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# Calorimetric investigations into enzymatic urea hydrolysis

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**Abstract**

Enzymatic urea hydrolysis has been investigated calorimetrically as a function of urea concentration, the buffer system and the pH value. It is shown that the reaction follows a complex mechanism. Unambiguous conclusions on the reaction sequences are made possible by extensive thermodynamic calculations. The most important conclusion from the experimental results and thermodynamic calculations is the absolute necessity of knowledge of the experimental conditions, i.e. urea concentration, pH value and buffer system, for the meaningful discussion of calorimetric data.

The molar reaction enthalpy of the enzymatic partial reaction is determined to be $\Delta_R H_1 = (-26.5 \pm 1.5)$ kJ per mole urea. Thermokinetic evaluation by means of a simple consecutive reaction model allows the velocity constants to be calculated. For the enzymatic partial reaction, parameters $K_m$ and $v_{max}$ are determined in phosphate buffer and HEPES buffer.

*Keywords:* Buffer; Calorimetry; Enzyme; Hydrolysis; pH; Urea

## 1. Introduction

Enzymatic urea hydrolysis has been the subject of numerous studies in recent years [1,2]. The interest in this enzyme-catalyzed reaction is due to its possible use for urea determination. At the same time, the well known inhibitory effect of heavy metals on enzyme activity [3,4] can be used for toxicity screening. Optimum enzyme

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activity is attained in the pH range between 6 and 8 in urea hydrolysis with urease. Calorimetric measurements with various buffer systems [5,6] produced molar reaction enthalpies which varied strongly from study to study, which in turn led to different interpretations of the reaction.

Particularly for analytical methods using thermal detection (thermal sensors), exact knowledge of the influence of the buffer system, pH value and urea concentration on the reaction, and thus on the reaction enthalpy and kinetics, is a necessary precondition for data evaluation.

This study presents the results of extensive calorimetric measurements on enzymatic urea hydrolysis in four different buffer systems with varying pH values. By detailed thermodynamic calculations, exact statements have been obtained on the establishment of the hydrolysis equilibria so that a satisfactory interpretation of the experimental results has become possible. The thermokinetic evaluation by a simple consecutive reaction model and the subsequent application of the Michaelis–Menten relation show the different influences that different buffer systems have on enzyme activity.

## 2. Experimental

Calorimetric measurements were carried out in an isoperibolic normal temperature calorimeter [7] or an LKB type 8700 calorimeter. Before the reaction in the calorimeter, the investigated systems were thermostatted to a constant temperature of 298.15 K.

The details of the calorimetric measurements and their evaluation have been communicated in an earlier publication [8]. The urease (EC 3.5.1.5.) employed (obtained from Serva) had a specific activity of 88 U $mg^{-1}$.

Phosphate buffers according to Sörensens, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 2-(*N*-morpholino)ethanesulphonic acid (MES) and tris(hydroxymethyl)-aminoethane (TRIS) were used as buffers. The urea concentration was varied in a range between 2.4 and 25 mmol per kg buffer solution.

## 3. Results and discussion

The experimental conditions (pH value and buffer system) of enzymatic urea conversion were selected mainly depending on the optimum pH value for urease activity. The catalytic effectiveness of urease is limited to a relatively narrow range of pH 6–8, with the optimum pH value depending on the type of buffer; with the exception of acetate buffer, it corresponds roughly to the $pK_s$ value of the buffer [9].

In spite of the narrow pH range and the therefore limited number of buffers suitable for measurement, the literature reflects different opinions on the type of final products of enzymatic urea conversion and considerable disagreement concerning the reaction enthalpy per mole of urea conversion $\Delta_R H_H$ [6,10,11].

