

A new, intrinsic, thermal parameter for enzymes reveals true temperature optima

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Running Title: A new thermal parameter for enzymes

Summary

Two established thermal properties of enzymes are the Arrhenius activation energy and thermal stability. Arising from anomalies found in the variation of enzyme activity with temperature, a comparison has been made of experimental data for the activity and stability properties of 5 different enzymes with theoretical models. The results provide evidence for a new and fundamental third thermal parameter of enzymes, T_{eq} , arising from a sub-second timescale, reversible, temperature-dependent equilibrium between the active enzyme and an inactive (or less active) form. Thus, at temperatures above its optimum, the decrease in enzyme activity arising from the temperature-dependent shift in this equilibrium is up to two orders of magnitude greater than occurs through thermal denaturation. This parameter has important implications for our understanding of the connection between catalytic activity and thermostability, and of the effect of temperature on enzyme reactions within the cell. Unlike the Arrhenius activation energy, which is unaffected by the source (“evolved”) temperature of the enzyme, and enzyme stability, which is not necessarily related to activity, T_{eq} is central to the physiological adaptation of an enzyme to its environmental temperature and links the molecular, physiological, and environmental aspects of the adaptation of life to temperature in a way that has not been previously described. We may therefore expect the effect of evolution on T_{eq} with respect to enzyme temperature/activity effects to be more important than on thermal stability. T_{eq} is also an important parameter to consider when engineering enzymes to modify their thermal properties by both rational design and by directed enzyme evolution.

Introduction

A graph of rate of product generation against temperature is sometimes presented to show the “temperature optimum” (T_{opt}) of an enzyme; however, it is a misconception that this optimum is an intrinsic enzyme property. The descending limb of this plot arises mostly from the denaturation of the enzyme and, since denaturation is both time and temperature dependent, shorter assays give a higher T_{opt} . In this classical description, the variation in enzyme activity with temperature can be described as follows:

$$V_{\text{max}} = k_{\text{cat}} \cdot [E_0] \cdot e^{-k_{\text{inact}} \cdot t} \quad \text{Equation (1)}$$

where V_{max} = maximum velocity of the enzyme; k_{cat} = the enzyme's catalytic constant; $[E_0]$ = total concentration of enzyme; k_{inact} = thermal inactivation rate constant; t = assay duration. Both rate constants, k_{cat} and k_{inact} , are dependent on temperature. This gives rise to temperature-activity graphs as shown in Figure 1a, where it can be seen that the apparent T_{opt} decreases with increasing time during the assay, but at zero time (i.e., under initial rate conditions), no temperature optimum exists (1). In this Classical Model the temperature-dependent behaviour of the enzyme arises from the activation energy of the reaction and the thermal stability of the enzyme.

However, the proposal (1) for a third temperature-dependent property of enzymes, involving the reversible equilibrium between active and inactive forms of an enzyme, implies a “true” temperature optimum. In this model (The Equilibrium Model), the active form of the enzyme (E_{act}) is in reversible equilibrium with an inactive form (E_{inact}) and it is the inactive form that undergoes irreversible thermal inactivation to the thermally-denatured state (X):



In this situation, the concentration of active enzyme at any time point is defined by:

$$[E_{act}] = \frac{[E_o] - [X]}{1 + K_{eq}} \quad \text{Equation (2)}$$

where K_{eq} is the equilibrium constant between active and inactive forms of the enzyme ($K_{eq} = [E_{inact}] / [E_{act}]$). Thus K_{eq} becomes a new temperature-dependent property of an enzyme, in addition to k_{cat} and k_{inact} , and its variation with temperature is given by:

$$\ln (K_{eq}) = \frac{\Delta H_{eq}}{R} \left[\frac{1}{T_{eq}} - \frac{1}{T} \right] \quad \text{Equation (3)}$$

where ΔH_{eq} is the enthalpic change associated with the conversion of active to inactive enzyme, and T_{eq} is the temperature at the mid-point of transition between the two forms. That is, T_{eq} is the temperature at which $K_{eq} = 1$ and $\Delta G_{eq} = 0$; therefore $T_{eq} = \Delta H_{eq} / \Delta S_{eq}$. [We previously (1) used the term T_m to designate this temperature, but now prefer the term T_{eq} , as it is the temperature at which the concentrations of E_{inact} and E_{act} at equilibrium]. In this Equilibrium Model the temperature-dependent behaviour of an enzyme can be explained only by the inclusion of an additional intrinsic thermal parameter, T_{eq} .

The effect of incorporating the parameters K_{eq} and T_{eq} into simulated progress curves (Figure 1b) yields major differences from the Classical Model shown in Figure 1a, showing an initial rate temperature optimum that is obviously independent of assay duration and enabling an experimental distinction between the two models.

To compare the experimental data with the models, five enzymes from a variety of sources were assayed for activity at different temperatures, using continuous assays to allow the simultaneous measurement of activity and thermal stability in the same cuvette, and therefore under identical

conditions. These measurements allow the generation of a unique temperature profile for each enzyme. Most of this work has been carried out on monomeric enzymes to avoid the potentially complicating effects of subunit dissociation.

The data presented support the Equilibrium Model hypothesis, involving K_{eq} as an intrinsic, temperature-dependent property of enzymes. The consequence is that, in such cases, T_{eq} must now be considered as a new thermal parameter that is a characteristic of any particular enzyme and which gives rise to a true temperature optimum.

Experimental Procedures

Enzymes and reagents

Alkaline phosphatase from calf intestinal mucosa was purchased from Roche Applied Science, Basel, Switzerland. Adenosine deaminase from bovine spleen, aryl acylamidase from *Pseudomonas fluorescens* and β -lactamase from *Bacillus cereus* were purchased from Sigma-Aldrich Inc. (St. Louis, MO., USA). Acid phosphatase from wheat germ was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). Reagents for the analysis of the activity of these enzymes were purchased from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany) and Oxoid Ltd (Basingstoke, UK). All other chemicals used were of analytical grade. Buffers were adjusted to the appropriate pH value at the assay temperature, using a combination electrode calibrated at this temperature.

Enzyme Assays

All enzymic activities were measured using continuous assays on a Thermospectronic™ Helios γ -spectrophotometer equipped with a Thermospectronic™ single cell peltier-effect cuvette holder. This system was networked to a computer installed with Vision32™ (Version 1.25, Unicam Ltd) software including the Vision Enhanced Rate Programme capable of recording absorbance changes over time intervals of down to 0.125 seconds. Substrate concentrations were maintained at approximately 10 times K_M to minimise the effects of any possible increases in K_M with temperature. Where these concentrations could not be maintained (e.g. due to substrate solubility), tests were conducted to confirm that there was no decrease in rate over the assay period due to substrate depletion. No evidence was found for either substrate or product inhibition under the experimental conditions described.

Adenosine deaminase [E.C. 3.5.4.4, adenosine aminohydrolyase] activity was measured by following the decrease in absorbance at 265nm ($\Delta\epsilon_{265} = 8.1 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) resulting from the deamination of adenosine to inosine (7). Reaction mixtures (1 mL) contained 0.1 M sodium phosphate pH 7.4, 0.12 mM adenosine and 0.003 units of enzyme. One unit is defined as the amount of enzyme that hydrolyses one μmole of adenosine to inosine per minute at 30°C.

Acid phosphatase [E.C. 3.1.3.2, orthophosphoric-monoester phosphohydrolase (acid optimum)] activity was measured using *p*-nitrophenylphosphate (*p*NPP) as substrate (8). Reaction mixtures (1 mL) contained 0.1 M sodium acetate pH 5.0, 10 mM *p*NPP and 0.024 units of enzyme. The release of *p*-nitrophenol was monitored at 410 nm ($\Delta\epsilon_{410} = 3.4 \text{ mM}^{-1}\cdot\text{cm}^{-1}$). One unit is defined as the amount of enzyme that hydrolyses one μmole of *p*NPP to *p*-nitrophenol per minute at 37°C.

Alkaline phosphatase [E.C. 3.1.3.1, orthophosphoric-monoester phosphohydrolase (alkaline optimum)] activity was measured using *p*NPP as substrate (9). Reaction mixtures (1 mL) contained 0.1 M diethanolamine/HCl pH 8.5, 0.5 mM MgCl_2 , 10 mM *p*NPP and 0.02 units of enzyme. The release of *p*-nitrophenol was monitored at 405 nm ($\Delta\epsilon_{405} = 18.3 \text{ mM}^{-1}\cdot\text{cm}^{-1}$). One unit is defined as the amount of enzyme that hydrolyses one μmole of *p*NPP to *p*-nitrophenol per minute at 37°C.

