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ENTHALPIMETRIC FLOW ANALYSIS OF UREA USING IMMOBILIZED UREASE

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ABSTRACT

An enthalpimetric determination of urea using immobilized urease in a flow system is described. Sensitivity of analysis is related to the mole reaction enthalpy (dH_R). This is estimated as $(61 \pm 3$ kJ mol^{-1} and 20 ± 1 kJ mol^{-1} in sodium phosphate and tris–HCl buffer respectively. Factors affecting the minimum amount of urea detected, linearity range and rate of analysis are discussed.

INTRODUCTION

Enthalpimetry of enzyme catalysed reactions has immense potential for application to the analyses of compounds of clinical and industrial interest. By far the greater number of reports have concerned the use of soluble enzymes [1–4]. The cost of enzyme may be higher than that of any other reagent in proportion to the quantity used.

Prior immobilization of the enzyme, i.e., physical entrapment in, adsorption on, or chemical binding to an inert support, can reduce the enzyme cost very significantly. This is chiefly because immobilization results in the ability to separate the enzyme from a given reaction mixture for re-use, increased stability, and therefore a prolonged useful life [5]. The easier recovery may encourage the use of higher concentrations of enzyme; for reasons discussed below, this is desirable. Such advantages of enzyme immobilization become particularly striking in enthalpimetric analysis under flow conditions.

This approach was recently illustrated by the use of immobilized cholinesterase in the enthalpimetric measurement of inhibitors [6]. An excellent study by Grime and Tan illustrates the complementary approach using soluble cholinesterase [7].

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The performance of an enzyme-based flow enthalpimetric analytical system will obviously depend on enzyme related factors (e.g., enzyme concentration, degree of affinity for the reactant and the uniqueness of the reaction catalysed) as well as on the thermochemistry of the underlying reaction. How such factors affect the sensitivity. i.e., heat change per mole of urea injected, linearity range and speed of sample analysis is the subject of this paper.

Urea has a long established significance in the clinical evaluation of kidney function [8]. The thermochemistry of urea hydrolysis, catalysed by soluble urease, has been studied under non-flow conditions [9]. A flow microcalorimetric study of the kinetics of urea hydrolysis catalysed by immobilized urease was recently reported [10].

THEORETICAL

The heat change (dQ: J) resulting from n mol of chemical change in a calorimetric reaction cell is given by eqn. 1, Table 1; V (cm^3), S (mol cm^{-3}) and dH_R (J mol^{-1}) are the total cell volume, concentration of reactant (hereafter termed the substrate) and the mol reaction enthalpy, respectively. It may be more convenient to measure the heat effect (dQ/dt: J s^{-1}) in flow analysis, e.g., by heat conduction calorimetry [11]. In eqn. 2, F (cm^3 s^{-1}) is the sample flow rate; the ratio V/F defines the reaction time or sample residence time (T, s) and dH^* (J mol^{-1}) is the observed sensitivity; $dH^* \leqslant dH_R$ depending on the experimental conditions.

TABLE 1

Relations applied to enzyme based flow enthalpimetry [a,b]

$dQ = VS\,dH_R = n\,dH_R$	(1)
$(dQ/dt) = FS\,dH^*$	(2)
$(S < K_m)$	(3)
$(dQ/dt) = k_1 SV\,dH^* = (V_{max}/K_m)SFT\,dH^*$	
$(S > K_m)$	(4)
$(dQ/dt) = k_0 V\,dH^* = V_{max}FT\,dH^*$	
$X = 1 - \mathrm{Exp}(-k_1 T) = 1 - \mathrm{Exp}(-(V_{max}/K_m)V/F))$	(5)
$\epsilon V'' = (dQ/dt)/V\,dH_R$	(6)
$V/F = T\epsilon^{-1}$	

[a] Ref. (12); eqn. (6) applies only to immobilized enzyme filled calorimetric cells. $V = 0.5$ cm^3.
[b] See text for the meaning of symbols.

Assuming Michaelis–Menten enzyme kinetics, the heat effect from an enzyme catalysed reaction is described by eqns. (3) and (4) for the first or zero-order limiting cases respectively [12]. In eqns. (3) and (4), K_m (mol cm^{-3}) is the Michaelis constant and V_{max} (mol cm^{-3} s^{-1}) is the maximum velocity of the enzyme catalysed reaction. V_{max} is proportional to the concentration of enzyme (E_t); $V_{max} = k_c E_t$. The constant k_c (s^{-1}) is a so-called turn-over number or number of catalytic cycles per molecule of enzyme per second [13]. Relation (3) clearly shows the dependence of the heat effect on concentration of substrate, concentration of enzyme, affinity for the substrate ($1/K_m$), sample flow rate and calorimeter cell volume.

Finally, eqn. (5) is a consequence of general first order kinetics and describes the fraction of substrate reacted (X) as a function of the reaction time; k_1 is the first order rate constant.

