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Note

Application of direct injection enthalpimetry to biochemical systems. Theoretical aspects and the urea–urease system

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The technique and practice of both thermometric titrimetry and direct injection enthalpimetry have been reviewed recently[1–3]. However as has been pointed out[3] very few reports exist in the literature of applications of the thermometric principles in the field of biochemistry.

In a recent paper[4] Beezer and Tyrrell have discussed the theoretical aspects of flow microcalorimetry as applied to biological problems. This paper describes a similar set of theoretical equations governing the response of a thermometric titration apparatus operated in the direct injection enthalpimetric approach, and, specifically it's application to enzyme catalysed reactions. Kinetic methods have been used extensively in thermometric analysis (see refs. 1 and 4 and refs. cited therein) and the principles of calorimetry have found quite widespread application in the study of chemical kinetics[5–11]. A majority[5–8] of the investigations relied upon the determination of maximum (or minimum) temperature attained in a reaction vessel under non-adiabatic conditions. Jordan and coworkers[10,11] have developed "thermokinetic analysis" techniques considerably of late. However these principles have not, as yet, been applied to biochemical problems.

Enzyme reactions proceed at a rate which is directly proportional to the enzyme activity, e_0, and, to the substrate concentration (S) if this is small enough. The rate is independent of substrate concentration at sufficiently high substrate concentrations. The well known Michaelis–Menten[12] equation describes this behaviour.

$$\frac{\mathrm{d}[\text{product}]}{\mathrm{d}t} = \frac{ke_0S}{K_m+S} \tag{1}$$

where k and K_m are constants. Enzyme activities may best be determined from measured reaction rates under the restriction $S \gg K_m$ whereas substrate concentrations may be found only under the restriction that $S \ll K_m$. Application of the direct injection enthalpimetric procedure then involves reactions exhibiting either zero or first order kinetics as limiting cases.

Fast reactions and some applications of kinetic methods in thermometric analysis have been adequately reviewed[1,4].

Application to slow reactions

(*a*) *Zero order reaction*

If the volume of the calorimetric cell is V_c ml and the zero order rate constant is k_0 mol ml^{-1} sec^{-1} then the rate of formation of product is $k_0 V_c$ mol sec^{-1}. The amount of product formed after time t sec is then $k_0 V_c t$ mol. If the heat of reaction is $-\Delta H_P$ J mol^{-1} then the heat evolved is equal to

$$-k_0 V_c t \Delta H_R$$

With a heat capacity of W J deg^{-1} the evolution of this amount of heat within the calorimetric system would result in a temperature rise, ΔT, of

$$\Delta T = \frac{k_0 V_c t \Delta H_R}{W} \text{ deg.} \tag{2}$$

(*b*) *First order reaction*

If the initial concentration of the reagent is S mol ml^{-1} and the first order rate constant is k_1 sec^{-1} then the amount of product formed after time t sec is, from the known kinetics,

$$S[1-\exp(-k_1 t)]V_c \text{ mol.}$$

Thus the heat evolved under these conditions will be

$$-\Delta H_R S[1-\exp(-k_1 t)]V_c \text{ J.}$$

Similarly the temperature rise produced in the calorimetric system will be

$$\Delta T = \frac{\Delta H_R S}{W}(1-\exp(-k_1 t)]V_c \text{ deg.} \tag{3}$$

Equation (3) indicates that a plot of ΔT vs. S should be linear for ΔT measured at a fixed time after initiation of the reaction. If, however, $k_1 t$ is small as will be true for situations in which k_1 is small and/or t is small (*i.e.* initial rates are measured) than

$$[1-\exp(-k_1 t)] = k_1 t$$

and eqn (3) can be modified to

$$\Delta T = \frac{\Delta H_R S}{W} k_1 t V_c \tag{4}$$

Equation (4) also implies that a plot of ΔT vs. S should be linear throughout the first order region only. Furthermore, a plot of $\Delta T/t$ vs. S should be linear over the first order period and a similar plot of $\Delta T/t$ vs. S for the zero order case should likewise be linear (eqn 2) but of different (zero) slope. From the slopes of these lines both the first order and zero order rate constants may be derived if ΔH_R and W are known (V_c can easily be measured). W may easily be determined by performing a (rapid)

reaction of known ΔH_R in the calorimetric vessel. ΔH_R may be determined from the zero order mode by use of subsidiary analytical data on the extent of reaction in time t.

Application to enzyme reactions

(*a*) *Excess substrate, zero order reaction*

Under these conditions $S \gg K_m$ and eqn (1) becomes

$$\frac{d[\text{product}]}{dt} = ke_0$$

and hence eqn (2) becomes

$$\Delta T = \frac{ke_0 V_c t \Delta H_R}{W} \text{ deg} \tag{5}$$

(*b*) *Substrate concentration small, $S \ll K_m$*

This restriction transforms eqn (1) to

$$\frac{d[\text{product}]}{dt} = \frac{ke_0 S}{K_m}$$

and

$$k_1 = \left(\frac{ke_0}{K_m}\right)$$

and hence eqn (4), when the condition that $k_1 t$ is small applies, becomes

$$\Delta T = \frac{\Delta H_R S}{W}\left(\frac{ke_0}{K_m}\right) t V_c. \tag{6}$$

Derivation of the Michaelis constant, K_m

From eqn (5) we may derive

$$\left(\frac{\Delta T}{t}\right)_Z = \frac{ke_0 V_c \Delta H_R}{W}$$

and from eqn (6)

$$\left(\frac{\Delta T}{t}\right)_F = \frac{\Delta H_R}{W}\left(\frac{ke_0}{K_m}\right) V_c S.$$

If now a quantity α is defined as $\frac{\left(\frac{\Delta T}{t}\right)_F}{S}$ then

$$\alpha = \frac{\left(\frac{\Delta T}{t}\right)_F}{S} = \frac{\Delta H_R}{W}\left(\frac{ke_0}{K_m}\right) V_c$$

which is a constant. Now, K_m the Michaelis constant will be given by $[(\Delta T/t)/\alpha]Z$. Furthermore since $(\Delta T/t)$ is directly proportional to the reaction rate (*cf.* eqn 4) then it is possible to treat the data by the Lineweaver–Burk method and plot $[1/(\Delta T/t)]$ vs. $(1/S)$ and hence obtain K_m.

