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APPLICATION OF MICROCALORIMETRY FOR ANALYSING INITIAL RATES OF ENZYMATIC REACTIONS

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ABSTRACT

The use of Calvet type heat conduction microcalorimetry in the flow-mix mode for the investigation of enzyme kinetics has been discussed. The criteria to choose the optimal values of enzyme concentration and flow rate have been proposed. As an example, the experimental data for sucrose hydrolysis by means of "Serva" invertase at 30°C, pH 4.8 have been presented using LKB 2277 microcalorimeter ("Bioactivity Monitor").

INTRODUCTION

The thermochemical method as a sensitive and quite universal method can largely be used for kinetic investigations both for chemical and enzymatic reactions.

The flow-mix mode of modern heat conductive microcalorimetry seems to be a convenient method for the measurement of initial rates of enzymatic reactions. Conditions for such measurements must be chosen accurately. Insufficient attention has been directed to this problem.

Calorimetry is often used for the determination of enzyme activity. For example, Grime et al. [1] have described the enthalpimetric determination of serum cholinesterase activity with typical errors of 1%. The heat effect in this case is significantly amplified by the concurrent protonation of Tris buffer. High values of substrate initial concentration $[S]_0$ have been used in this work ($[S]_0 \gg K_m$), initial rate v_0 has a value near to maximum rate V. Using the integrated form of the Michaelis–Menten equation, Grime, Lockhart and Tan [2] have determined the value of K_m for the α-chymotrypsin-catalysed hydrolysis of ATEE. Corrected enthalpimetric progressive curves have been used for calculation. The summary heat effect including Tris buffer protonation is great (about 48 kJ mol^{-1}) and the method is quite sensitive.

Calorimetric measurements of enzymatic reactions are also popular for substrate concentration determination. "The best" conditions in this case can be found empirically. Carunchio et al. [3] have elaborated the method

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for urea determination and they have found that the flow-mix mode is 10^4 fold more sensitive than the flow-through mode. This conclusion is not commented on in this work. The large enzyme concentrations have been used here and it's easy to calculate that, using flow-through mode, the reaction was practically completed before entering the measuring cell.

Whiting and Carr have made systematic investigations for application of DSC in kinetic analysis of first- and zero-order reactions, especially in the isothermal mode [4]. The authors conclude that DSC may be applied to only a limited number of enzyme reactions.

In our opinion the thermochemical methods can and must be systematically used for enzyme kinetics analysis. Modern Calvet type microcalorimeters seem to be preferred for this purpose. In the present work one of the methods will be discussed, the use of flow-mix systems for initial rates measurements. It must be pointed out that it's one of the few methods overall where the direct measurement of the rate of reaction (intensivity of process) is available.

The scheme of flow-mix cell is presented in Fig. 1.

Solutions of substrate S and enzyme E are pumped into the measuring cell with flow rates q_1 and q_2, respectively. The reaction mixture leaves the cell with flow rate $q = q_1 + q_2$ [ml s^{-1}]. The observed summary heat flow is caused by different effects—by the transformation of substrate and the enthalpy change due to dilution of substrate and enzyme solutions.

The mean residence time in the cell τ_r is equal to

$$\tau_r = \frac{V_c}{q}[S] \tag{1}$$

where V_c is the effective volume of cell in ml.

The initial concentrations of S and E after mixing are $[S]_0$ [M or mmol ml^{-1}] and $[E]_0$ [mg ml^{-1}].

If the thermal effects of dilution are equal to Q_1 for substrate [kJ mol^{-1} or J $mmol^{-1}$] and Q_2 for enzyme [J mg^{-1}], then we can find the heat flow N_{dil} as the sum of two dilution effects. It must be considered that the amounts of

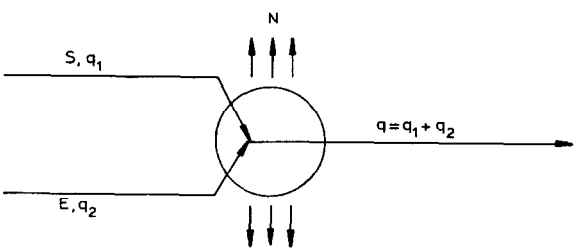

Fig. 1. Scheme of the flow-mix cell.