Table 1
Comparison between $\Delta_R H_H$ for different buffer systems and pH values obtained in this study and in the literature

| Buffer | pH | $-\Delta_R H_H$/(kJ mol$^{-1}$) | Ref. |
|---|---|---|---|
| Phosphate buffer | 7.6 | 50.9 ± 1.4 | This work |
| | 7.5 | 61.3 ± 1.0 | [6] |
| | 6.86 | 58.6 ± 1.1 | This work |
| | 6.7 | 48.3 ± 1.0 | [6] |
| | 6.5 | 61.0 ± 0.9 | This work |
| | 6.0 | 52.9 ± 2.0 | |
| Tris buffer | 7.0 | 16.4 ± 0.7 | This work |
| | 7.0 | 7.1 ± 1.0 | [5] |
| | 8.0 | 8.4 ± 1.0 | [5] |
| Citrate buffer | 6.7 | 21.9 ± 0.6 | [6] |
| MES | 6.3 | 46.0 ± 1.2 | This work |
| HEPES | 7.0 | 42.0 ± 1.1 | This work |

Table 2
Reaction enthalpy $\Delta_R H_A$ (ammonium carbamate hydrolysis) in different buffer systems

| Buffer | pH | $\Delta_R H_A$/(kJ mol$^{-1}$) |
|---|---|---|
| Phosphate | 6.86 | −32.7 ± 0.1 |
| HEPES | 7.0 | −14.7 ± 0.1 |
| MES | 6.3 | −18.2 ± 0.1 |
| TRIS | 8.0 | 8.7 ± 0.4 |

Table 1 gives a comparison of the values obtained in this study for the reaction enthalpy per mol of urea conversion $\Delta_R H_H$ with literature data. The values unambiguously show that the different values of $\Delta_R H_H$ were determined for different buffer systems and pH values. This suggests a complex reaction mechanism (overall reaction) consisting of several partial reactions and/or different reaction products.

The following eight partial reactions have to be taken into account for the overall reaction:

$$CO(NH_2)_2 + H_2O \xrightarrow{\text{Urease}} NH_2COO^- + NH_4^+ \quad (1)$$

$$NH_2COO^- + H_2O \longrightarrow NH_3(aq) + HCO_3^- \quad (2)$$

$$NH_3(aq) + H^+ \longrightarrow NH_4^+ \quad (3)$$

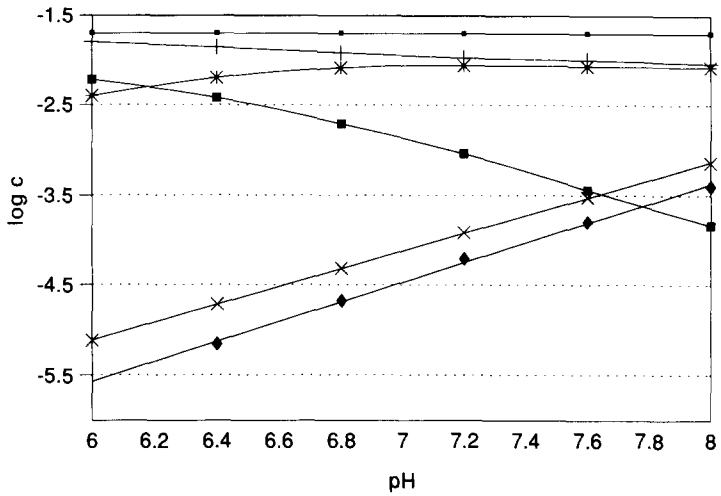


Fig. 1. Species distribution as a function of the pH value when $c^0_{urea} = 0.01$ mmol $l^{-1}$: ■, $CO_2$; □, $NH_4^+$; ×, $NH_3$; +, $H^+$; ◆ $CO_3^{2-}$; *, $HCO_3^-$.

$$HCO_3^- + H^+ \longrightarrow CO_2(aq) + H_2O \quad (4)$$

$$HCO_3^- \longrightarrow CO_3^{2-} + H^+ \quad (5)$$

$$CO_2(aq) \longrightarrow CO_2(g) \quad (6)$$

$$NH_3(aq) \longrightarrow NH_3(g) \quad (7)$$

$$H^+ + B^- \longrightarrow HB \quad (8)$$

Equation (8) represents the buffer reaction.

