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Thermochimica Acta 322 (1998) 1–7

thermochimica  
acta

## Thermokinetic method for faster enzyme-catalyzed reactions

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Received 17 January 1998; received in revised form 29 June 1998; accepted 30 June 1998

### Abstract

This paper describes the application of dynamic microcalorimetry to determine the thermokinetics of short-time enzymatic reactions. A thermokinetic double-parameter method for faster enzyme-catalyzed reactions is proposed on the basis of the double-parameter theoretical model of the conduction calorimeter. By analyzing the calorimetric curves of faster enzyme-catalyzed reactions, this method can be conveniently used to calculate both molar reaction enthalpy ( $\Delta_r H_m$ ) and kinetic parameters ( $K_m$ ,  $\nu_{max}$ ) of these reactions. Thermokinetics of the oxidation of xanthine catalyzed by xanthine oxidase in the presence of excess oxygen has been studied using microcalorimetry. This faster enzyme-catalyzed reaction obeyed the Michaelis–Menten kinetics, and the Michaelis constant ( $K_m$ ) for xanthine and the overall molar reaction enthalpy ( $\Delta_r H_m$ ) of this oxidation were determined by this method to be  $1.04 \times 10^{-3} \text{ mol dm}^{-3}$  and  $-5.25 \text{ kJ mol}^{-1}$ , respectively, at 298.15 K and pH 7.5. The reliability of the double-parameter method for faster enzyme-catalyzed reactions was verified by the experimental results. © 1998 Elsevier Science B.V.

**Keywords:** Double-parameter method; Faster enzyme-catalyzed reaction; Microcalorimetry; Thermokinetics; Xanthine oxidase

### 1. Introduction

Conduction calorimeters have been largely used for thermokinetic studies of both long-term and short-time reactions due to their high sensitivity and generality [1–10]. However, it is well known that a conduction calorimeter is an inertial ‘black box’ that transforms and distorts the input function (the heat power of an investigated reaction at time  $t$ ) into an output function (the recorded calorimetric signal of the reaction at the same time) [11–13]. In other words, the output signal is delayed relative to the reaction in the calorimeter cells owing to the thermal inertia of the

instrument, and thus the measured calorimetric curves are unable to represent the real thermokinetic curves correctly. When a fast reaction is taking place in a conduction calorimeter, this distortion will be serious. Scientists have expressed deep concerns at the elimination of this distortion since Tian first applied a simple thermokinetic equation, which was later named after him, to describe the dynamic properties of the conduction calorimetric system involving a slow reaction in 1923 [14–16]. In 1967, Camia [17] gave the fundamentals of applying a simplified form of the Fourier–Kirchhoff equation to the description of fast reactions in conduction calorimeters. Since that publication, many hundreds of publications [18–27] have been presented on this subject, and international

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efforts have been made to evaluate the methods of determining thermokinetics of fast reactions. These results were recently published [28]. Thereafter several modifications of previously proposed methods, as well as new methods of calculating thermokinetics, were suggested [29–32].

There are at least 10 methods for the determination of thermokinetics of fast reactions [30–32], many of which are based on the ‘black box’ notion and apply the Laplace transform or the Fourier transform to correct the output signal for the large thermal inertia usually present at fast reactions. These include the dynamic optimization method, harmonic analysis method, state variable method, thermal curve interpretation method, multi-body method, general heat-transfer equation method [2] (i.e. the analogue and numerical correction method [30]), flux method, double-parameter method [6] and analog thermo-analytical curve method [33]. Although many studies on the thermokinetics of fast reactions have been reported, little information on the thermokinetics of fast enzyme-catalyzed reactions is available at the present time.