Aryl acylamidase [E.C. 3.5.1.13, aryl-acylamide amidohydrolyase] activity was measured by following the increase in absorbance at 382nm ($\Delta\epsilon_{382} = 18.4 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) corresponding to the release of *p*-nitroaniline from the *p*-nitroacetanilide (*p*NAA) substrate (10). Reaction mixtures contained 0.1 M Tris/HCl pH 8.6, 0.75 mM *p*NAA and 0.018 units of enzyme. One unit is

defined as the amount of enzyme required to catalyse the hydrolysis of one μmole of *p*NAA per minute at 37°C.

β -lactamase [E.C. 3.5.2.6, β -lactamhydrolase] activity was measured by following the increase in absorbance at 485nm ($\Delta\epsilon_{485} = 20.5 \text{ mM}^{-1}\cdot\text{cm}^{-1}$) associated with the hydrolysis of the β -lactam ring of nitrocefin (11). Reaction mixtures contained 0.05 M sodium phosphate pH 7.0, 0.1 mM nitrocefin and 0.003 U of enzyme. One unit is defined as that which will hydrolyse the β -lactam ring of one μmole of Cephalosporin per minute at 25°C.

Data collection

For each enzyme, progress curves (absorbance versus time) at a variety of temperatures were collected; the time interval was set so that an absorbance reading was collected every second. Assay reactions were initiated by the addition of microlitre amounts of enzyme that had no significant effect on the temperature. Three progress curves were collected at each temperature, each for a five-minute period. Where the slope for these triplicates deviated by more than 10%, the reactions were repeated. Temperature was recorded using a Cole-Palmer Digi-Sense® thermocouple thermometer accurate to $\pm 0.1\%$ of the reading and calibrated using a Cole-Parmer NIST-traceable high-resolution glass thermometer. The temperature probe was placed inside the cuvette adjacent to the light path during temperature equilibration prior to the initiation of the reaction and again immediately after completion of each enzyme reaction. Measurements of temperature were also taken at the top and bottom of the cuvette to check for temperature gradients. Where the temperature measured before and after the reaction differed by more than 0.1°C, the reaction was repeated.

Data analysis

For each enzyme, the catalytic rates (expressed as $\mu\text{M.s}^{-1}$) were calculated at 1s time intervals along the three progress curves at each temperature. The averages of each time-point value were used to generate 3D plots of rate (v) versus temperature (T in Kelvin) versus time (t in seconds) [SigmaPlot® 2001 for Windows, Version 7.101, SPSS Inc.]. Data were smoothed using a Loess transformation, a curve-fitting technique based on local regression that applies a tricube weight function to elicit trends from noisy data (12). The first data point was obtained at approximately 2 seconds, owing to the lag between the addition of enzyme to the reaction mixture and the start of data collection. The data for zero time were obtained during the smoothing process, which extrapolates back to zero using the trend elicited from the data.

The data for each enzyme were analysed to provide values of $\Delta G_{\text{cat}}^{\ddagger}$ (the activation energy of the catalytic reaction), $\Delta G_{\text{inact}}^{\ddagger}$ (the activation energy of the thermal inactivation process), ΔH_{eq} (the enthalpy change for the transition between active and inactive forms of the enzyme) and T_{eq} (the temperature for the mid-point of this transition). Initial estimates of these parameters were calculated from 2D analyses. Firstly, for the data at $t = 0$ (where there is no thermal inactivation), Eyring plots of $\ln(v/T)$ versus $1/T$ give values of $\Delta H_{\text{cat}}^{\ddagger}$ and $\Delta S_{\text{cat}}^{\ddagger}$ (from the slope and intercept, respectively), from which $\Delta G_{\text{cat}}^{\ddagger}$ can thus be calculated at any temperature of assay. Secondly, at each assay temperature, plots of $\ln(v)$ versus time were used to calculate rate constants (k_{inact}) for the thermal inactivation process; Eyring plots of these data [$\ln(k_{\text{inact}}/T)$ versus $1/T$] similarly give values of $\Delta H_{\text{inact}}^{\ddagger}$ and $\Delta S_{\text{inact}}^{\ddagger}$, and hence of $\Delta G_{\text{inact}}^{\ddagger}$. Values of K_{eq} were calculated from an Arrhenius plot [$\ln(v)$ versus $1/T$] for the data at $t = 0$. That is, according to the Equilibrium Model (1), in the absence of any thermal inactivation, the deviation from

linearity in the Arrhenius plot is attributed to a shifting of the equilibrium from active to inactive forms; a comparison of the observed values with those from the extrapolated linear portion can thus be used to calculate the value of K_{eq} at any temperature. Using equation (3), values of ΔH_{eq} and T_{eq} were subsequently determined from a plot of $\ln(K_{eq})$ versus $1/T$.

Using these estimates of the thermodynamic parameters, the complete data set for each enzyme (rate versus time versus temperature) was fitted to the Equilibrium Model equations to derive the values given in Table 1. The fits were performed using Scientist (Micromath) software, employing a non-linear least squares minimisation of the numerically integrated rate equations utilising Powell's algorithm.

Protein determination

Protein concentrations were determined from A_{280} measurements or by the colorimetric methods of Bradford and Biuret (13).

Results

The overall dependence of velocity on temperature with time can be described in our model by the relationship:

$$V_{\max} = \frac{k_B T \cdot e^{\left(-\frac{\Delta G_{cat}^\ddagger}{RT}\right)} \cdot E_0 \cdot e^{\left(\frac{\Delta H_{eq} \left(\frac{1}{T_{eq}} - \frac{1}{T}\right)}{R}\right)} \cdot t}{h \left(1 + e^{\left(\frac{\Delta H_{eq} \left(\frac{1}{T_{eq}} - \frac{1}{T}\right)}{R}\right)}\right)} \quad \text{Equation (4)}$$

where k_B is Boltzmann's constant and h is Planck's constant. A comparison of the simulated plots for the Classical and Equilibrium models (Fig.1A & 1B) with the experimentally-determined 3D plots of rate versus time versus temperature (Fig. 1C and 2A-D) shows that all the enzymes studied conform to the Equilibrium Model; that is, they display clear temperature optima at time = 0 when there can be no loss of activity due to thermal inactivation. Analysis of the data gives values for ΔG_{cat}^\ddagger , $\Delta G_{inact}^\ddagger$, ΔH_{eq} and T_{eq} for each enzyme (Table 1), assuming that the data can be described by the Equilibrium Model. Taking adenosine deaminase as the example, these parameters were used to simulate the 3D plot (Fig. 1D), and a comparison with

the plot of the experimental data (Fig. 1C) shows excellent agreement. Similarly good agreement has been obtained with the 4 other enzymes (data not shown).

Setting $t = 0$ in equation (4) and differentiating with respect to T gives

$$\frac{dV_{\max}}{dT} = \frac{T + \frac{\Delta G_{cat}^{\ddagger}}{R}}{\frac{\Delta H_{eq}}{R} - \frac{\Delta G_{cat}^{\ddagger}}{R} - T} - e^{\left(\frac{\Delta H_{eq} \left(\frac{1}{T_{eq}} - \frac{1}{T} \right)}{R} \right)} \quad \text{Equation (5)}$$

When dV_{\max}/dT is set to zero (i.e. at the maximum of the rate/temperature profile in the observed range of T), $T = T_{\text{opt}}$. It can be demonstrated that for the range of parameter values typically shown for enzymes:

$$\frac{1}{T_{eq}} - \frac{1}{T_{opt}} \approx -\frac{R}{\Delta H_{eq}} \ln \left(\frac{\Delta H_{eq} - \Delta G_{cat}^{\ddagger}}{\Delta G_{cat}^{\ddagger}} \right) \quad \text{Equation (6)}$$

Further manipulation provides the relationship:

$$T_{\text{opt}} \approx T_{\text{eq}}(1 - \alpha T_{\text{eq}}) \quad \text{Equation (7)}$$

where

$$\alpha \approx \frac{R}{\Delta H_{eq}} \ln \left(\frac{\Delta H_{eq} - \Delta G_{cat}^{\ddagger}}{\Delta G_{cat}^{\ddagger}} \right)$$

and α is small (such that $\alpha T_{\text{eq}} \ll 1$). Thus in general, for enzymes whose thermal activity dependence follows the Equilibrium Model, T_{opt} will be close in value to T_{eq} and always smaller (by the term αT_{eq}^2). Over the range of values of $\Delta G_{cat}^{\ddagger}$ and ΔH_{eq} encountered in this study, T_{opt} and T_{eq} follow an essentially linear relationship.

A plot of relative activity at zero time versus temperature (Fig. 3) illustrates that all five enzymes display true temperature optima of catalytic activity as defined by the Equilibrium Model, and that the values of T_{opt} are essentially in accordance with the determined values of T_{eq} (Table 1). For β -lactamase and adenosine deaminase, the difference between T_{opt} and T_{eq} is greater than for the other enzymes; both enzymes have relatively low ΔH_{eq} values, which will have a major influence on this difference (Equation 7). It should be stressed that $\Delta G_{\text{inact}}^{\ddagger}$ is not a factor in the position of the peaks illustrated in Figure 3 as the curves are determined at “time zero” where there is no thermal inactivation process. As also noted by Thomas and Scopes (2), in all cases reported here the T_{opt} is approximately 20-40°C above the optimum growth temperature/body temperature of the source organisms.

