urease–Cd(II) system [12,13], by means of Lineweaver–Burk and Hanes linear transformations.

The effect of boric acid on the activity of urease has been studied previously. The reaction progress curves were recorded with the use of calorimetric and phenol-hypochlorite techniques and the results obtained with both techniques were in agreement [14].

In this study, the kinetics of inhibition of jack bean urease by 2-mercaptoethanol was investigated by calorimetric technique using integration methods. The value of the inhibition constant determined, K_i, was compared with the values, determined by Dixon et al. [15], with spectrophotometric and pH-stat techniques.

2. Symbols and basic equations

S_0, S	substrate (urea) concentrations; initial and remaining after time t, mmol dm^{-3}
P_0, P	product concentrations; initial and formed in time t, mmol dm^{-3}, P=[NH_3]/2
K_M	Michaelis constant, mmol dm^{-3}
v	reaction rate, mmol dm^{-3} s^{-1}, v=dP/dt
K_i	inhibition constant, mmol dm^{-3}
ε	fraction of conversion
ΔT, ΔT_{max}	corrected heat increments; transient and final, °C
m, n	coefficients of straight lines, slope and intercept.

The following relationships hold:

$$P + S = S_0,\ S = S_0(1-\varepsilon) \text{ and } \varepsilon = \Delta T/\Delta T_{max} \quad (1)$$

An uninhibited enzymatic reaction is described by the hyperbolic Michaelis–Menten equation Eq. (2):

$$v = -\frac{dS}{dt} = \frac{v_{max}S}{K_M + S} \quad (2)$$

For a competitively inhibited enzymatic reaction, Eq. (2) becomes:

$$v = \frac{v_{max}S}{K_M\left(1+\frac{I}{K_i}\right) + S} \quad (3)$$

Eq. (3) can be integrated in two different ways: one leads to Eq. (4) and the other to Eq. (5):

$$\frac{P}{v_{max}} - \frac{K_M}{v_{max}}\left(1+\frac{I}{K_i}\right)\ln\left(1-\frac{P}{S_0}\right) = t \quad (4)$$

$$K_M\left(1+\frac{I}{K_i}\right) + \frac{1}{2}(S+S_0) = -\frac{v_{max}\int_0^t S dt}{S-S_0} \quad (5)$$

Introduction of calorimetric parameters (Eq. (1)) to Eqs. (4) and (5) gives Eqs. (4a) and (5a):

$$\frac{S_0}{v_{max}}\frac{\Delta T}{\Delta T_{max}} - \frac{K_M}{v_{max}}\left(1+\frac{I}{K_i}\right) \times \ln\left(1-\frac{\Delta T}{\Delta T_{max}}\right) = t \quad (4a)$$

$$K_M\left(1+\frac{I}{K_i}\right) + \frac{S_0}{2}\left(\frac{\Delta T}{\Delta T_{max}}+1\right) = -\frac{v_{max}\int_0^t \Delta T dt}{\Delta T - \Delta T_{max}} \quad (5a)$$

K_i can be determined from two successive progress curves monitoring the reactions in the absence, and presence, of an inhibitor, respectively. In this study, four linearization procedures based on the integrated rate equation Eqs. (4a) and (5a) were applied. Each procedure changes the function, ΔT vs. t, into a straight line Y=mX+n, in which $Y$and X are dependent on reaction time and concentration.

3. Experimental

3.1. Materials

The jack-bean urease used was of the Sigma type III, of specific activity 33 units/mg protein. One unit is the amount of enzyme that liberates 1.0 μmol of NH_3 from urea per minute at pH 7 and 25°C. 2-Mercaptoethanol (Analar grade) was purchased from Sigma. Urea and all other chemicals (Analar grade) were obtained from POCh, Gliwice, Poland.

3.2. Enzymatic reaction

The hydrolysis of urea catalyzed by jack-bean urease was studied in phosphate buffer pH 7.0 (100 mmol dm^{-3}, 2 mmol dm^{-3} EDTA) at 25°C. The initial concentration of urea was 10 mmol dm^{-3}. The concentrations of 2-mercaptoethanol studied were 0.3 and 0.8 mmol dm^{-3}. The reaction was initiated by the addition of 0.5 cm^3 urease solution (20 mg cm^{-3}) to 100 cm^3 of assay mixtures.