In a previous study [7], a single-parameter method for slower enzyme-catalyzed reactions based on Tian’s equation was suggested and verified by experimental data. In this paper, a double-parameter method for faster enzyme-catalyzed reactions is proposed on the basis of a double-parameter theoretical model of the conduction calorimeter, and it can be conveniently used to calculate both molar reaction enthalpy ( $\Delta_r H_m$ ) and kinetic parameters ( $K_m, \nu_{max}$ ). In order to test the validity of this method, we have applied microcalorimetry to the study on the oxidation of xanthine catalyzed by xanthine oxidase (XOD, EC 1.2.3.2) in the presence of excess oxygen. This oxidation was a well-studied faster enzyme-catalyzed reaction and often utilized as a source of superoxide anion ( $O_2^-$ ) in assays of superoxide dismutase [34–41]. The Michaelis constant calculated by the proposed method is in agreement with that in the literature [34], therefore, validating the double-parameter method.

## 2. Theory and method

For a faster reaction occurring in a conduction calorimeter, the thermal resistance between the sam-

ple and the sample container,  $R_1$ , cannot be neglected, and the thermal delay between the temperature sensor and the reaction system must be considered. Therefore, according to the double-parameter theoretical model of the conduction calorimeter [6] and Hemminger’s equation [16], we obtain

$$P = K\Delta_t + K(1/\alpha + 1/\beta)(d\Delta_t/dt) + (K/\alpha\beta)(d^2\Delta_t/dt^2) \quad (1)$$

where  $P$  is the heat power of the reaction at time  $t$ ,  $\Delta_t$  the peak height at time  $t$ ,  $\alpha$  and  $\beta$ , called double parameters, the cooling constants of the calorimetric system and the sensor, respectively,  $K$  the heat-transfer constant of the calorimeter, and all of these model parameters ( $\alpha$ ,  $\beta$ , and  $K$ ) can be calibrated by the introduction of electrical energy [6]. Eq. (1) is the second-order approximation of the general heat-transfer equation when the calorimeter is treated as a linear inertial object of second order [2,30].

For a slower reaction taking place in a conduction calorimeter,  $R_1$  approaches zero and the influence of the delay of sensing can be neglected, namely  $(d\Delta_t/dt)/\beta \ll \Delta_t$  [6] or  $(d^2\Delta_t/dt^2)/\alpha = -d\Delta_t/dt$ .

Assuming

$$\alpha = k \quad (2)$$

Eq. (1) turns into Tian’s equation [14–16]:

$$P = K\Delta_t + (K/k)(d\Delta_t/dt) \quad (3)$$

here  $k$ , called a single parameter, is the Newton’s cooling constant of the calorimeter system and can also be easily determined by electric calibration [6]. Obviously, the commonly used Tian’s equation (Eq. (3)) is of the first order when the calorimeter can be treated as a linear inertial object of first order [2,30].

When an extremely slow process, e.g. the germination of seeds and the loss of potency [5], is taking place in a conduction calorimeter, the influence of all of the thermal lags within the calorimeter system can be ignored, namely  $(d\Delta_t/dt)/\alpha \ll \Delta_t$ . Combining with  $\beta > \alpha$  [6], Eq. (1) simplifies to the zero-parameter equation:

$$P = K\Delta_t \quad (4)$$

Under this condition, the conduction calorimetric curve for this type of process reduces to the corresponding adiabatic calorimetric curve. Eq. (4) is the

zero-order approximation of the general heat-transfer equation.

Initial rate studies have been found particularly useful for investigating the kinetics of enzyme-catalyzed reactions because of the three advantages of such studies [42]. For an enzyme-catalyzed reaction obeying the Michaelis–Menten equation, according to the thermokinetic single-parameter method [7], the earlier stage of the reaction follows zero-order reaction kinetics and the heat power in this stage is the initial heat power of the reaction,  $P_0$ . Thus, we have

$$1/P_0 = (K_m/P_{\max})(1/[S]_0) + 1/P_{\max} \quad (5)$$

This is a linear equation called the Lineweaver–Burk equation in which  $K_m$  is the Michaelis constant,  $P_{\max}$  the maximum heat power and  $[S]_0$  the initial substrate concentration. The values of  $K_m$  and  $P_{\max}$  can be calculated from the intercept of  $1/P_0$  and the slope  $K_m/P_{\max}$ , respectively. Then, the maximum reaction rate,  $\nu_{\max}$ , can be determined by the following equation:

$$\nu_{\max} = P_{\max}/(\pm\Delta_r H_m)V \quad (6)$$

where  $V$  is the total volume of the reacting system (in this paper,  $V=6.00$  ml), the positive sign applies to endothermic reactions and the negative sign to exothermic cases, and the overall molar reaction enthalpy,  $\Delta_r H_m$ , can be determined from the total area under the calorimetric curve,  $A$ , according to the formula reported [9]. Eqs. (1), (5) and (6) are the mathematical model of the thermokinetic double-parameter method for faster enzyme-catalyzed reactions.