substrate and enzyme, entering in the cell in a second, are $q\,[S]_0$ [mmol s^{-1}] and $q \cdot [E]_0$ [mg s^{-1}], respectively.

$$N_{dil} = q[S]_0 Q_1 + q[E]_0 Q_2 = q([S]_0 Q_1 + [E]_0 Q_2)[W] \tag{2}$$

It is easy to see that N_{dil} is proportional to flow rate (to the quantity of mixed solutions in time unit).

Usually, the concentration of enzyme is small and the corresponding heat effect of dilution is negligible. Consequently, N_{dil} has only a weak dependence on enzyme concentration.

The summary heat flow caused by enzymatic reaction depends on enzyme concentration, its activity and on the heat effect of the reaction Q_r [kJ mol^{-1} or J $mmol^{-1}$]. Let us assume that the rate of reaction during the residence time in the cell changes unremarkably and is equal to the initial rate of enzymatic reaction [mol l^{-1} s^{-1} or mmol ml^{-1} s^{-1}]. (This problem will be discussed in further detail.) Then it's evident that the heat flow corresponding to the course of reaction N_r can be expressed as follows

$$N_r = v_0 V_c Q_r [W] \tag{3}$$

The initial rate v_0 is proportional to enzyme concentration $[E]_0$ [mg ml^{-1}] and to the effective activity of enzyme A [mcat mg^{-1}] at the temperature and pH used. We can assume that the value of A differs only slightly from maximum rate V and that the initial rate of reaction belongs to Michaelis–Menten kinetics. In this case

$$v_0 = [E]_0 \cdot A \cdot \frac{[S]_0}{K_m + [S]_0} [\text{mcat ml}^{-1}] \tag{4}$$

Consequently

$$N_r = [E]_0 \cdot A \cdot \frac{[S]_0}{K_m + [S]_0} \cdot V_c \cdot Q_r [W] \tag{5}$$

and in the first approximation this heat flow does not depend on flow rate. (In fact, the effective volume of cell V_c depends slightly on flow rate.)

The summary heat flow in the flow-mix measuring cell is equal to the sum of N_{dil} and N_r

$$N = [E]_0 \cdot A \frac{[S]_0}{K_m + [S]_0} \cdot V_c Q_r + q([S]_0 Q_1 + [E]_0 Q_2)[W] \tag{6}$$

For each series of experiments, primarily the values of two parameters can and must be optimized—the flow rate and enzyme concentration $[E]_0$.

As we can see from eqn. (6), the rôle of dilution effects reduces at low flow rates. The economy of solutions is also evident in this case and in certain circumstances may be important. On the other hand, we must take into consideration the volume of tubes between the solutions' vessels and the measuring cell which for LKB-2277 microcalorimeter is equal to 1.5–3 ml. If

the optimal flow rates are chosen as recommended by LKB, $2.8–5.6 \times 10^{-3}$ ml s^{-1} (10–20 ml h^{-1}) the solutions arrive at the cell after 6–15 min. The thermal inertia must also be considered (the value of the first time constant for our calorimeter in the flow-mix cell with empty glass ampoules is equal to 134 s.) To economize the time necessary for experiments too low flow rates are not recommended.

The principal advantage of the Calvet type calorimeter in our case is the possibility of measuring directly the rate of reaction because the output signal is proportional to this one. The conditions for enzyme kinetic study must be chosen in a way as to guarantee the measurement of the rates of enzymatic reaction which are really near to the initial rates.

In the flow-mix cell $v_0 \cdot V_c$ [mmol s^{-1}] of substrate will react in one second, and the amount of substrate entering in the cell at the same time is $q \cdot [S]_0$ [mmol s^{-1}].