3.3. Experimental techniques

The progress of the urea hydrolysis in the absence, and presence, of 2-mercaptoethanol was observed in the isoperibol calorimetric set. The rate of temperature change was measured at 2-s intervals with an accuracy of 0.0001°C. The starting temperature was 25.000±0.001°C. The observed differential increments, ΔT, were corrected for heat exchange. The details of the calorimetric apparatus has been presented previously [16].

4. Results

Three reaction curves of ΔT vs. t were recorded: one in the absence of the inhibitor, and the other two in the presence of 0.3 and 0.8 mmol dm^{-3} 2-mercaptoethanol. The temperature increments were corrected for heat loss (Fig. 1). The axis on the right-hand side of Fig. 1 expresses fraction conversion ε. The corrected progress curves were approximated with polynomials of the fourth degree.

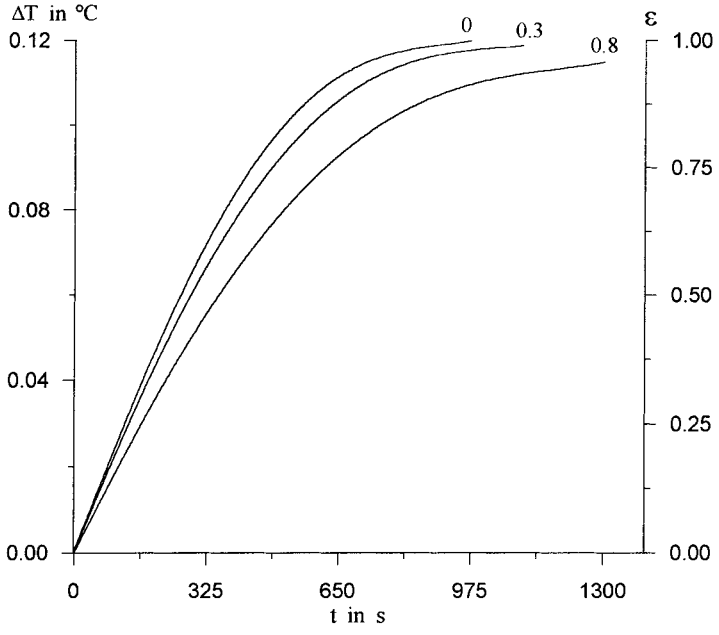

Fig. 1. Reaction progress curves of urease-catalyzed hydrolysis of urea recorded by the calorimetric technique; curve 0 for the uninhibited reaction; curves 0.3 and 0.8 for the reactions inhibited with 0.3 and 0.8 mmol dm^{-3} 2-mercaptoethanol. Curves approximated with polynomials of the fourth degree.