### 3. Experimental

#### 3.1. Reagents

Doubly distilled water was used throughout. Analytical grade disodium orthophosphate and monosodium orthophosphate were used in the preparation of the buffer solution. The pH of the solution was adjusted to 7.5 by means of a pH meter, mixing slowly two solutions with concentrations both  $3.3 \times 10^{-2}$  mol dm<sup>-3</sup>.

The solid xanthine oxidase was obtained from the Shanghai Institute of Biochemistry of Academia

Sinica and purified. A XOD solution of 1.16 g dm<sup>-3</sup> was prepared by dissolving the solid XOD in the buffer solution and the stock solution was stored in a refrigerator.

The substrate, xanthine, was purchased from Sigma and a  $1.50 \times 10^{-3}$  mol dm<sup>-3</sup> substrate solution was made by dissolution of xanthine in the buffer solution.

All solutions were freshly prepared and saturated with purified oxygen before each set of experiments.

#### 3.2. Instrumentation

The heat of the oxidation of xanthine catalyzed by xanthine oxidase in the presence of excess oxygen was determined at 298.15 K (25.00°C) and pH 7.5 using a LKB-2107 batch microcalorimeter system. The output voltage signal was recorded in the form of a calorimetric curve by means of the LKB-2210 dual-pen integration recorder [9].

## 4. Results

#### 4.1. Determination of the model parameters

Aliquots of 4.00 and 2.00 ml of the phosphate buffer solutions were held in compartments II and I of the reaction cell, respectively, and the contents of the reference cell were the same as those in the reaction cell. The heat-transfer constant ( $K$ ) and the cooling constants ( $\alpha$ ,  $\beta$ , and  $k$ ) calculated by the double- and single-parameter theoretical models of the conduction calorimeter from the electric calibrating data of the above calorimetric system, are listed in Tables 1 and 2. In Table 1,  $R$  is the resistance of the calibration heater,  $I$  the strength of a current flowing through the resistance,  $t'$  the calibrating time and  $A_{\text{cal}}$  the total area under the calibration calorimetric curve.

From these tables, it can be seen that the calculated values of the model parameters ( $K$ ,  $\alpha$ ,  $\beta$  and  $k$ ) for various experiments are of good reproducibility and they are also self-consistent for a single experiment. Therefore, the validity of the double- and single-parameter theoretical model for the conduction calorimeter was verified by the results of electric calibration. In Tables 1 and 2, the value of  $\alpha$  is close to that of  $k$ , which is in agreement with Eq. (2).

Table 1  
Results of electrical energy calibration at 298.15 K

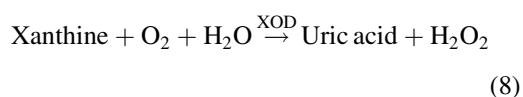
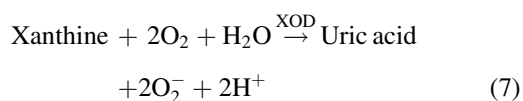
No.	$I/mA$	$R/\Omega$	$t'/s$	$A_{cal}/(mV s)$	$K/(J V s^{-1})$	$\alpha \times 10^3/s^{-1}$	$\beta \times 10^2/s^{-1}$	$k \times 10^3/s^{-1}$
1	3.000	49.766	50	1.4430	15.520	7.956	2.370	8.139
2	4.000	49.766	80	4.1190	15.465	8.359	2.412	8.479
3	4.000	49.766	120	6.1560	15.522	8.372	2.425	8.494
4	4.000	49.766	160	8.1894	15.557	8.197	2.394	8.316
5	3.000	49.766	300	8.6718	15.495	8.085	2.381	8.203
Average values					15.512	8.194	2.396	8.326
Standard errors					0.068	0.178	0.022	0.160