Although the application of the Equilibrium Model has the potential to be complicated by temperature-induced subunit dissociation in the case of oligomeric enzymes, we find no evidence to suggest that this treatment is restricted to monomeric enzymes; the data for the dimeric alkaline phosphatase adhere equally well to the Equilibrium Model as do the other, monomeric, enzymes investigated here.

Discussion

The results and their analysis indicate that the experimental velocity data as a function of temperature can be described by the Equilibrium Model, suggesting K_{eq} as an intrinsic, temperature-dependent property of enzymes, and supporting the hypothesis that these enzymes possess a third thermal parameter (T_{eq}), alongside the Arrhenius activation energy and the activation energy for thermal stability.

Currently, we have no evidence bearing on the molecular basis of the equilibrium between E_{act} and E_{inact} , although it is clearly a fast process relative to thermal denaturation. All the variation of activity with temperature at zero time [Figs 2 and 3] occurs as a result of changes in the $E_{\text{act}}/E_{\text{inact}}$ equilibrium, and is thus attained over timescales shorter than the mixing process, say less than 1 second, whereas the measured rate of irreversible thermal inactivation (conversion from E_{inact} to denatured state) is at least two orders of magnitude slower over the same temperature range. For example, in the case of aryl acylamidase [Fig 2A], at 51°C the activity at time zero is approximately 40% lower than at the "optimum" temperature [46°C], whereas the activity/time line at this temperature shows that it takes approximately 60 seconds for 40% denaturation to occur.

Since the Native/Denatured transition is generally a two-state process for single-domain proteins (14), E_{inact} is unlikely to be significantly unfolded. A reversible conformational change is most likely, and we speculate that the differing effects of temperature on the various weak interactions stabilising protein structure offer an opportunity for a shift of structure with changing temperature, leading to a change in activity. The existence of conformational sub-states in equilibrium over sub-second timescales is widely accepted (15, 16), and it has recently been suggested that adaptation to thermal stability may involve a change in the scale of fluctuations about the average state (17).

T_{eq} is important in describing the effect of temperature on enzymes, and in particular on the role of temperature as a selection pressure on enzyme structure and function. There is no evidence

connecting the Arrhenius activation energy of an enzyme to its thermal environment, and although there is a strong correlation between thermal stability and the environmental temperature of the source organism, it is known that enzyme thermal stability also reflects resistance to other cellular conditions such as the action of proteases (18). T_{eq} is central to the physiological adaptation of an enzyme to its environmental temperature and links the molecular, physiological, and environmental aspects of the adaptation of life to temperature in a way that has not been previously possible. We predict that T_{eq} will be a better expression of the effect of environmental temperature on the evolution of the enzyme than thermal stability; and thus we might expect differences in the overall shapes of the curves such as those shown in Figure 2 to describe the fit of an enzyme to its thermal environment, especially at the high temperature part of the graph, since the shape of the low temperature part of the graph will be determined largely by the Arrhenius activation energy. T_{eq} thus provides an important new parameter for matching an enzyme's properties to its cellular and environmental function.

In terms of protein engineering, T_{eq} provides an additional parameter that determines the *thermoactivity* of an enzyme and so must be considered when designing enzymes for particular functions. Much enzyme engineering is directed at stabilising enzymes against denaturation. The results here suggest that engineering to manipulate the E_{act}/E_{inact} equilibrium [i.e., T_{eq}] may be equally productive, and that T_{eq} must also be shifted to higher temperatures to obtain the expected catalytic benefits of enhanced enzyme thermostability. It will be important to distinguish between mutations that affect stability from those that affect T_{eq} .

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References

1. Daniel, R. M., Danson, M. J. and Eisinger, R. (2001) *Trends Biochem. Sci.* **26**, 223-225
2. Thomas, T. M. and Scopes, R. K. (1998) *Biochem. J.* **330**, 1087-1095
3. Gerike, U., Russell, N. J., Danson, M. J. and Hough, D. W. (1997) *Eur. J. Biochem.* **248**, 49-57
4. Buchanan, C. L., Connaris, H., Hough, D. W., Reeve, C. D. and Danson, M. J. (1999) *Biochem. J.* **343**, 563-570
5. Arnott, M. A., Michael, R. A., Thompson, C. R., Hough, D. W. and Danson, M. J. (2000) *J. Mol. Biol.* **304**, 655-666
6. Medina, D. C., Hanna, E., MacRae, I. J., Fisher, A. J. and Segel, I. H. (2001) *Arch. Biochem. Biophys.* **393**, 51-60
7. Pfromm, N. (1967) *Arch. Biochem. Biophys.* **119**, 141-146
8. Hollander, V. P. (1971) in *The Enzymes*, Vol. 4 (ed. Boyer, P. D.) 449-498 (Academic Press, New York)
9. Fernley, H. N. (1971) in *The Enzymes*, Vol. 4 (ed. Boyer, P. D.) 417-447 (Academic Press, New York)
10. Hammond, P. M., Price, C. P. and Scawen, M. D. (1983) *Eur. J. Biochem.* **132**, 651-655

11. O'Callaghan, C. H., Morris, A., Kirby, S. M. and Shingler, A. H. (1972) *Antimicrob. Agents Chemother.* **1**, 283-288
12. Cleveland, W. S. (1993) *Visualizing Data*, Hobart Press, New Jersey
13. Scopes, R. K. (1994) in *Protein Purification: Principles and Practice*, 3rd Edition, (ed. Cantor, C. R.) 44-50 (Springer Verlag: San Diego)
14. Creighton, T. E. (1993) *Proteins*, 2nd Ed., W H Freeman, New York
15. Brooks, C. L., Karplus, M. and Pettitt, B. M. (1988) *Proteins*, John Wiley, New York
16. Svensson, A-K. E., O'Neill, J. C. and Matthews, C. R. (2003) *J. Mol. Biol.* **326**, 569-583
17. Wintrode, P. L., Zhang, D., Vaidehi, N., Arnold, F. H. and Goddard III, W. A. (2003) *J. Mol. Biol.* **327**, 745-757
18. Daniel, R. M., Dines, M. and Petach, H. H. (1996) *Biochem. J.* **317**, 1-11

Figure Legends

Fig. 1 - The temperature dependence of enzyme activity

(A) *Simulated plot for the Classical Model (1).* The variation of enzyme activity with temperature (290-340K) and time during the assay (0-200s) was simulated using Equation (1). The variation of the two rate constants in Equation (1) with temperature is given by $k_{\text{cat}} = (k_B T/h) e^{-(\Delta G_{\text{cat}}^{\ddagger}/RT)}$ and $k_{\text{inact}} = (k_B T/h) e^{-(\Delta G_{\text{inact}}^{\ddagger}/RT)}$ where k_B = Boltzmann's constant; R = Gas constant; T = absolute temperature; h = Planck's constant; $\Delta G_{\text{cat}}^{\ddagger}$ = activation energy of the catalysed reaction; $\Delta G_{\text{inact}}^{\ddagger}$ = activation energy of the thermal inactivation process. The following parameter values were used: $\Delta G_{\text{cat}}^{\ddagger} = 80 \text{ kJ}\cdot\text{mol}^{-1}$; $\Delta G_{\text{inact}}^{\ddagger} = 95 \text{ kJ}\cdot\text{mol}^{-1}$; total enzyme concentration = 100nM.

(B) *Simulated plot for the Equilibrium Model (1).* The variation of enzyme activity with temperature (280-360K) and time during the assay (0-200s) was simulated using the equation $V_{\text{max}} = k_{\text{cat}}[E_{\text{act}}]$, where the concentration of E_{act} at any time point is defined by Equation (2) and the variation of K_{eq} with temperature is given by Equation (3). The rate of formation of X is given by $d[X]/dt = k_{\text{inact}}\{[E_0] - [E_{\text{act}}] - [X]\}$. The following parameter values were used: $\Delta G_{\text{cat}}^{\ddagger} = 80 \text{ kJ}\cdot\text{mol}^{-1}$; $\Delta G_{\text{inact}}^{\ddagger} = 95 \text{ kJ}\cdot\text{mol}^{-1}$; total enzyme concentration = 100nM; $\Delta H_{\text{eq}} = 100 \text{ kJ}\cdot\text{mol}^{-1}$; $T_{\text{eq}} = 320\text{K}$.

(C) *Experimental data for adenosine deaminase.* The enzyme was assayed spectrophotometrically at 265nm as described in Materials and Methods; the data are plotted as rate ($\mu\text{M}\cdot\text{s}^{-1}$) versus temperature (K) versus time during assay (s).