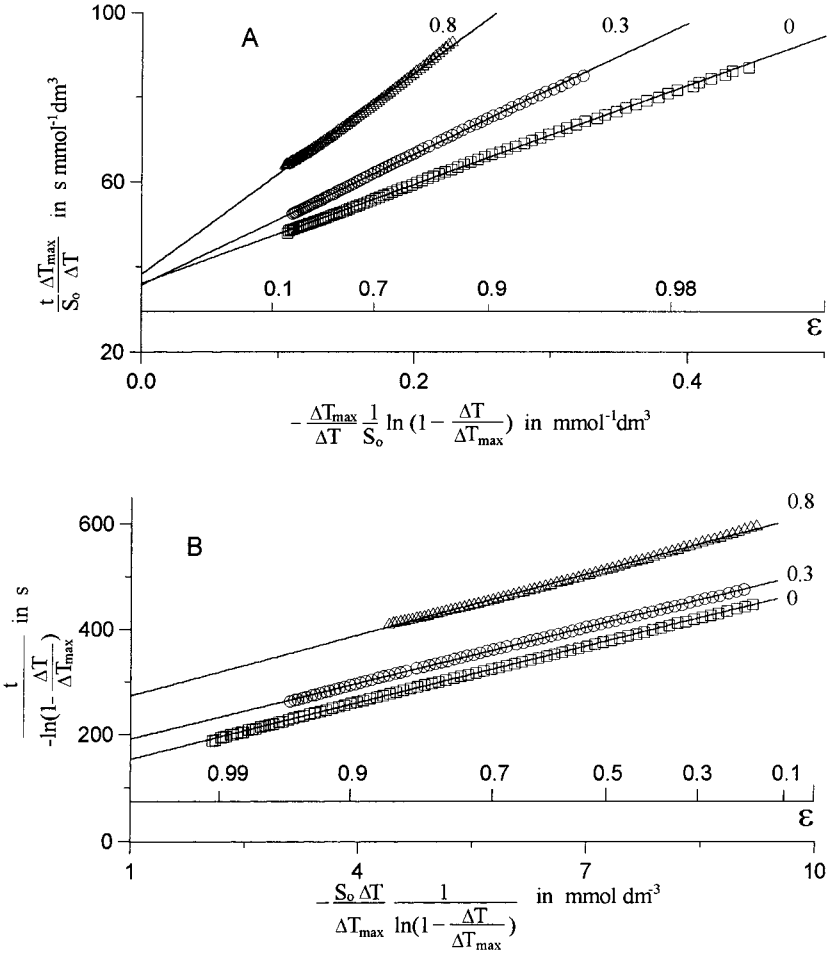

Fig. 2. Replots of the progress curves of Fig. 1 obtained by the integration methods of: (a) Jennings–Niemann (I), (b) Jennings–Niemann (II). 0 – uninhibited reaction; 0.3, 0.8 – inhibited reactions. The added ε-axes show the sections of the progress curves from which the linear replots were obtained.

4.1. Linearization procedures

1. The Jennings–Niemann procedure (I) [17].

The procedure employs an integrated rate equation Eq. (4a) in the form:

$$\frac{t}{S_0}\frac{\Delta T_{max}}{\Delta T} = -\frac{K_M}{v_{max}}\left(1+\frac{I}{K_i}\right)\frac{\Delta T_{max}}{\Delta T}\frac{1}{S_0} \times \ln\left(1-\frac{\Delta T}{\Delta T_{max}}\right)+\frac{1}{v_{max}} \qquad (6)$$

where S_0, ΔT_{max} are the values fixed for all three studied systems; S_0=10 mmol dm^{-3}; and ΔT_{max}= 0.1200°C.

A plot of $(t/S_0)(\Delta T_{max}/\Delta T)$ vs. $-(\Delta T_{max}/\Delta T)(1/S_0)\ln(1-(\Delta T/\Delta T_{max}))$ is linear with a slope $m = (K_M/v_{max})(1+(I/K_i))$.

The linear replots of the polynomial progress curves 0, 0.3 and 0.8 are presented in Fig. 2(a). The value of K_i was calculated from Eqs. (7) and (8) with I=0.3 and 0.8, respectively:

$$\left(\frac{K_i}{I}\right)_{0.3} = \frac{m_0}{m_{0.3}-m_0};\ K_i = 0.93\ \text{mmol dm}^{-3} \qquad (7)$$

$$\left(\frac{K_i}{I}\right)_{0.8} = \frac{m_0}{m_{0.8}-m_0};\ K_i = 0.76\ \text{mmol dm}^{-3} \qquad (8)$$

The relationships:

$$X = -\frac{1}{S_0}\frac{\ln(1-\varepsilon)}{\varepsilon};\ X_{\varepsilon\to 0} = \frac{1}{S_0}$$

$$Y = \frac{1}{S_0}\frac{t}{\varepsilon};\ Y_{\varepsilon\to 0} = \frac{1}{v_0}$$

indicate that variables X and Y are definite at ε=0, and are indefinite at ε=1. The point X=0 has no physical sense.