Table 2  
An example of analysis of the cooling curve of electrical energy calibration (No. 1 in Table 1)

$i$	$\Delta T/\mu V$	Data groups	$\Delta t'/s$	$\alpha \times 10^3/s^{-1}$	$\beta \times 10^2/s^{-1}$	$k \times 10^3/s^{-1}$
1	8.050	(1,5,9,13)	82.5	7.953	2.350	—
2	7.000	(2,6,10,14)	82.5	8.008	2.392	—
3	5.950	(3,7,11,15)	82.5	7.956	2.475	—
4	4.950	(4,8,12,16)	82.5	7.907	2.264	—
5	4.000	(1,5)	82.5	—	—	8.477
6	3.505	(2,6)	82.5	—	—	7.384
7	3.000	(3,7)	82.5	—	—	8.300
8	2.500	(4,8)	82.5	—	—	8.280
9	2.050	(5,9)	82.5	—	—	8.102
10	1.795	(6,10)	82.5	—	—	8.111
11	1.545	(7,11)	82.5	—	—	8.043
12	1.290	(8,12)	82.5	—	—	8.020
13	1.060	(9,13)	82.5	—	—	7.995
14	0.925	(10,14)	82.5	—	—	8.036
15	0.800	(11,15)	82.5	—	—	7.978
16	0.670	(12,16)	82.5	—	—	7.941
Average values				7.956	2.370	8.139
Standard errors				0.041	0.088	0.176

#### 4.2. Determination of thermokinetic parameters

Xanthine oxidase is a very important enzyme in the purine metabolism that catalyzes xanthine (the substrate) to uric acid (the product) in the presence of oxygen [41]. It reduces oxygen both univalently to superoxide anion (Eq. (7)) and divalently to hydrogen peroxide (Eq. (8)) [35,37,38]:



The partition between these two processes is differentially affected by the concentrations of reactants. Fridovich et al. [35,38] reported that high concentration of  $\text{O}_2$ , low concentration of xanthine and high pH favored univalent reduction.

The oxidation of xanthine catalyzed by xanthine oxidase in the presence of excess oxygen at 298.15 K and pH 7.5 was studied to test the correctness and applicability of the proposed double-parameter method. Both of the reactions were faster enzymatic reactions and obeyed typical Michaelis–Menten kinetics [35–39]. Therefore, this double-parameter method can be used to analyze the calorimetric curves of the above reactions. Under the experimental conditions in this work, the oxidation

Table 3

Overall molar reaction enthalpies of xanthine oxidation catalyzed by xanthine oxidase in the presence of excess oxygen at 298.15 K and pH 7.5

$[S]_0 \times 10^4 / (\text{mol dm}^{-3})$	$A / (\text{mV s})$	$Q_\infty / \text{mJ}$	$-\Delta_r H_m / (\text{kJ mol}^{-1})$
2.500	0.5514	8.553	5.70
3.250	0.6984	10.834	5.56
5.000	1.0476	16.250	5.42
6.250	1.2522	19.424	5.18
8.250	1.5618	24.227	4.89
10.00	1.8294	28.378	4.73
Average value			5.25
Standard error			0.38

almost went to completion in the range from 5 to 15 min.

The results of the measurement of  $\Delta_r H_m$  at different substrate concentrations are given in Table 3. Table 4 presents the results of the determination of the initial heat powers in two experiments, the experiments a and b, by means of zero- (Eq. (4)), single- (Eq. (3)), and double-parameter methods (Eq. (1)), respectively. It can be seen from Table 4 that the values of  $P_0$  calculated by zero- and single-parameter methods have poor self-consistency and considerable dispersivity (the standard relative errors in the two cases are

larger than 25% and 100%, respectively). Conversely, the values of  $P_0$  calculated by the double-parameter method are better self-consistent and less dispersive (the standard relative errors in the case are smaller than 10%). These results show that the double parameter model is better for describing the initial dynamic properties of the calorimetric system involving a faster enzyme-catalyzed reaction, and is applicable to thermokinetic studies of faster enzymatic reactions.