Depending on the selected pH value, the degree of hydrolysis will be changed, and the above pH-dependent partial reactions will take place to different extents. Due to the different protonation enthalpies (reaction (8)) of the different buffers [12], the selection of the buffer system has a strong influence on the reaction enthalpy $\Delta_R H_H$ of the overall reaction.

Experimentally it was possible to investigate calorimetrically the ammonium carbamate hydrolysis (Eq. (2)) separately from reaction (1). The results obtained for the reaction enthalpy $\Delta_R H_A$ for the conversion of 1 mol ammonium carbamate according to the partial reactions (2)–(8) are given in Table 2. With these values of the reaction enthalpy $\Delta_R H_A$ and those for the overall reaction of urea conversion $\Delta_R H_H$ it is possible to calculate the molar reaction enthalpy of the enzymatic partial reaction $\Delta_R H_1$. Independently of the selected buffer system and the pH value, $\Delta_R H_1 = (-26.5 \pm 1.5)$ kJ $mol^{-1}$ was determined for the enzymatic reaction (1). This value is in good agreement with the literature data [13].

Our results have not confirmed ammonium carbamate to be the final product of urea hydrolysis in TRIS buffer as proposed in Ref. [6]. Instead, the species of the reactions (3)–(7) should occur as final products of urea hydrolysis.

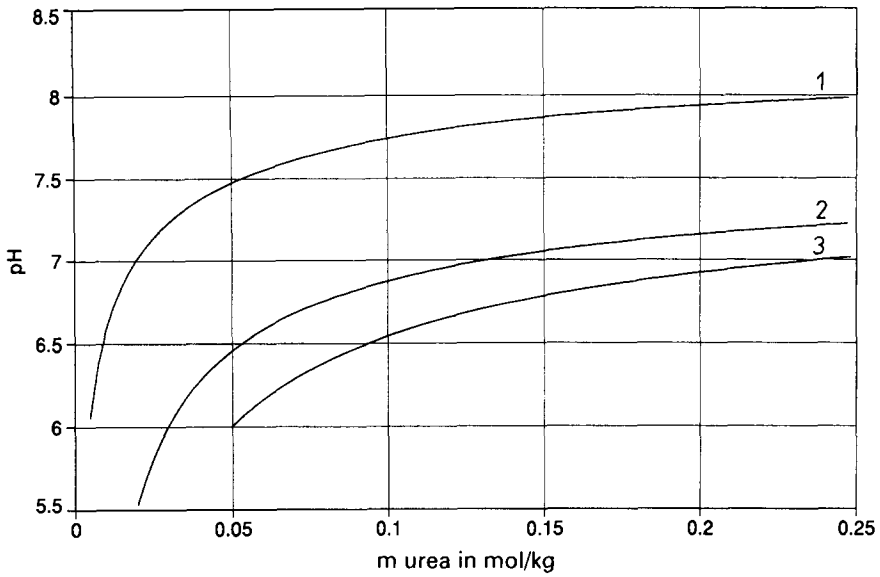


Fig. 2. pH value of $CO_2$ release as a function of the urea concentration and for various external $CO_2$ pressures: curves 1, 0.1 bar; curve 2, 0.6 bar; curve 3, 1 bar.