2. The Jennings–Niemann procedure (II) [17].

The procedure uses Eq. (4a) rearranged in the following form:

$$-\frac{t}{\ln(1-(\Delta T/\Delta T_{\max}))} = -\frac{1}{v_{\max}}\frac{S_0\Delta T}{\Delta T_{\max}}\frac{1}{\ln(1-(\Delta T/\Delta T_{\max}))} + \frac{K_M}{v_{\max}}\left(1+\frac{I}{K_i}\right) \quad (9)$$

A plot of

$$-\frac{t}{\ln(1-(\Delta T/\Delta T_{\max}))} \text{ vs. } -\frac{S_0\Delta T}{\Delta T_{\max}}\frac{1}{\ln(1-(\Delta T/\Delta T_{\max}))}$$

is linear with an intercept $n = (K_M/v_{\max})(1+(I/K_i))$.

Fig. 2(b) shows the replots of the polynomial curves 0, 0.3 and 0.8. The values of K_i were calculated from Eq. (10) for I=0.3 and from Eq. (11) for I=0.8:

$$\left(\frac{K_i}{I}\right)_{0.3} = \frac{n_0}{n_{0.3}-n_0};\ K_i = 0.92\,\text{mmol dm}^{-3} \quad (10)$$

$$\left(\frac{K_i}{I}\right)_{0.8} = \frac{n_0}{n_{0.8}-n_0};\ K_i = 0.80\,\text{mmol dm}^{-3} \quad (11)$$

The following relationships are held:

$$X = -S_0\frac{\varepsilon}{\ln(1-\varepsilon)};\ X_{\varepsilon\to 0} = S_0$$

$$Y = -\frac{t}{\ln(1-\varepsilon)};\ Y_{\varepsilon\to 0} = \frac{S_0}{v_0}$$

At ε=1, variables X and Y are indefinite.

3. The Yun–Suelter procedure [18].

The procedure uses Eq. (4a) in the form:

$$\frac{\Delta T_{\max}}{S_0}\frac{t_j-t_i}{\Delta T_j-\Delta T_i} = \frac{K_M}{v_{\max}}\left(1+\frac{I}{K_i}\right) \times \ln\left(\frac{\Delta T_{\max}-\Delta T_i}{\Delta T_{\max}-\Delta T_j}\right) + \frac{1}{v_{\max}} \quad (12)$$

where ΔT_j and ΔT_i are corrected temperature increments corresponding to times t_j and t_i of the reaction, respectively. Each point of the curve in the Yun–Suelter coordinate system represents two points of the progress curve (t_i; ΔT_i) and (t_j; T_j), located at any chosen fixed-time interval ($\Delta t = t_j - t_i$=60 s).

A plot of

$$\frac{\Delta T_{\max}}{S_0}\frac{t_j-t_i}{\Delta T_j-\Delta T_i} \text{ vs. } \ln\left(\frac{\Delta T_{\max}-\Delta T_i}{\Delta T_{\max}-\Delta T_j}\right)$$

is linear with a slope $m = (K_M/v_{\max})(1+(I/K_i))$

The linear replots of the polynomial progress curves 0, 0.3 and 0.8 are presented in Fig. 3(a). The values of K_i were calculated from the following relationships:

$$\left(\frac{K_i}{I}\right)_{0.3} = \frac{m_0}{m_{0.3}-m_0};\ K_i = 0.99\,\text{mmol dm}^{-3} \quad (13)$$

$$\left(\frac{K_i}{I}\right)_{0.8} = \frac{m_0}{m_{0.8}-m_0};\ K_i = 0.81\,\text{mmol dm}^{-3} \quad (14)$$

The relationship: $X = 1/(S_0(1-\varepsilon))$ holds good. Variables X and Y are definite at ε=0, and are X=1/S_0 and Y=1/v_0. For ε=1, X is indefinite. The point X=0 at which the lines intersect has no physical sense.