The degree of conversion X of substrate can be expressed as follows

$$X = \frac{v_0 \cdot V_c}{q \cdot [S]_0} = \frac{[E]_0 \cdot V_c \cdot A \cdot [S]_0}{q \cdot [S]_0 \cdot (K_m + [S]_0)} = \frac{[E]_0 \cdot V_c \cdot A}{q(K_m + [S]_0)} \tag{7}$$

We can choose the maximum permitted degree of conversion for each experiment. For example, for Michaelis–Menten kinetics and high substrate concentrations values of X up to 0.2 may be used in some cases to have the rates of reaction near to the initial rates. If the products of reaction are strong inhibitors, the permitted value of X may be lower than 0.01 (1% transformation of substrate).

On the basis of eqn. (7) the next principal equation can be proposed to find an appropriate ratio of enzyme concentration and flow rate to guarantee the permitted value of conversion X_p of substrate.

$$\frac{[E]_0}{q} \leq \frac{X_p \cdot (K_m + [S]_0)}{A \cdot V_c} \left[\text{mg s (ml ml)}^{-1}\right] \tag{8}$$

It's evident that the approximate values of K_m and activity A must be known to choose suitable conditions for experiments.

An alternative possibility may be inevitable if the reaction heat effect is very small. In this case the greater enzyme concentration or the smaller flow rate must be chosen and the corrections to the values of measured initial rates must be done. A good knowledge of the reaction integral kinetics is necessary in this case.

EXPERIMENTAL

Apparatus

A LKB 2277 microcalorimeter ("Bioactivity Monitor") with "Colora" prethermostat was used in flow-mix mode at 30°C. The solutions of sub-

strate, enzyme and buffer were pumped into the measuring cell by means of LKB 2132 microperpex pump. The volume of the cell V_c is about 0.50 ml. For the preparation of the enzyme and substrate solutions, identical buffer solutions were used, namely 0.05 M acetate buffer, pH 4.8.

All substrate solutions were prepared daily from sucrose of chemically pure grade. Enzyme solution of "Serva" β-fructofuranosidase (invertase) with concentration 0.33 mg ml^{-1} was stored at 5°C and used after dilution for the experiments. This enzyme preparation has an excellent stability and there was no inactivation after one month.

RESULTS AND DISCUSSION

The experiments of sucrose hydrolysis have been carried out over the concentration range 8–80 mM.

In Fig. 2 the dependence of sucrose solutions dilution effects (with buffer) on flow rate q are presented. It can be concluded that this effect N_{dil} is really approximately proportional to flow rate (see eqn. 2).

The flow rates $q_1 = q_2 = 13.2$ ml h^{-1} were chosen for initial rates measurements. In this case the dilution effects are small and the residence time of reaction mixture in the cell τ_r is about 70 s.

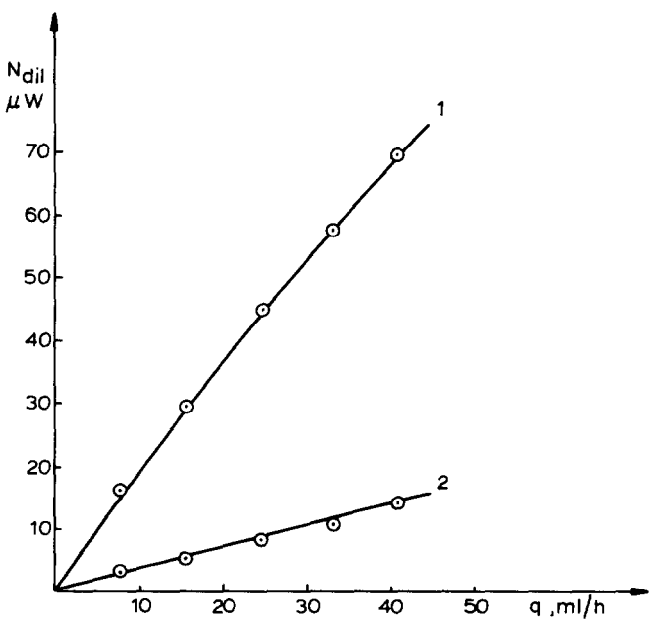

Fig. 2. Dilution effects of sucrose solutions with buffer. Concentration of sucrose: (1) 0.2 M, (2) 0.1 M.