Plots of  $1/P_0^{(2)}$  versus  $1/[S]_0$  at different enzyme concentrations with the corresponding data calculated by the double-parameter method are shown in Fig. 1 from which it can be seen that the curves are linear. The calculated results of the kinetic parameters ( $K_m$ ,  $P_{\max}$ , and  $\nu_{\max}$ ) are summarized in Table 5. From Table 5, it can be found that the value of measurement of  $K_m$  has better repeatability and  $\nu_{\max}$  is approximately proportional to the enzyme concentration. Furthermore, the Michaelis constant calculated by the double-parameter method is in fair agreement with the literature value [34]. Therefore, the correctness and validity of this thermokinetic double-parameter method applied to the studies of faster enzyme-catalyzed reactions should be considered as proved. The experimental results also mean that the XOD-cata-

Table 4

Determination of initial heat powers of the oxidation of xanthine catalyzed by xanthine oxidase in the presence of excess oxygen at 298.15 K and pH 7.5 by zero-, single-, and double-parameter methods

$t/s$	$\Delta/\mu\text{V}$		$-(d\Delta/dt) \times 10^2 / (\mu\text{V s}^{-1})$		$(d^2\Delta/dt^2) \times 10^4 / (\mu\text{V s}^{-2})$		$P_0^{(0)} \times 10^2 / \text{mW}$		$P_0^{(1)} \times 10^2 / \text{mW}$		$P_0^{(2)} \times 10^2 / \text{mW}$	
	a	b	a	b	a	b	a	b	a	b	a	b
60	5.950	6.800	5.500	6.500	8.333	10.533	9.230	10.548	-1.017	-1.561	1.838	2.370
75	5.200	5.950	4.500	5.167	6.110	7.777	8.066	9.230	-0.317	-0.397	1.460	2.245
90	4.600	5.250	3.667	4.167	5.000	6.113	7.136	8.144	0.304	0.381	1.769	2.386
105	4.100	4.700	3.000	3.333	3.890	4.447	6.360	7.291	0.771	1.081	1.811	2.335
120	3.700	4.250	2.500	2.833	2.777	3.333	5.739	6.593	1.082	1.315	1.582	2.028
135	3.350	3.850	2.167	2.333	2.223	2.777	5.197	5.972	1.159	1.626	1.447	2.238
150	3.050	3.550	1.833	2.000	2.223	2.220	4.731	5.507	1.316	1.781	1.830	2.179
165	2.800	3.250	1.500	1.667	1.667	2.223	4.343	5.041	1.549	1.936	1.849	2.562
180	2.600	3.050	1.333	1.333	1.390	1.390	4.033	4.731	1.550	2.248	1.744	2.443
Average values							6.093	7.006	0.711	0.935	1.703	2.310
Standard relative errors							29.0%	28.3%	125%	133%	9.57%	6.80%

Conditions: For the experiment a, the initial concentrations of xanthine and xanthine oxidase in cell are  $5.000 \times 10^{-4} \text{ mol dm}^{-3}$  and  $9.667 \times 10^{-3} \text{ g dm}^{-3}$ , respectively, and for the experiment b, the corresponding concentrations are  $8.250 \times 10^{-4} \text{ mol dm}^{-3}$  and  $9.667 \times 10^{-3} \text{ g dm}^{-3}$ , respectively.  $P_0^{(0)}$ ,  $P_0^{(1)}$ , and  $P_0^{(2)}$  are the initial heat powers calculated by zero-, single-, and double-parameter methods, respectively.