Table 3
Molar reaction enthalpies of the partial reactions (1)–(8)

| Partial reaction | $\Delta_R H$/(kJ mol$^{-1}$) | Ref. |
|---|---|---|
| (1) | −24.27 | [13] |
| (2) | 12.584 | [14] |
| (3) | −52.216 | [14] |
| (4) | −7.594 | [14] |
| (5) | 14.853 | [14] |
| (6) | 20.334 | [14] |
| (7) | 35.428 | [14] |
| (8) | | |
| Phosphate | 4.7 | [15] |
| HEPES | 16.4 | [12] |
| MES | 12.7 | [12] |
| TRIS | 47.3 | [12] |

For comparison with the experimental reaction enthalpies, the species distribution was calculated as a function of the pH value (6–8) on the basis of the tabular standard values of urea and the hydrolysis products (Eqs. (1) to (8)) [14]. The calculation was done by the program CHEMSAGE [15] on the basis of a minimization of the free enthalpy, taking into account the Debye–Hückel activities and the result is shown in Fig. 1. It is evident that reactions (1)–(3) proceed almost to

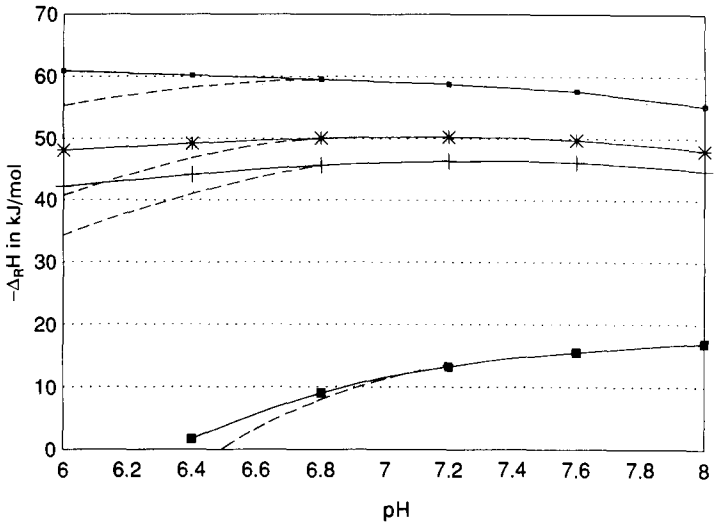


Fig. 3. Reaction enthalpy $\Delta_R H_H$ as a function of the pH value and buffer system: □, phosphate; +, HEPES; *. MES; ■, TRIS.

completion in the pH range investigated whereas the subsequent partial reactions (4)–(8) strongly depend on the pH value. Especially interesting is the $CO_2$ development proposed in Ref. [6]. Figure 2 represents the calculated dependence of the pH value at which the release of $CO_2$ begins as a function of the urea concentration and for various external $CO_2$ pressures. Thus, $CO_2$ release appears to be impossible for the maximum urea concentration in the solution that was reached in this study. Therefore, the decrease of the exothermic character of $\Delta_R H_H$ (particularly for small pH values (pH < 6.8)) in the phosphate buffer probably has different causes.

According to our thermokinetic evaluation, the hydrolysis rate of urea decreases considerably in the phosphate buffer below about pH 6.8 (see also Fig. 7). The reaction times for the complete establishment of the equilibrium become so long (more than 1.5 h) that there is a danger of incomplete registration of the calorimetric effect, so that an apparently lower reaction enthalpy is measured. On the basis of the species distribution calculated with CHEMSAGE and the known molar reaction enthalpies of the partial reactions (Table 3) it was possible to calculate the reaction enthalpy of the overall reaction $\Delta_R H_H$ as a function of the pH value and buffer system; the results are shown in Fig. 3. In addition, Fig. 3 shows the influence of $CO_2$ desorption on the reaction enthalpy at $p(CO_2) = 0.6$ bar.

The dependence represented in the figures and their comparison with the experimental values (Table 1) allow the following conclusions to be drawn:

(1) The calculated values are always 1–2 kJ $mol^{-1}$ higher than the experimental values. This discrepancy is probably caused by not taking into account mixing and/or excess enthalpies in the calculation.

(2) There is a marked influence of the protonation/buffer reaction (8) which leads to different slopes of the $\Delta_R H$–pH curve.