(D) *Simulated data for adenosine deaminase.* The experimental data from (C) were analysed as described in Materials and Methods to give estimated values of $\Delta G_{\text{cat}}^{\ddagger}$, $\Delta G_{\text{inact}}^{\ddagger}$, ΔH_{eq} , and T_{eq} . Using these values, the complete data set was fitted to the Equilibrium Model to give final values, which were then used to simulate the plot of rate ($\mu\text{M.s}^{-1}$) versus temperature (K) versus time during assay (s).

Fig. 2 - Experimentally-determined temperature dependence of enzymic activity.

Enzymes were assayed spectrophotometrically as described in Materials and Methods, and the data plotted as rate ($\mu\text{M.s}^{-1}$) versus temperature (K) versus time during assay (s).

(A) aryl acylamidase; **(B)** acid phosphatase; **(C)** β -lactamase; **(D)** alkaline phosphatase.

Figs. 3A & 3B - Temperature dependence of enzymic activity at time zero

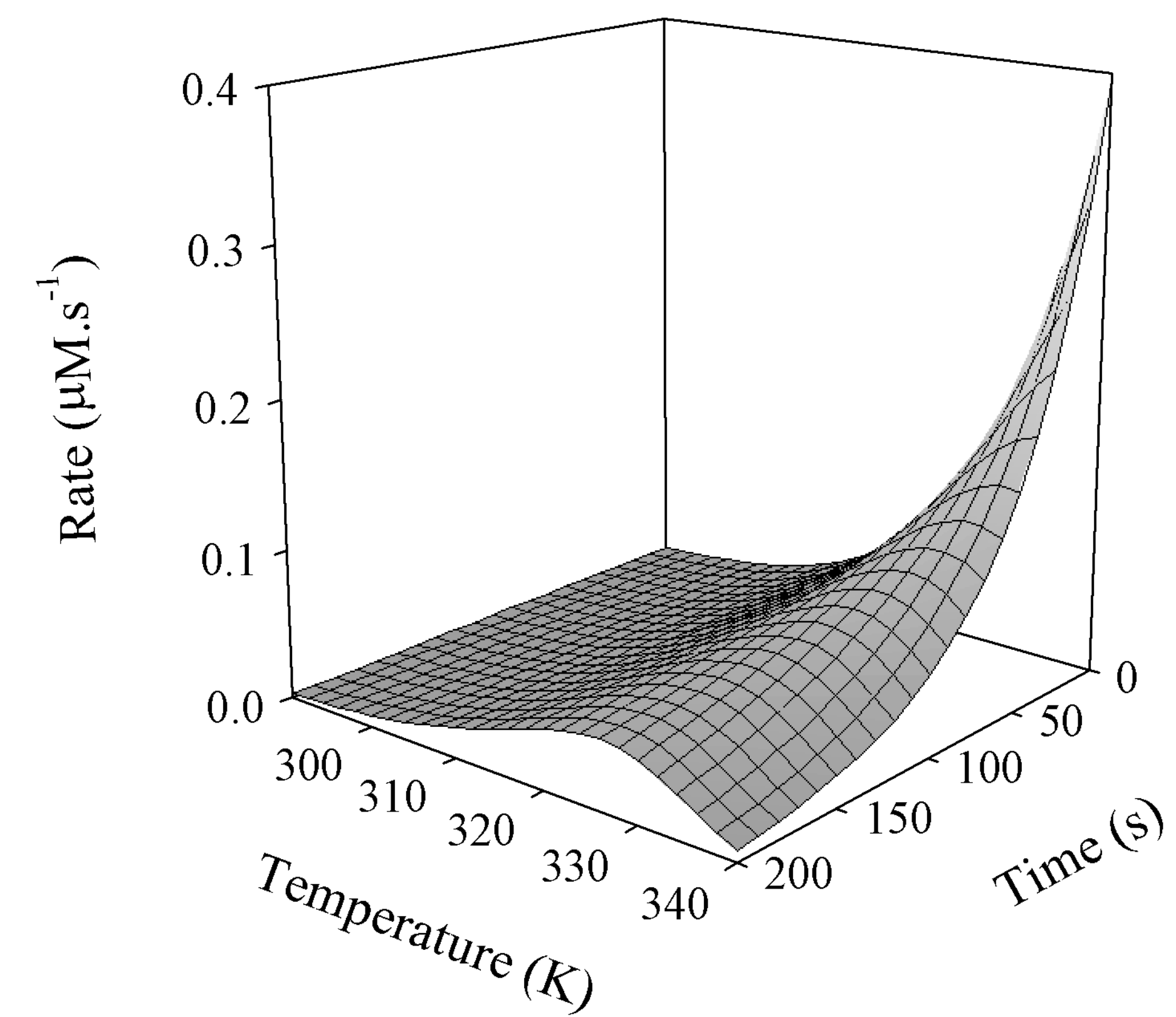
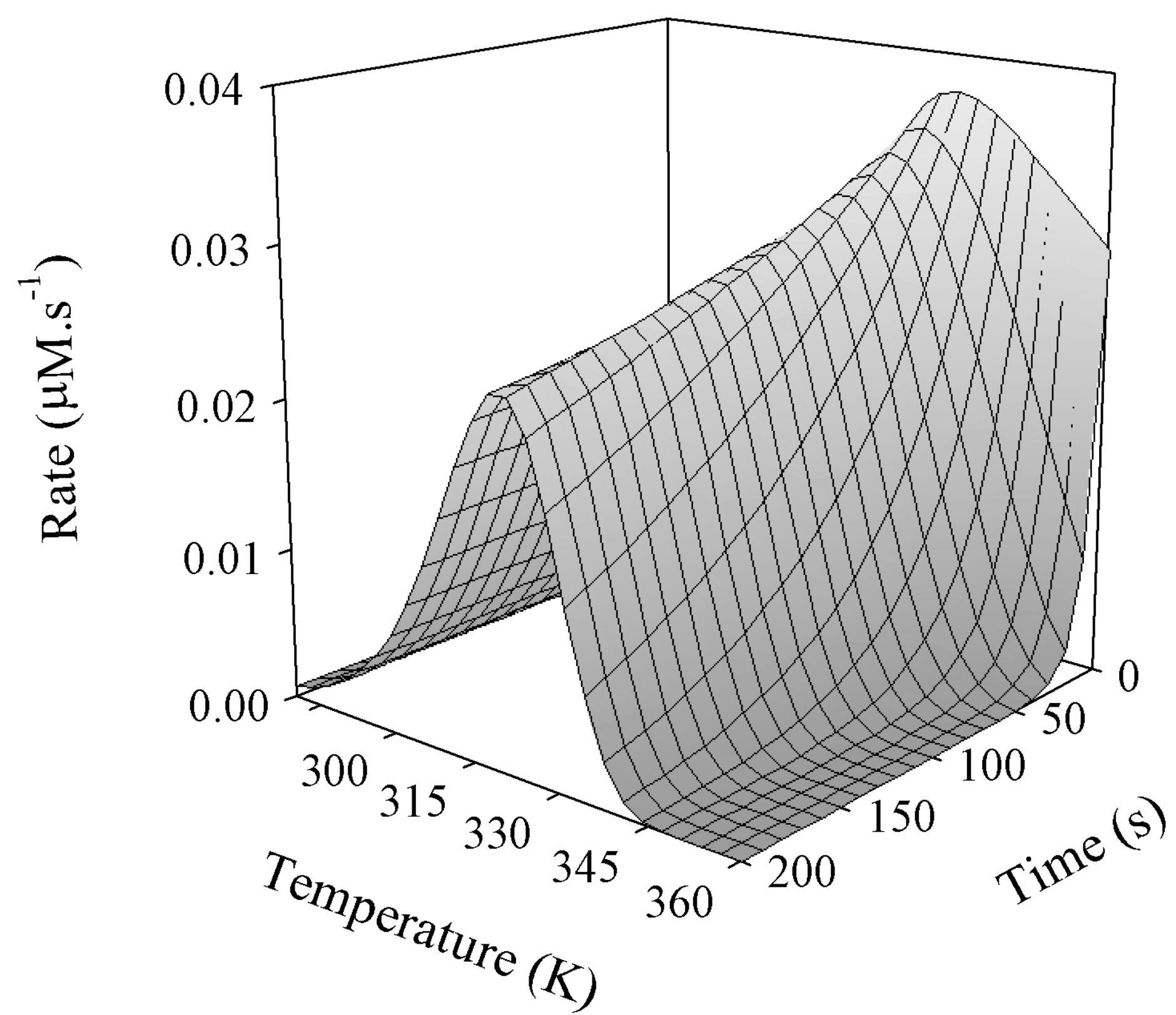
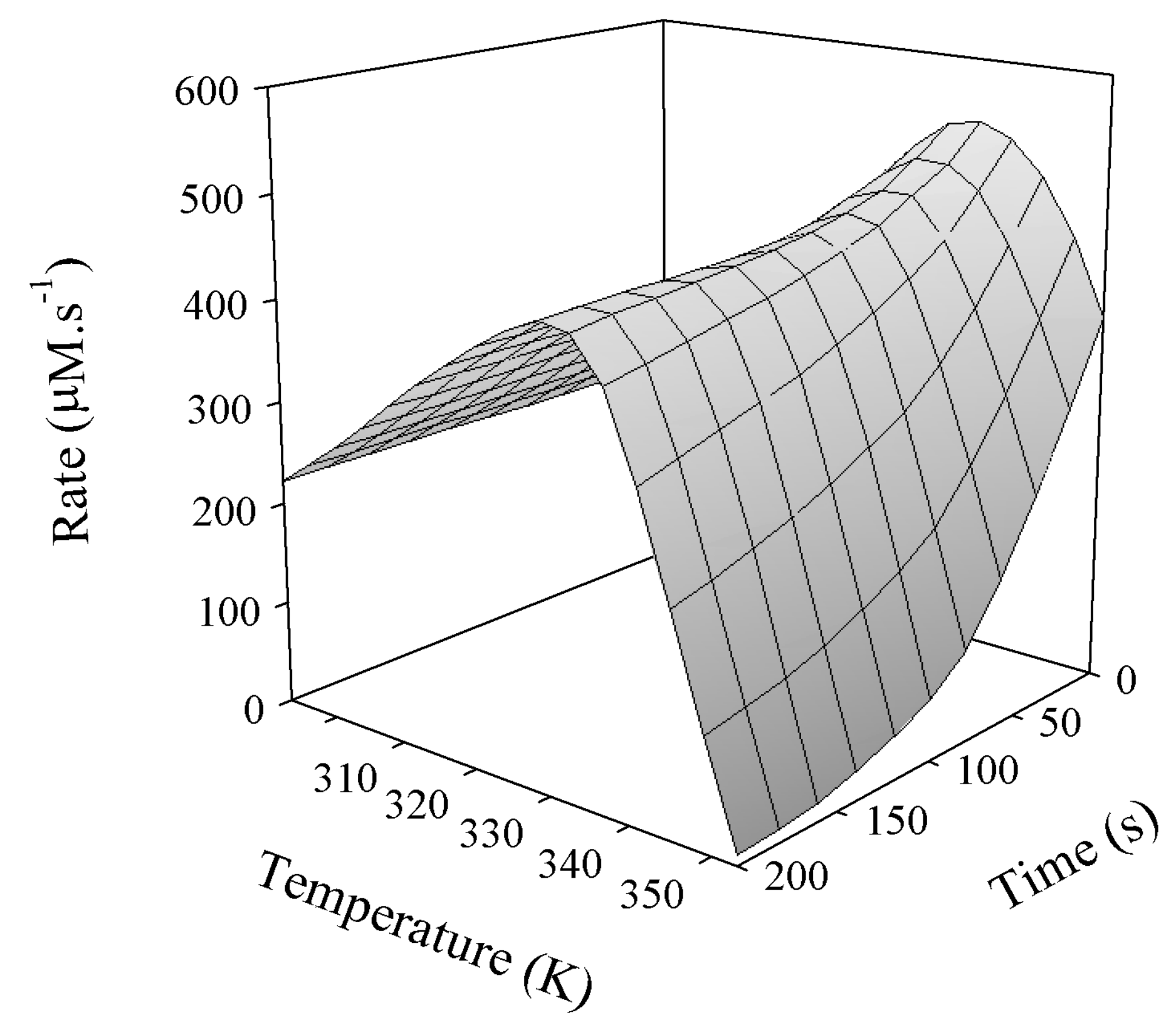
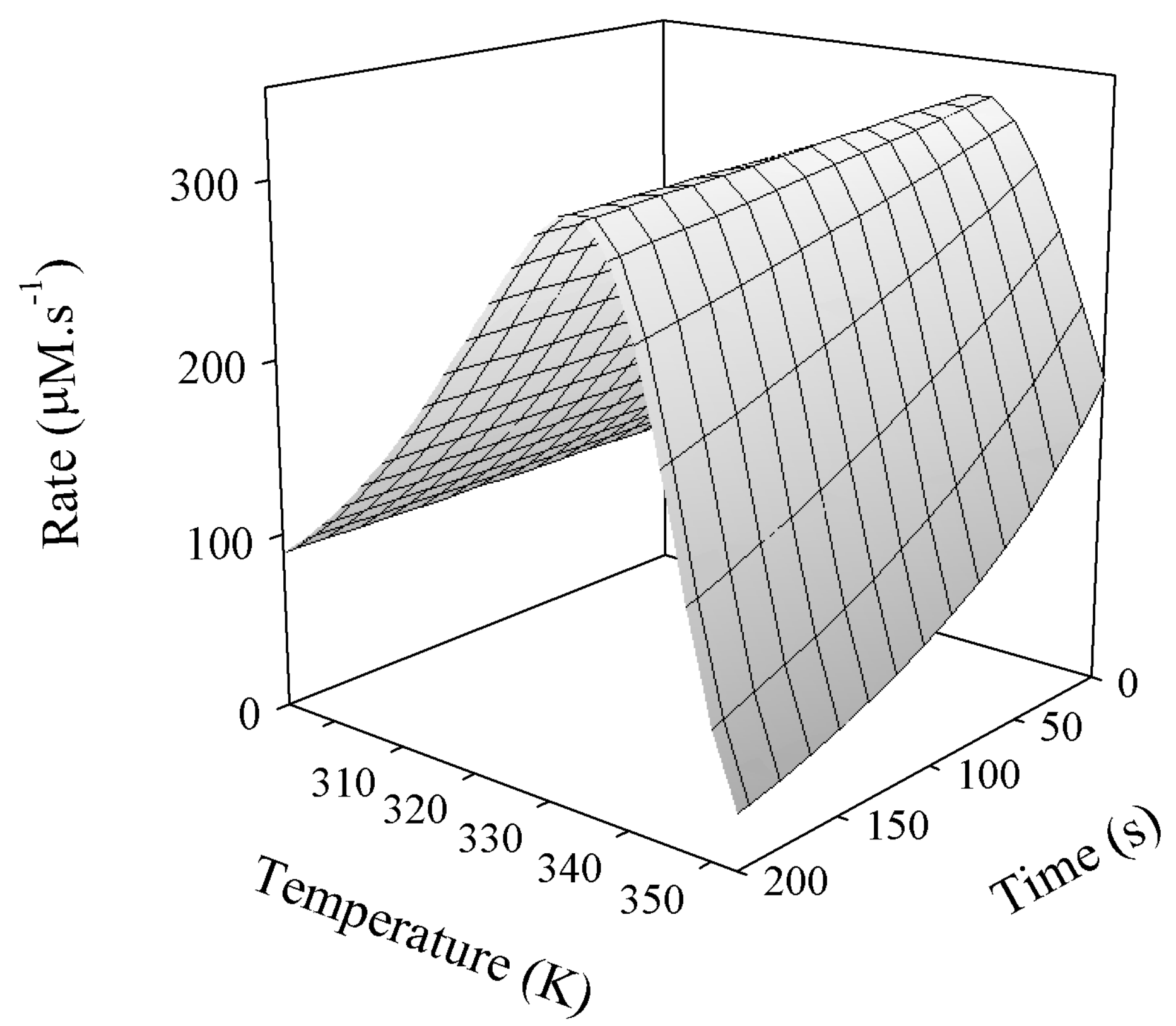
From the experimental data in Figure 1C and Figure 2A-D, the enzymic activities at time zero were plotted against temperature to demonstrate the dependence of enzymic activity on temperature in the absence of thermal inactivation. For clarity, the data have been divided into two graphs. The activity data for each enzyme are scaled from 5-100% relative to the maximum activity for that enzyme. **(A)**: (●) acid phosphatase; (○) adenosine deaminase; (■) aryl acylamidase. **(B)**: (□) β -lactamase; (▲) alkaline phosphatase. The solid curves through the points are fits to the data using equation (4) with time (t) set to zero.