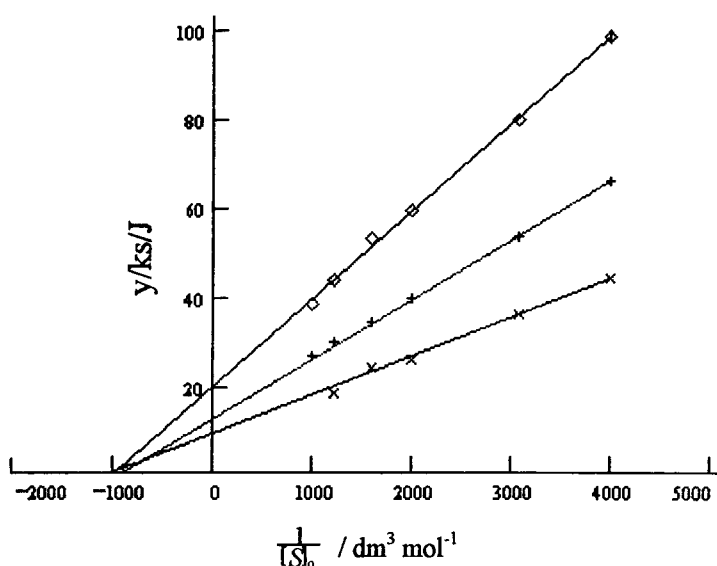


Fig. 1. Lineweaver–burk plot of  $1/P_0^{(2)}$  (y) against  $1/[S]_0$  at 298.15 K at xanthine oxidase concentrations of ( $\diamond$ )  $9.667 \times 10^{-3} \text{ g dm}^{-3}$ , (+)  $1.450 \times 10^{-2} \text{ g dm}^{-3}$ , ( $\times$ )  $1.993 \times 10^{-2} \text{ g dm}^{-3}$ .

Table 5

$K_m$ ,  $P_{\max}$ , and  $\nu_{\max}$  values of xanthine oxidation catalyzed by xanthine oxidase in the presence of excess oxygen at 298.15 K and pH 7.5

$[E]_0 \times 10^3 / (\text{g dm}^{-3})$	$K_m \times 10^3 / (\text{mol dm}^{-3})$	$P_{\max} \times 10^2 / \text{mW}$	$\nu_{\max} \times 10^6 / (\text{mol dm}^{-3} \text{ s}^{-1})$
9.667	1.02	5.17	1.64
14.50	1.10	8.16	2.59
19.33	0.985	11.04	3.50
Average value	1.04	—	—
Literature value	1.05	—	—

lyzed oxidation of xanthine in the presence of excess oxygen follows the Michaelis–Menten kinetics.

## 5. Discussion

### 5.1. Advantages of the thermokinetic double-parameter method

Compared with most of the methods for the determination of thermokinetics of fast reactions, this thermokinetic double-parameter method has two advantages. The first advantage is that the physical senses of the model parameters for the double-parameter method are definite, and the second advantage is that the determination of these parameters is simple.

In addition, to calculate the thermokinetic parameters with this thermokinetic double-parameter method, it is not necessary to know the peak area at time  $t$ . For the reduced-extent method [6], however, we must measure the peak area. This double parameter method is especially suitable for studying faster enzyme-catalyzed reactions that cannot be investigated by the traditional thermokinetic single-parameter method based on Tian's equation [7].

### 5.2. On the reaction mechanism

As seen from Table 3, the overall reaction of XOD-catalyzed oxidation of xanthine is slightly exothermic (this small heat effect can only be measured by microcalorimetry), and the overall molar reaction

enthalpy of this oxidation is remarkably dependent on the initial concentration of xanthine. From the data of the bond enthalpies for the corresponding reacting components [43], it can be concluded that Eq. (7) is an endothermic reaction whereas Eq. (8) is exothermic. These results show that the concentration of xanthine has noticeable influence on the partition between Eqs. (7) and (8), which support the mechanism for the oxidation of xanthine catalyzed by xanthine oxidase proposed by Fridovich et al. [35,38].

In conclusion, the thermokinetic double-parameter method for faster enzyme-catalyzed reactions proposed in this paper is simple and its validity and feasibility have been demonstrated.

### Acknowledgements

The support for this project from the National Natural Science Foundation of China is gratefully acknowledged.

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