EXPERIMENTAL

Reagents

Urease (EC.3.5.1.5; Type VII from Jack beans), urea, aminopropyltriethoxysilane (APTES), and glutaraldehyde were commercial samples (Sigma U.K.). Controlled porosity glass (CPG: 200–400 mesh, 55 μm mean diameter; 216 m^2 g^{-1}) and all other compounds were supplied by the British Drug House (BDH) Ltd., Poole, England.

Methods

Urease was chemically bound to CPG as follows [6]. The CPG was cleansed by washing in hot dilute nitric acid (30% v/v) and thoroughly rinsing with distilled water. The CPG was modified by allowing to stand with APTES (1% v/v) in dry acetone at room temperature until the mixture evaporated to dryness. The aminosilane modified glass was activated by treating with glutaraldehyde (1% v/v) for 1 h. To immobilize, urease (1 mg cm^{-3}) was incubated with CPG, and suspended in sodium phosphate buffer (0.2 M, pH 7.0; 10^{-3} M EDTA), for 18 h at 5°C. The CPG–enzyme conjugate resulting was then exposed to trishydroxymethylaminomethane–HCl (tris–HCl) buffer (0.2 M, pH 8.0; 10^{-3} M EDTA) to block remaining free aldehyde groups.

The heat effect from the urea hydrolysis was measured using the LKB 10700-1 flow microcalorimeter as described previously [6]. The carrier stream was tris (0.2 M, pH 8.0; 10^{-3}M EDTA) or sodium phosphate buffer (0.2 M, pH 7.0; 10^{-3} M EDTA). Samples (0.5 cm^{-3} or 8.0 cm^{-3}) of urea were injected via a three-way valve into the carrier stream propelled at 5–50 cm^{-3} h^{-1} using a syringe pump (Razel).

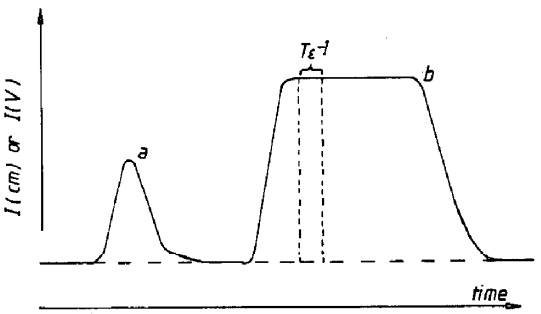

Fig. 1. Instrument response (I, volt (V)) with discrete (a) and continuous (b) sample injection. (a) $dQ = E_a$ (J cm^{-2})$\times C_{sp}$ (cm s^{-1}) $\int I\,dt$ (cm s), where C_{sp} is the chart recorder speed. (b) $dQ = E_b$ (J s^{-1} V^{-1})$\times I \times T\epsilon^{-1}$. E_a and E_b are the transient and steady state calibration constants for the calorimeter [18].

The nominal rate of urea hydrolysis in the immobilized urease reactor ($\epsilon v''$) was calculated using eqn. (6) and the steady state (dQ/dt) from continuous (8.0 cm^3) sample injection (Fig. 1); ϵ is the fraction of reactor volume occupied by the immobilized enzyme support. Enzyme kinetic parameters ($\epsilon V''_{max}$ and K''_m) incorporating diffusion effects were determined using the Lineweaver–Burke linearization plot [10,13]. The activity of immobilized urease in the calorimeter flow cell could be reduced by passing dry air through it for varying lengths of time. Sensitivity (dH^*) was estimated from eqn. (2), Table 1.

RESULTS AND DISCUSSION

The sensitivity of urea determination, by flow enthalpimetry (dH^*), is given as a function of the reactor nominal immobilized urease activity ($\epsilon V''_{max}$) and sample residence time ($T\epsilon^{-1}$) for sodium phosphate and tris buffers, Table 2. The highest sensitivity observed in both cases is, within experimental error, the same as the highest estimates of dH_R for urea hydrolysis given in the literature, Table 3. Thus the dH_R value is correctly considered as the maximum sensitivity in (flow) enthalpimetric analysis.

Urea hydrolysis, catalysed by immobilized urease, results in a quantitative heat effect. Maximum sensitivity of enthalpimetric analysis should therefore arise where there is complete hydrolysis of all injected substrate within the reaction time. From eqn. (5), it is seen that for $k_1 T \geqslant 3.0$, $X \geqslant 0.95$. That is, the sensitivity of analysis will be enhanced by a high reactor immobilized urease activity and by a long sample residence time. These expectations are supported by the results given in Table 2.

The sensitivity of analysis can also be related to more "remote" parameters, e.g., immobilized enzyme column radius (r), lenght (l) (cf. eqn. (6)

TABLE 2

Residence time and enzyme concentration effects on sensitivity [a] of urea determination

$\epsilon V''_{max}$	K''_m	$T\epsilon^{-1}$ (s)	X	dH^* (kJ mol^{-1}) [b]
1.48	66.4	177	0.98	60
1.48	66.4	79	0.84	52
1.00	46.1	49	0.66	32
0.08	29.6	159	0.34	16
0.40 [c]	32.0	158	0.86	17
0.32	44.4	98	0.51	9
0.20	73.3	98	0.23	3

[a] V''_{max} (10^{-6} mol cm^{-3} s^{-1}), K''_m (10^{-6} mol cm^{-3}); $V = 0.5$ cm^3; X was determined using eqn. (5) conditions.