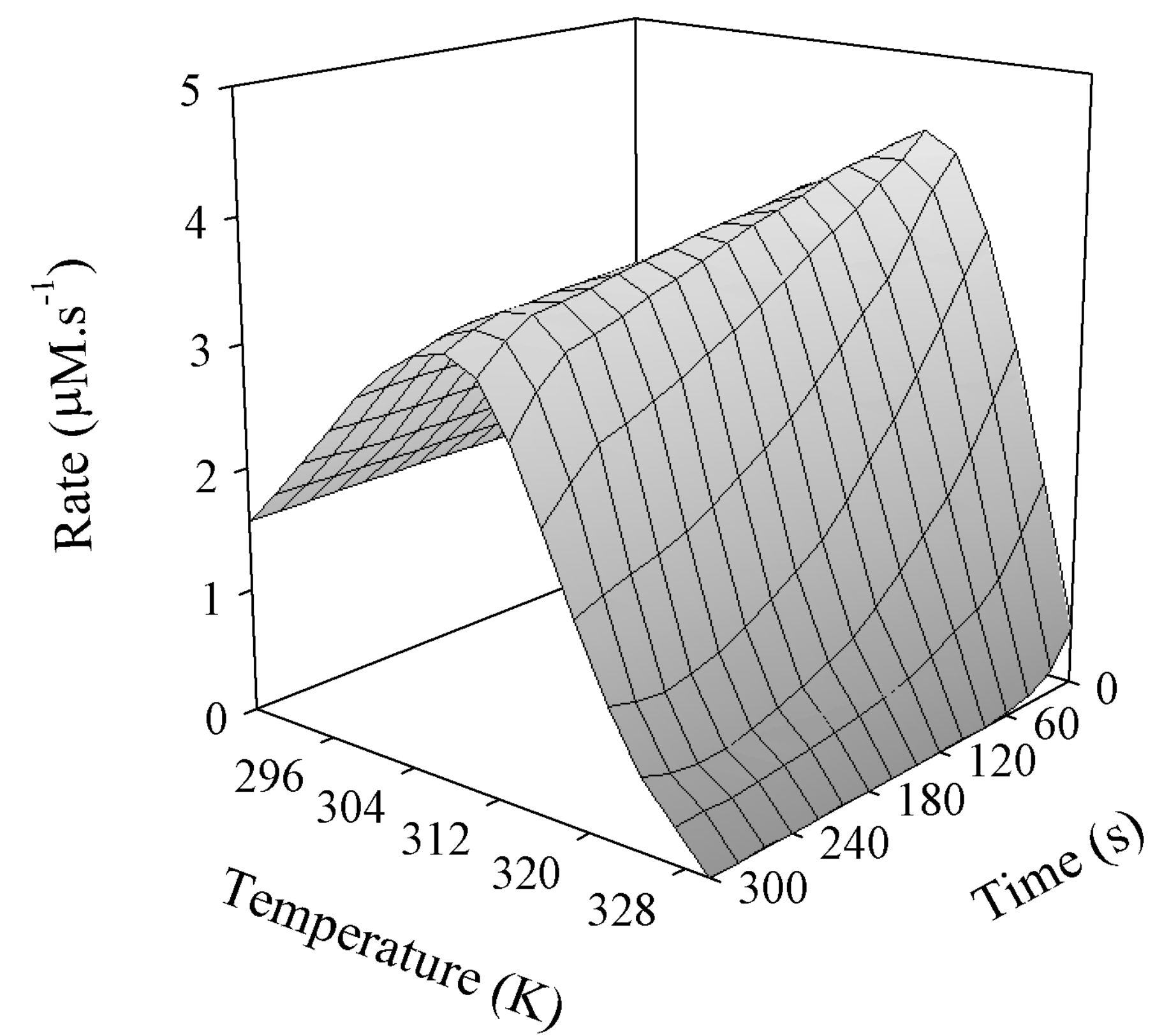
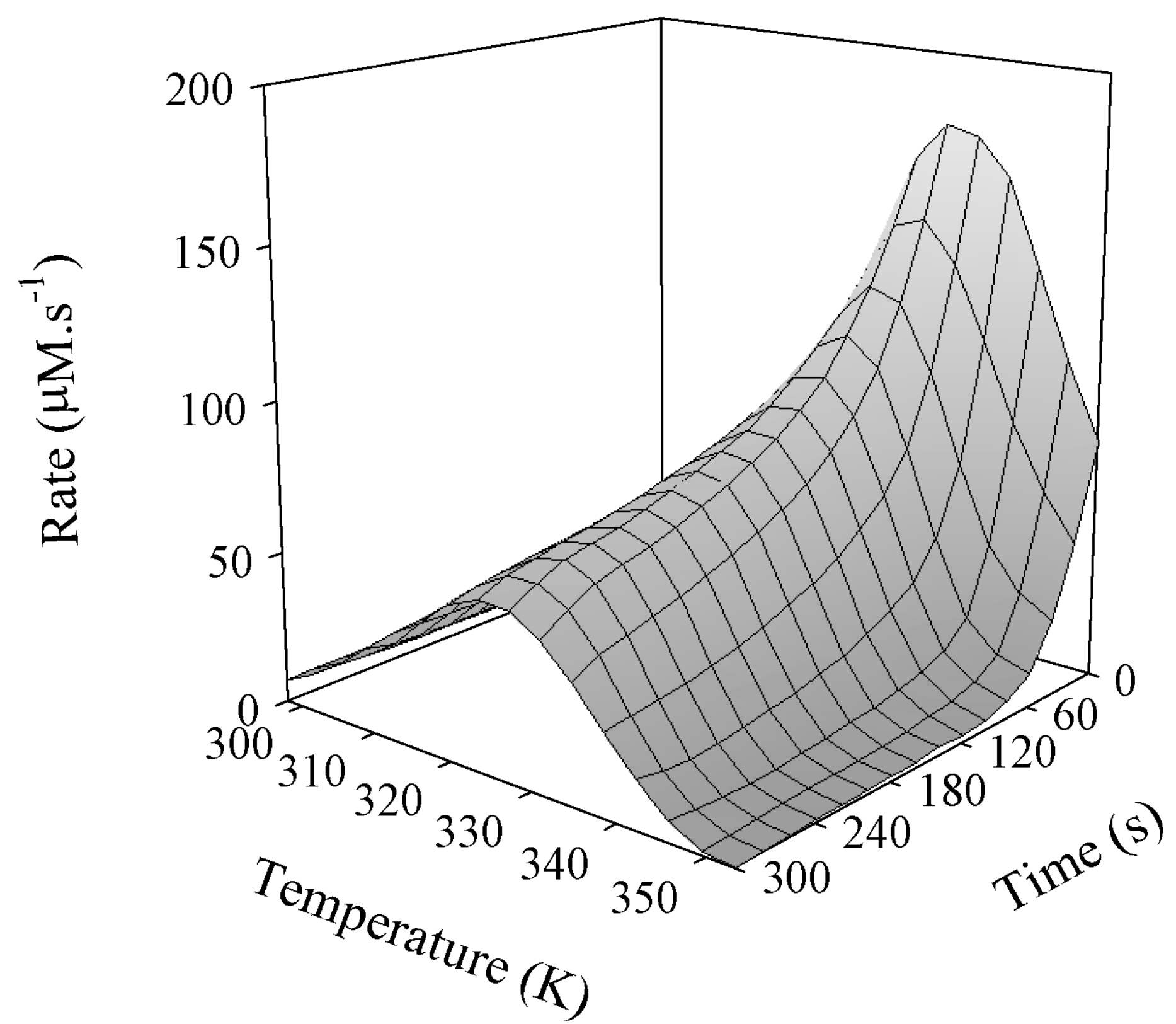
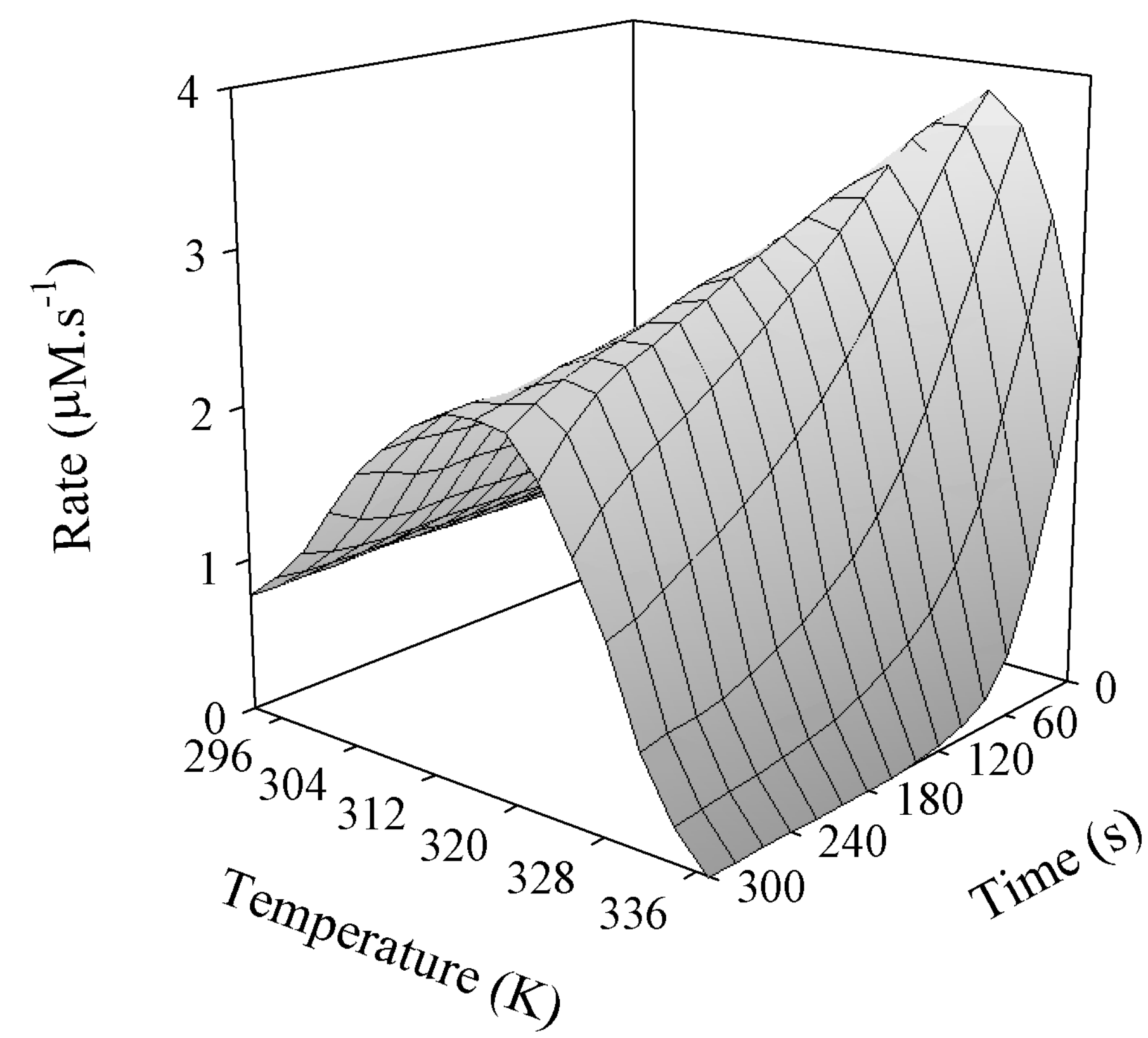
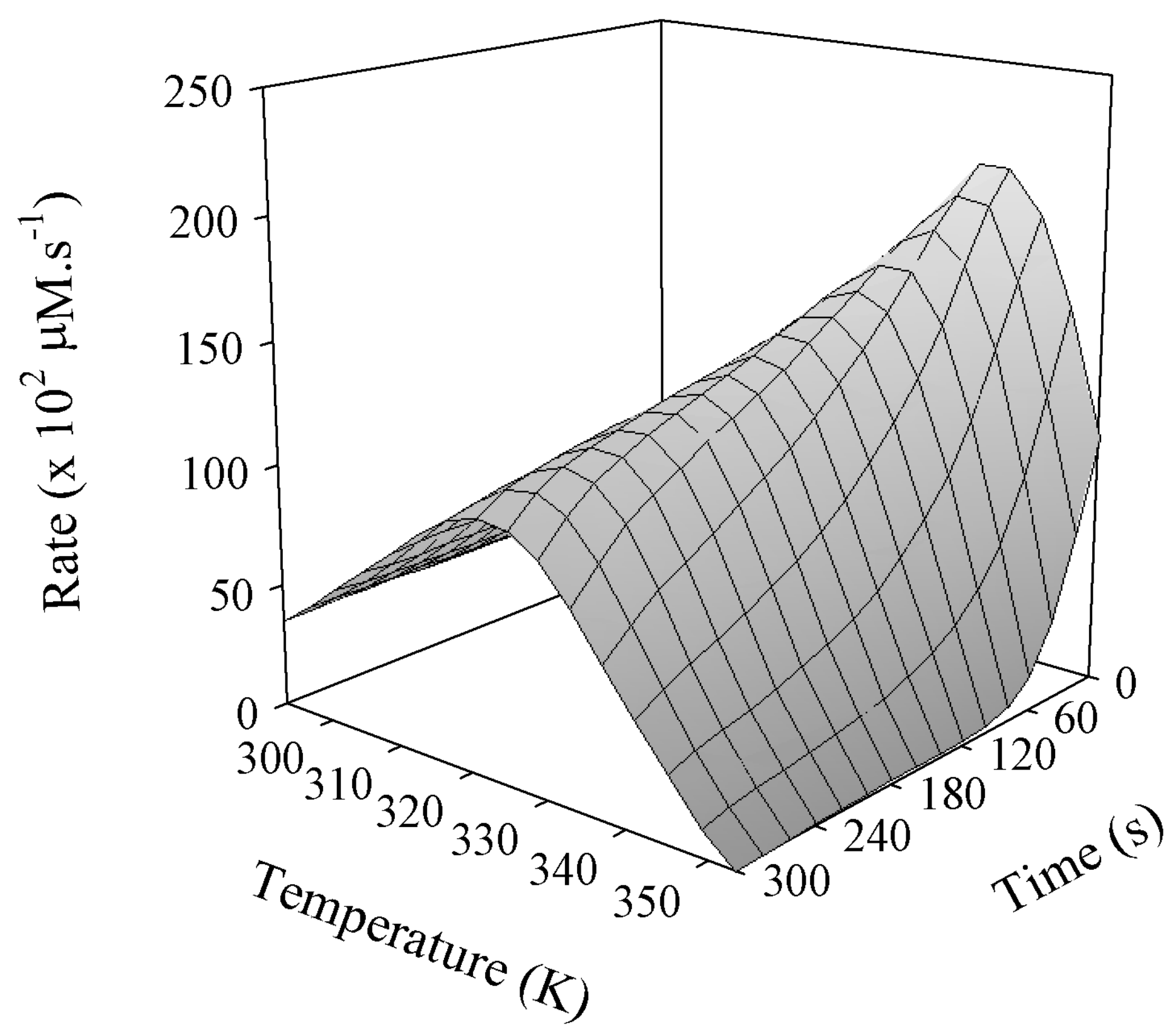
Table I - Summary of experimentally-determined thermodynamic parameters