4. The Booman–Niemann procedure [19].

The following form of the integrated rate equation Eq. (5a) is used:

$$\frac{\Delta T_{\max}t - \int_0^t \Delta T\,dt}{\Delta T} = \left(S_0 - \frac{S_0\Delta T}{\Delta T_{\max}}\right)\frac{1}{2v_{\max}} + \frac{2K_M(1+(I/K_i)) + S_0}{2v_{\max}} \quad (15)$$

The integrals $\int_0^t \Delta T\,dt$ were calculated by integrating the approximating polynomials. A plot of

$$\frac{\Delta T_{\max}t - \int_0^t \Delta T\,dt}{\Delta T} \text{ vs. } \left(S_0 - \frac{S_0\Delta T}{\Delta T_{\max}}\right)$$

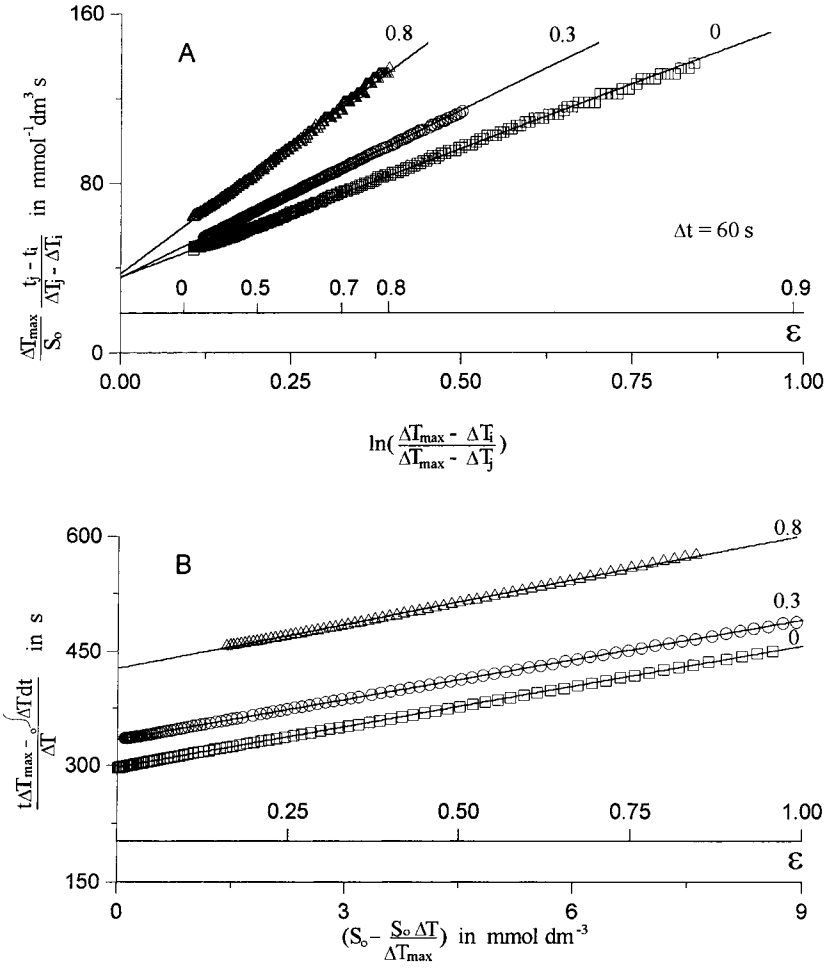

Fig. 3. Replots of the progress curves of Fig. 1 obtained by the integration methods of: (a) Yun–Suelter, (b) Booman–Niemann. 0 – uninhibited reaction; 0.3, 0.8 – inhibited reactions. The added ε-axes show the sections of the progress curves from which the linear replots were obtained.

is linear with a slope $m = 1/2v_{max}$ and intercept $n = (2K_M(1 + (I/K_i)) + S_0)/(2v_{max})$.

Fig. 3(b) shows the linear replots of the polynomials curves 0, 0.3 and 0.8. The values of K_i were calculated from the following relationship:

$$\left(\frac{K_i}{I}\right)_{0.3} = \frac{n_0 - m_0 S_0}{n_{0.3} - n_0};\ K_i = 0.99\,\text{mmol}\,\text{dm}^{-3} \tag{16}$$

$$\left(\frac{K_i}{I}\right)_{0.8} = \frac{n_0 - m_0 S_0}{n_{0.8} - n_0};\ K_i = 0.74\,\text{mmol}\,\text{dm}^{-3} \tag{17}$$

The Booman–Niemann function is the only function, of all those applied here, which is definite at both limits $0 \leq \varepsilon \leq 1$, $X = S_0(1-\varepsilon)$.