(3) The endothermic and exothermic effects of the partial reactions (1)–(8) almost completely compensate each other in the phosphate, MES and HEPES buffers so that the mean slope $\delta\Delta_R H/\delta\mathrm{pH} \approx 1$ kJ mol$^{-1}$ pH$^{-1}$ is comparatively flat. There is a considerable slope, however, in the TRIS buffer and particularly in the range below pH 7.6.

For the kinetic evaluation of the calorimetric measurements the overall reaction was divided into the following two reactions:

$$\text{Urea} \xrightarrow{k_1} \text{Ammonium carbamate} \xrightarrow{k_2} \text{Products} \tag{9}$$

The first reaction is the enzyme-catalyzed reaction (1) while the second reaction summarizes the equilibria (2)–(8), the conversion of which depends in all cases on the selected reaction conditions (buffer system, pH value).

If a reaction of first order is assumed for both stages, we obtain for the concentration of the reactants

$$c_H = c_H^0 \, e^{-k_1 t} \tag{10}$$

$$c_p = c_H^0[k_1/(k_2 - k_1)](e^{-k_1 t} - e^{-k_2 t}) \tag{11}$$

$$c_A = c_H^0\{1 + [1/(k_1 - k_2)](k_2 \, e^{-k_1 t} - k_1 \, e^{-k_2 t})\} \tag{12}$$

where $c_H$ is the concentration of urea at time $t$, $c_H^0$ is the total urea concentration, $c_P$ is the concentration of products and $c_A$ is the concentration of ammonium carbamate at time $t$.

In accordance with the two partial reactions, the adiabatic total calorimetric effect $\Delta T_{max}$ is subdivided into

$$\Delta T_{max} = \Delta T_{max1} + \Delta T_{max2} \tag{13}$$

where $\Delta T_{maxi}$ is the maximum value of $\Delta T$ for the partial reaction $i$ and $\Delta T_i(t)$ is the adiabatic temperature difference for the partial reaction $i$ at time $t$.

Conversion at time $t$ being proportional to $\Delta T$, we obtain for the first step (ammonium carbamate formation)

$$(c_H^0 - c_H)/c_H^0 = \Delta T_1(t)/\Delta T_{max1} \tag{14}$$

taking into account Eq. (10)

$$\Delta T_1(t) = \Delta T_{max1}(1 - e^{-k_1 t}) \tag{15}$$

By analogy, it holds for the second stage that

$$c_A/c_H^0 = \Delta T_2(t)/\Delta T_{max2} = \{1 + [1/(k_1 - k_2)](k_2 \, e^{-k_1 t} - k_1 \, e^{-k_2 t})\} \tag{16}$$

and

$$\Delta T_2(t) = \Delta T_{max2}\{1 + [1/(k_1 - k_2)](k_2 \, e^{-k_1 t} - k_1 \, e^{-k_2 t})\} \tag{17}$$

Taking into account Eq. (13), we then obtain for the entire reaction

$$\Delta T(t) = \Delta T_{max1}(1 - e^{-k_1 t}) + \Delta T_{max2}\{1 + [1/(k_1 - k_2)](k_2 \, e^{-k_1 t} - k_1 \, e^{-k_2 t})\} \tag{18}$$