Experimental

The technique of direct injection enthalpimetry has been described previously[13] as has the apparatus[14]. Enzyme (urease, Sigma Type III from Jack Bean) solutions (10 mg ml^{-1}) were made up in phosphate buffer $M/15$, pH 7) and stored on ice throughout the working period. The charge in the calorimetric cell was 20 ml of the appropriate urea solution in buffer. 0.5 ml of enzyme solution (equivalent to approximately 0.4 mg ml^{-1} in the resulting mixture) was added, rapidly from a syringe, in the manner previously described[13]. ΔT was measured in arbitrary units, over a fixed time t. Adiabaticity was assumed to exist for 2 min only following injection of enzyme solution. $(\Delta T/t)$ was measured over the first minute of reaction.

Results and discussion

A representative set of data are presented in Table 1.

TABLE 1

$S = [urea]$ (M)	$(\Delta T/t)$ *arbitrary units*	$(\Delta T/t)/S$
1.0	16.5	16.5
0.5	16.5	33.0
0.4	16.0	40.0
0.3	12.5	41.7
0.25	12.0	48.0
0.20	11.0	55.0
0.10	10.5	105
0.075	9.5	127
0.05	8.5	170
0.04	7.5	187
0.030	6	200
0.025	5	250
0.01	3	300
0.0075	2.5	334
0.005	2.0	400
0.0025	—	—

The column headed $(\Delta T/t)$ indicates that no constant value is achieved hence, no true first order region has been investigated. It is in the region between true first order and zero order kinetic reactions that the double reciprocal plot (Lineweaver–Burk[12]) of $[1/(\Delta T/t)]$ vs. $(1/S)$ should be most useful. Treatment of the data in this fashion yields a straight line (regression coefficient 0.9918). Measurement of slope and intercept yield a value for K_m of 0.027 M. This value is compared with those values available in the literature for the urea–urease system in phosphate buffer in Table 2.

TABLE 2
VALUES OF K_m IN PHOSPHATE BUFFER

K_m (M)	*M phosphate buffer*	*pH*	*Ref.*
0.027	0.067	7.0	this work
0.026	0.158	7.0	15
0.05	0.267	7.0	15
0.06	0.75	6.9	16

The value of K_m derived is clearly of the right order of magnitude in comparison with the other values listed in Table 2 (in all systems described in Table 2 Jack Bean urease was used). This result suggests that treatment of experimental data in this fashion is justified. A plot of $(\Delta T/t)$ vs. S exhibits the existence of an apparent first order period (linear portion of graph $S \leqslant 0.01\ M$) however this must be an artefact since first order kinetics should only be exhibited for situations in which $S < 0.01\ K_m$ and such concentrations were not accessible to experimentation. Such pseudo first order behaviour in regions where $S \geqslant 0.1\ K_m$ has been observed previously[16]. The experimental thermograms exhibit no curvature over a 2 minute period demonstrating the existence of a pseudo first order process. ΔH_R for the urea–urease system has recently been determined[16] as 33 kJ mol^{-1}.

It should be noted that although rather large amounts of enzyme are described as necessary in this paper this is only a dictate of apparatus design, since, W, the heat capacity of the apparatus and contents can be defined as

$$W = V_c C_c + C_{cal}.$$

V_c and C_c refer to the volume and heat capacity per ml of the titrand solution respectively and C_{cal} refers to the heat capacity of all the associated glassware, stirrer *etc.* In the condition that $V_c C \gg C_{cal}$ then W reduces to

$$W = V_c C_c.$$

Substitution of this equation into eqns (2) and (4) yields expressions in which ΔT is independent of the volume of solution in the calorimetric cell *i.e.* miniaturisation is possible. The limit of this miniaturisation being determined both by the condition that $V_c C_c \gg C_{cal}$ and physically by the dimension of a cell in which must be located a stirrer, thermistor etc. 2 ml is the smallest cell reported[17].

The limitations of the method are that ΔH_R must be large enough, for the enzyme catalysed reaction, to give an appreciable temperature rise over the period of measurement (*ca.* 1–2 min) and that enzyme solutions may be prepared sufficiently concentrated to satisfy the requirements[1,13] of the direct injection enthalpimetric technique. Unfortunately ΔH_R, is not known for a great many substrate–enzyme reactions.

The advantages of the method are those inherent in the technique itself[1,3] namely simplicity, generality and cheapness. Additionally the reaction property

measured ΔH_R, (or its equivalent ΔT in a constant heat capacity system) is general and makes no demand for optical purity, pH change etc. The possibility exists therefore of using the technique for substrate and enzymic activity assays in situations in which conventional methods[18] are inappropriate. It likewise appears that inhibitor concentration levels may be determinable for the degree to which they inhibit the zero order response of a standard reaction. Such techniques have been employed[19,20] using flow microcalorimeters a more sensitive (and expensive) and precise apparatus.

Further work is continuing on enzyme systems studied by the thermometric technique.

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