The range (expressed with ε) applied in the linearization procedures is given in respective figures.

The values of the inhibition constant K_i obtained using the above presented linearization procedures are listed in Table 1.

Several thiol compounds were shown to be competitive inhibitors of urease [15,20–24]. The pH dependence of inhibition constant and spectroscopic studies demonstrate that –SH group forms a charge transfer complex with nickel ion(s) present in the

Table 1
Inhibition constants of urea hydrolysis catalyzed by urease (jack bean) inhibited by 0.3 and 0.8 mmol dm^{-3} 2-mercaptoethanol, obtained by integration methods

Procedure	K_i; mmol dm^{-3}	
	I=0.3 mmol dm^{-3}	I=0.8 mmol dm^{-3}
Jennings–Niemann (I)	0.93	0.76
Jennings–Niemann (II)	0.92	0.80
Yun–Suelter	0.99	0.81
Booman–Niemann	0.99	0.74
Mean value	0.87±0.10	

active site. The protonated thiol is inactive as an inhibitor.

The inhibition constants of different ureases by 2-mercaptoethanol are compared in Table 2.

5. Discussion

The inhibition constant K_i was determined from the total reaction curves recorded for urease-catalyzed hydrolysis of urea in the absence, and presence of 2-mercaptoethanol by the calorimetric technique (ΔT vs. t). Four linear transformations of the integrated Michaelis–Menten equation (linearization procedures) were chosen for calculations. The Yun–Suelter procedure transforms the initial section of the progress curves; the other procedures are effective in the central section of the progress curves. The Booman–Niemann procedure was found to be effective in the broadest section.

The linear replots of the polynomial progress curves representing the system without and with 2-mercaptoethanol (0.3 and 0.8 mmol dm^{-3}) either intersect at one point corresponding to $1/v_{max}$ (Fig. 2(a) and Fig. 3(a)) or have the same slope, as $1/v_{max}$ (Fig. 2(b)) or to $1/(2v_{max})$ (Fig. 3(b)); v_{max}=2.7±0.1 mmol dm^{-3} s^{-1}. The foregoing proves that 2-mercaptoethanol is a competitive inhibitor of urease.

The mean value of the inhibition constant of 2-mercaptoethanol is equal to 0.87±0.10 mmol dm^{-3}, and is in agreement with the values obtained by Dixon et al. with spectrophotometric and pH-stat methods.

The calorimetric technique provides a convenient continuous recording of reaction progress curves, suitable for application in integration methods of kinetic study of enzymatic reactions. The disadvantage of the technique, however, is that it is sensitive to the duration of the experiment. In the presence of a strong inhibitor the reaction time becomes long and the heat loss may become uncontrollable. That is why the progress curve recorded for the reaction without the inhibitor was transformed into a straight line in the range broader than the curves recorded for the reactions with the inhibitor.

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Table 2
Inhibition constants of ureases by 2-mercaptoethanol

Urease	Technique	pH buffer	Total inhibitor, K_i mmol dm^{-3}	Thiolate anion K_i^a μmol dm^{-3}	Ref.
	pH-stat differential	7.1 HEPES+phosphate	0.72±0.26	–	[15]
Jack bean (plant)	UV-visible spectra	7.12 N-ethylmorpholine-HCl	0.95±0.05	–	[15]
	calorimetric integration	7.0 phosphate	0.87±0.01	2.28±0.20	this paper
Klebsiella aerogenes (bacterial)	ammonia selective-electrode differential	7.75 HEPES	0.55±0.08	7.7	[24]

K_i^a values for the thiolate anion are calculated from the literature value of acid dissociation constant of 2-mercaptoethanol, equal to 9.58 [25].

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