TABLE 1

Thermochemical determination of initial rates of sucrose hydrolysis at 30°C, pH 4.8 (0.05 acetate buffer) with "Serva" invertase (flow rates $q_1 = q_2 = 13.2$ ml h^{-1})

$[S]_0$ (M)	$[E]_0$ (mg ml^{-1})	N_{dil} (μW)	N (μW)	N_r (μW)		Approx. value of substrate conversion X
				for $[E]_0$	calc. for $[E]_0$ = 0.01 mg ml^{-1}	
0.0081	0.0131	2.6	26.0	23.4	17.9	0.04
0.0102	0.0131	3.0	31.3	28.3	21.6	0.04
0.0102	0.0131	3.0	31.8	28.8	22.0	0.04
0.0203	0.0131	5.2	44.8	45.0	34.4	0.03
0.0253	0.0220	5.5	95.2	89.7	40.8	0.04
0.0507	0.0131	12.4	83.6	71.4	54.5	0.02
0.0509	0.0220	12.5	132.2	119.7	54.4	0.03
0.0509	0.0220	12.5	132.4	119.9	54.5	0.03
0.0509	0.0220	12.5	134.8	122.3	55.6	0.03
0.0761	0.0131	26.2	106.2	80.0	61.1	0.01

For the measurements the concentrations of enzyme $[E]_0$ 0.013–0.022 mg ml^{-1} were used. The activity of "Serva" β-fructofuranosidase preparation at 30°C pH 4.8 was about 1.67 μcat mg^{-1} (100 E mg^{-1}). The permitted

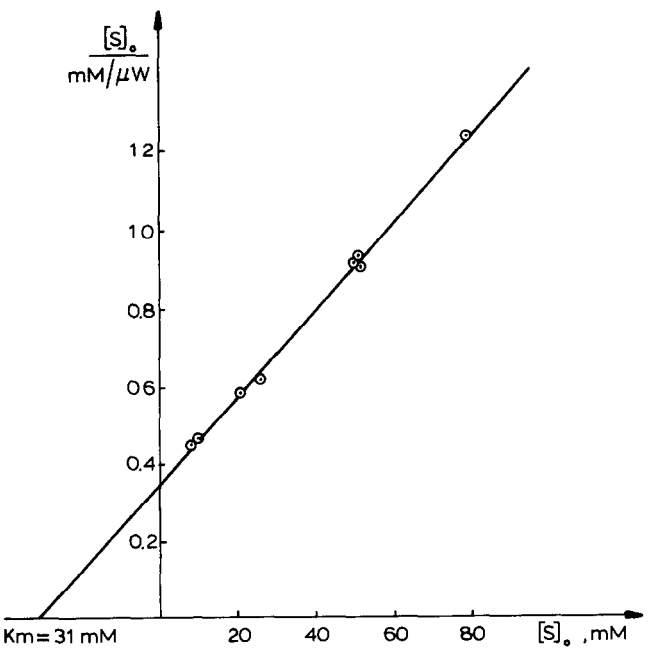

Fig. 3. Hanes plot of initial rates measurements.

maximum value of substrate conversion X has been chosen at 0.05 and the suitable values of $[E]_0$ were calculated using eqn. (8).

The results of measurements are presented in Table 1. The approximate values of substrate conversion have been calculated using eqn. (7). It's evident that the observed heat flow values N_r really correspond to initial rates of enzymatic hydrolysis of sucrose.

In Fig. 3 the results are illustrated by a Hanes plot, K_m value is about 31 mM. The calculation by the least-squares method described by Cornish-Bowden [5] gave the value $K_m = 0.0310 \pm 0.0011$ M. The value of V can not be expressed simply in enzyme activity units because the heat effect of hydrolysis reaction must be known (see eqn. 3).

We have proved the flow-mix mode for estimating of K_m values for many enzyme catalysed reactions. In our opinion the method is reliable and permits measurement of the initial rates in strictly controlled conditions.

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