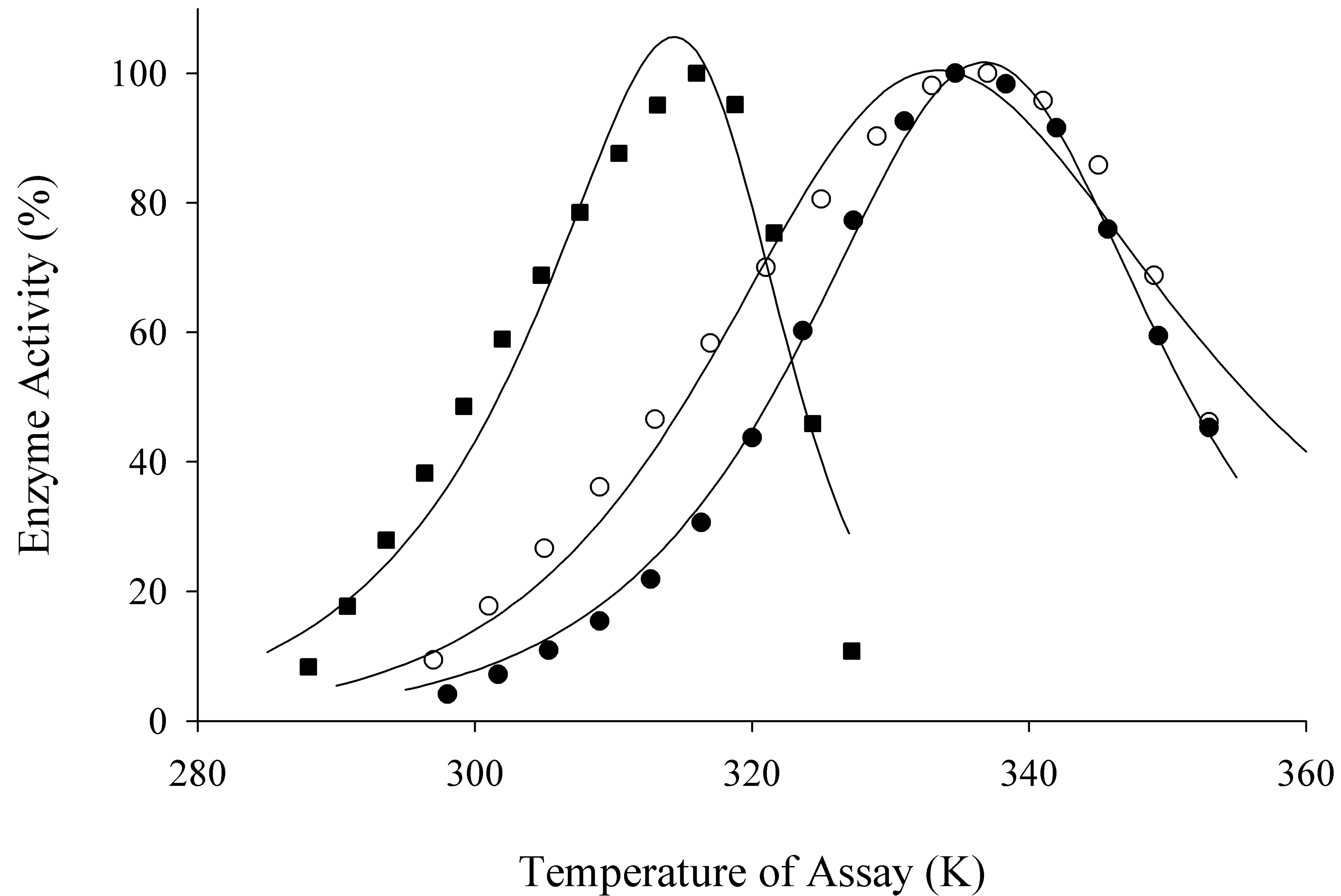
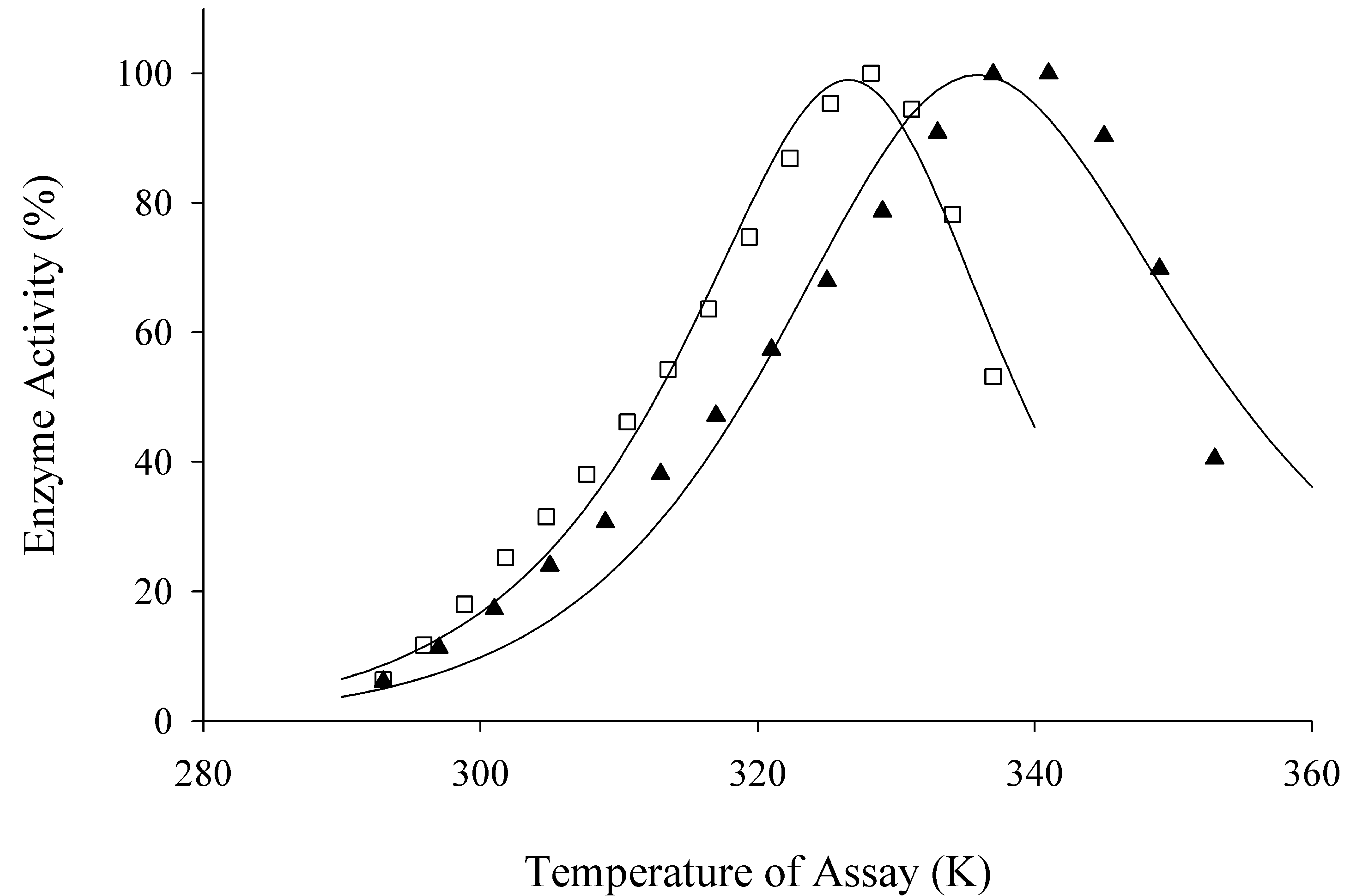
T_{opt} values were determined from the data in Fig. 3 using the peaks of the fitted curves. Values of T_{eq} , $\Delta G_{\text{cat}}^{\ddagger}$, $\Delta G_{\text{inact}}^{\ddagger}$, and ΔH_{eq} were determined as described in the Materials and Methods.