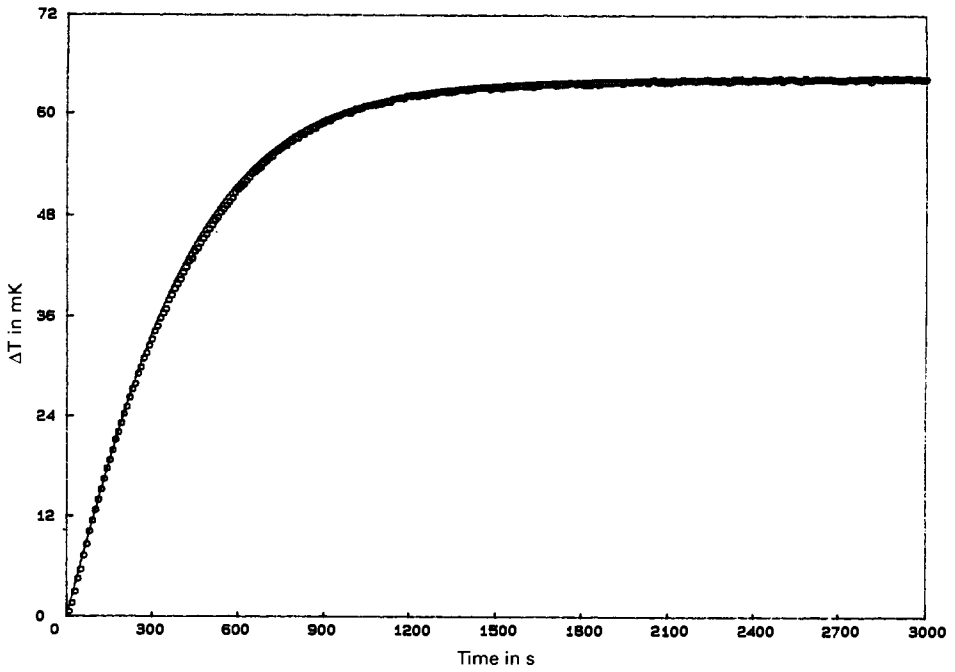


Fig. 4. Adiabatic $T$–time curve when $c_{urea} = 10.15$ mmol/(kg solvent) and HEPES buffer is used; □, exp. values; —, calculated.

After the introduction of a factor $f$ for the fraction of the two partial reactions in the overall reaction

$$\Delta T_{max1} = f\Delta T_{max} \tag{19}$$

$$\Delta T_{max2} = (1-f)\Delta T_{max} \tag{20}$$

Eq. (18) can be simplified to give

$$\Delta T(t) = \Delta T_{max} f(1 - e^{-k_1 t}) + [(1-f)\Delta T_{max}] \{1 + [1/(k_1 - k_2)](k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\} \tag{21}$$

The factor $f$ can be calculated from the proportion of the molar reaction enthalpy of ammonium carbamate hydrolysis to the molar reaction enthalpy of the overall reaction.

In the kinetic evaluation by Eq. (21) of the adiabatic calorimetric measurement curves, which had been corrected for the heat flow, gradual differences were observed in the adaptation depending on the buffer system used. These differences were probably caused by the limited validity of the initial assumption, i.e. that both partial reactions are of first order. Therefore, all further kinetic calculations were done mainly for the measurements in the HEPES buffer. A factor of $f = 0.65$ was obtained for the HEPES buffer.