[b] Sodium phosphate buffer (0.2 M, pH 7.0; 10^{-3} M EDTA).

[c] Tris–HCl buffer (0.2 M, pH 8.0; 10^{-3} M EDTA).

$T\epsilon^{-1} = \pi r^2 l/F$) and packing type. Column dimensions are usually fixed for a given calorimeter. The type of immobilization support, however, should present a large specific surface area for enzyme immobilization.

With the present flow system discrete samples (0.5 cm^3) of urea could be analysed at a rate of 4 h^{-1} with high sensitivity, Figs. 1 and 2. This rate of sample determination is very much greater than obtainable using a batch calorimeter, where time is required for temperature equilibration after successive sample loading. However, it is easier to obtain maximum sensitivity using a batch calorimeter. The substrate reaction is simply allowed time to go to completion.

The linear range for urea determination was $0.5–80 \times 10^{-6}$ mol ($r = 0.997$) with discrete samples and $0.7 \times 10^{-7}–15 \times 10^{-6}$ mol ($r = 0.994$) with continuous sample injection. There is also an increase in the maximum integral heat output, Fig. 2, as well as reduced sample requirement with the discrete sample injection regime. The first two effects are possibly due to the

TABLE 3

Some published values of dH_R for the urease reaction

dH_R (kJ mol^{-1}) [a]	dH_R (kJ mol^{-1}) [b]	Ref.
–	33.0	14 [c]
–	56.6	15
7.12	–	16 [c]
6.6	–	17
18.7 ± 0.6	61.2 ± 0.8	9
20 ± 1.0	61 ± 3.0	This work [c]

[a] Tris buffer (pH 7.8–8.0).

[b] Sodium phosphate buffer (pH 6.8–7.0).

[c] Determinations by flow enthalpimetry.

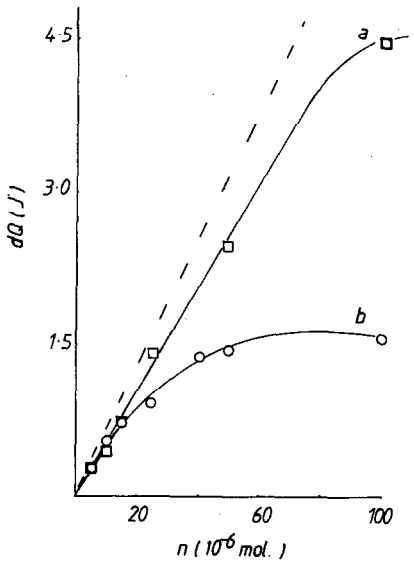

Fig. 2. Flow enthalpimetric determination of standard urea samples in phosphate buffer (0.2 M, pH 7.0, 10^{-3} M EDTA) with discrete (a) or continuous (b) sample injection. Maximum dH^* value of 61 kJ mol^{-1} (- - - - -), $F = 0.005$ cm^{-3} s^{-1}.

dispersion and dilution of small sample volumes by the carrier stream of buffer. The result of such dispersion would be that (a) the urea concentration does not reach steady state in the reactor (in contrast to continuous sample injection) and (b) there is increased sample residence time owing to spreading of sample along longer lengths of flow tubing. Continuous sample injection might be adopted with advantage where there is no limit to sample availability, as a lower amount of urea will therefore be detected.

Relation (5) also describes the conditions prescriptive for the accurate determination of dH_R by flow enthalpimetry. Small (i.e., ± 2.0 kJ mol^{-1}) differences in literature dH_R values may be attributed to the use of slightly different buffer concentration and pH in the various studies, Table 3. Larger differences than this, however, are perhaps most readily explained by reference to X and factors affecting this. The reaction enthalpy may be underestimated where an insufficiently high enzyme concentration and/or sample flow rate is used. The 41 kJ mol^{-1} difference in dH_R for urea hydrolysis in tris–HCl and sodium phosphate buffer corresponds closely to a 42.4 kJ mol^{-1} difference in the values of the enthalpies or (de)protonation of these buffers [9].

In some instances, the dH_R values could not be accurately determined by extrapolating from values of dH^* and X, the calculated fraction of urea hydrolysed. With a low column immobilized urease activity or sample flow rate, dH^* was smaller than expected from the value of X. It seemed that there was uneven flow through the enzyme reactor with substrate not

making contact with all the enzyme present. The presence of air bubbles would have a similar result. However, at high flow rate, individual particles of enzyme support were seen to be suspended in the upward flowing carrier stream; this presumably improves the contact of substrate with the immobilized enzyme.

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