Enzyme	Origin	Growth Temp. °C	T_{opt} °C	T_{eq} °C	$\Delta G_{\text{cat}}^{\ddagger}$ (kJ·mol ⁻¹)	$\Delta G_{\text{inact}}^{\ddagger}$ (kJ·mol ⁻¹)	ΔH_{eq} (kJ·mol ⁻¹)
Aryl acylamidase	<i>P. fluorescens</i>	25	41	44	37	93	165
β -lactamase	<i>Bacillus cereus</i>	30	53	75	38	91	103
Acid phosphatase	Wheat germ	15-25*	64	65	31	138	183
Adenosine deaminase	Bovine spleen	39	59	73	26	100	146
Alkaline phosphatase	Bovine intestine	39	63	70	18	108	220

* Spring germination temperatures

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ADDITIONS AND CORRECTIONS

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A new intrinsic thermal parameter for enzymes reveals true temperature optima.

Michelle E. Peterson, Robert Eisingthal, Michael J. Danson, Alastair Spence, and Roy M. Daniel

PAGE 20720:

Table I: It has become apparent to us that incorrect units were used for the fitting of experimental data to the “Equilibrium Model” and that the values for $\Delta G_{\text{cat}}^{\ddagger}$, $\Delta G_{\text{inact}}^{\ddagger}$, ΔH_{eq} , and T_{eq} published in this paper are therefore incorrect; a corrected Table I is shown below. The main conclusion of the paper is unaffected, namely that “The results and their analysis indicate that the experimental velocity data as a function of

temperature can be described by the Equilibrium Model, suggesting K_{eq} as an intrinsic, temperature-dependent property of enzymes, and supporting the hypothesis that these enzymes possess a third thermal parameter (T_{eq}), alongside the Arrhenius activation energy and the activation energy for thermal stability.”

However, based on the original table we stated: “For β -lactamase and adenosine deaminase, the difference between T_{opt} and T_{eq} is greater than for the other enzymes; . . . ” This statement no longer applies. In addition, as a result of the corrected values, the statement that “ T_{opt} will be close in value to T_{eq} and always smaller” should read: “ T_{opt} will almost always be close in value to T_{eq} but may be smaller or larger, depending on the relative values of $\Delta G_{\text{cat}}^{\ddagger}$ and ΔH_{eq} .”

T1

TABLE ONE							
Summary of experimentally determined thermodynamic parameters							
T_{opt} values were determined from the data in Fig. 3 using the peaks of the fitted curves. Values of T_{eq} , $\Delta G_{\text{cat}}^{\ddagger}$, $\Delta G_{\text{inact}}^{\ddagger}$, and ΔH_{eq} were determined as described under “Experimental Procedures.”							
Enzyme	Origin	Growth temp.	T_{opt}	T_{eq}	$\Delta G_{\text{cat}}^{\ddagger}$	$\Delta G_{\text{inact}}^{\ddagger}$	ΔH_{eq}
		°C	°C	°C	$\text{kJ}\cdot\text{mol}^{-1}$	$\text{kJ}\cdot\text{mol}^{-1}$	$\text{kJ}\cdot\text{mol}^{-1}$
Aryl-acylamidase	<i>P. fluorescens</i>	25	38	36	74	92	133
β -Lactamase	<i>B. cereus</i>	30	53	53	69	94	146
Acid phosphatase	Wheat germ	15–25 ^a	66	63	79	95	133
Adenosine deaminase	Bovine spleen	39	62	56	65	99	101
Alkaline phosphatase	Bovine intestine	39	68	60	57	97	86

^a Spring germination temperatures.

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