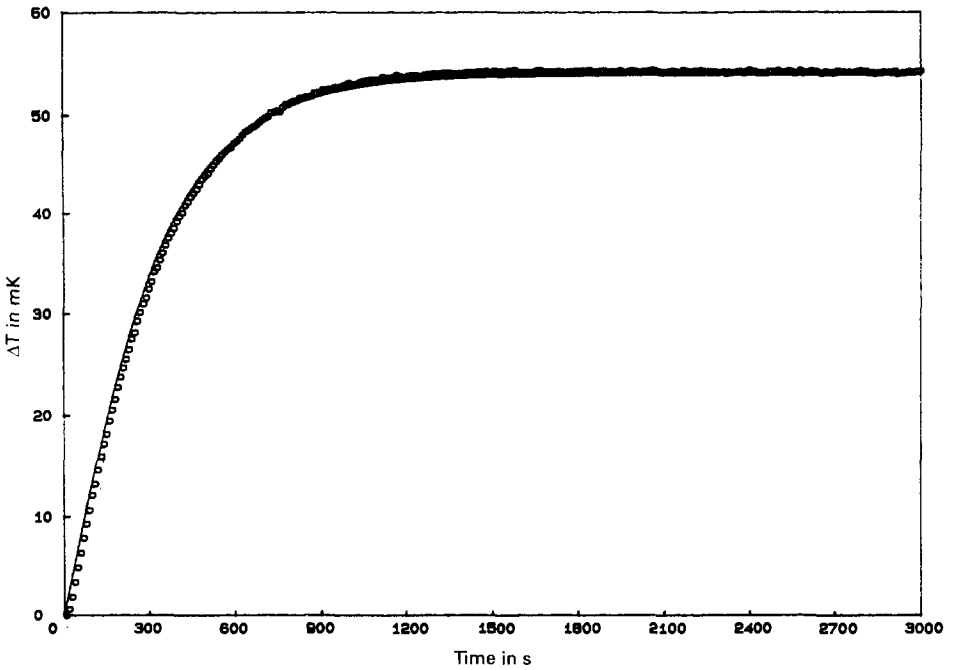


Fig. 5. Adiabatic $T$–time curve when $c_{urea} = 9.104$ mmol/(kg solvent) and HEPES buffer is used: □, exp. values; —, calculated.

Table 4
Results of the computation for velocity constants $k_1$ and $k_2$ in HEPES buffer

| Molality of urea (mmol per kg solvent) | $\Delta T_{max}$/mK | $k_1/(10^3\ s^{-1})$ | $k_2/(10^3\ s^{-1})$ |
|---|---|---|---|
| 4.619 | 29.35 | 6.0 | 7.0 |
| 7.081 | 43.98 | 5.0 | 8.0 |
| 9.104 | 53.96 | 4.0 | 8.0 |
| 10.150 | 64.34 | 3.1 | 7.0 |
| 12.760 | 79.0 | 2.9 | 8.0 |
| 24.160 | 146.25 | 1.7 | 7.0 |

Using this factor and the calculated value of $\Delta T_{max}$, attempts were made to fit the velocity constants $k_1$ and $k_2$ of the calorimetric curves converted to adiabatic conditions by the method of least squares. Figures 4 and 5 show that our kinetic model approximates the measured values well. As a result of the computation, the velocity constants $k_1$ and $k_2$ for measurement in the HEPES buffer are given in Table 4. As was expected, $k_1$ systematically decreases with increasing initial urea concentration whereas $k_2$ is approximately constant.

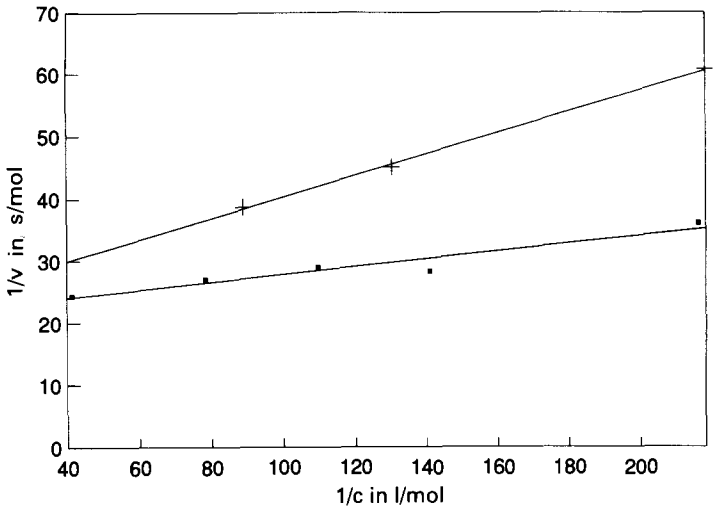


Fig. 6. Lineweaver–Burk diagram for □, HEPES buffer at pH 7.0; +, phosphate buffer at pH 6.86.

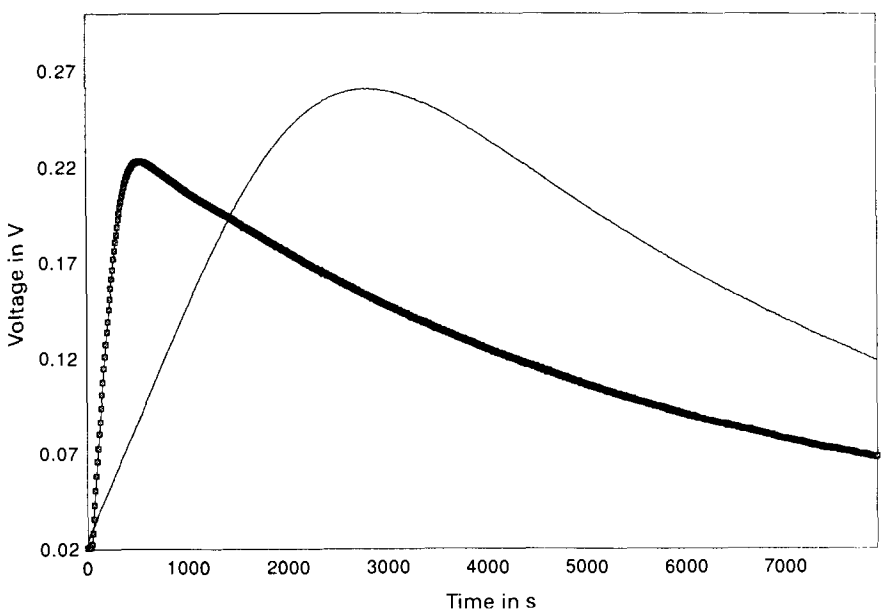


Fig. 7. Calorimetric curves when $c_{urea} = 0.01$ mmol/(kg solvent) in □, MES buffer; +, phosphate buffer.

The decrease of $k_1$ of the enzymatic partial reaction with increasing initial urea concentration is in agreement with the Michaelis–Menten relation

$$v^0 = v_{max} c^0/(K_m + c^0) \tag{22}$$

$$k_1 c_H^0 = v_{max} c_H^0/(K_m + c_H^0) \quad (23)$$

$$k_1 = v_{max}/(K_m + c_H^0) \quad (24)$$

where $v_{max}$ is the maximum velocity, $K_m$ is the Michaelis constant and $v^0$ is the initial reaction rate.

Figure 6 shows a possible evaluation of the measurements in the HEPES and phosphate buffers in the Lineweaver–Burk representation. The following kinetic parameters of the enzymatic reaction are obtained. In HEPES buffer at pH = 7.0 $K_m = (2.9 \pm 0.4)$ mmol $l^{-1}$ and $v_{max} = (46.4 \pm 3.0)$ μmol $l^{-1}$ $s^{-1}$, whilst in phosphate buffer at pH 6.86 $K_m = (7.4 \pm 0.3)$ mmol $l^{-1}$ and $v_{max} = (43.2 \pm 1.8)$ μmol $l^{-1}$ $s^{-1}$. While the maximum rate in the two buffer systems is in good agreement within error limits as was expected, the $K_m$ value for the enzymatic reaction in the phosphate buffer is approximately twice as high as in the HEPES buffer. The dependence of the $K_m$ value on the selected buffer system and pH value is in agreement with literature data [17]. The lower $K_m$ value and the resulting higher rate of the enzymatic reaction in the HEPES buffer ($pK_s = 7.24$) [12] as compared with the reaction in the phosphate buffer ($pK_s = 7.21$) [16] are probably due to different stabilization potential of the intermediate products of enzymatic catalysis.

With respect to investigations into the inhibiting effect of heavy metals on enzymatic urea hydrolysis which are now being prepared, the kinetics at pH 6.0 were of special interest. Related calorimetric measurements were performed in MES and phosphate buffers. Figure 7 shows the two calorimetric curves. There is a marked difference in reaction time. The quantitative evaluation of the measurement in the phosphate buffer is possible only with great uncertainty. The $pK_s$ value in the MES buffer is 6.08 [12]. For this value, urease has a high activity in the MES buffer so that it is essentially suitable for calorimetric measurements with an addition of heavy metal ions.

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