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A SIMPLE, RAPID METHOD FOR THE GENERATION OF LIGAND BINDING ISOTHERMS

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ABSTRACT

Using the protonation of tris(hydroxymethyl)aminomethane (Tris) as a standard reaction, a technique is established for the generation of reaction isotherms by continuous titration in a flow microcalorimeter. The procedure uses an exponential dilution device of simple design that provides a constant internal volume, efficient mixing of the contents and is a closed system. The method has considerable advantages over conventional calorimetric procedures and the precision of enthalpy measurements is estimated to be within ±2%.

INTRODUCTION

The ubiquity of enthalpy changes associated with all chemical reactions and the recent development of calorimeters capable of operating on a micro-scale has permitted the calorimetric technique to be employed in a wide variety of chemical and biological studies [1–3]. The flow microcalorimeter is one of several types of heat-leakage calorimeters commercially available, incorporating a design which ensures rapid heat exchange between the calorimeter vessel and an essentially isothermal heat sink surrounding it. This instrument measures the rate of heat generated in the calorimeter vessel and can be operated in two modes: the flow-through mode, in which the reacting solution flows through the calorimeter after the reaction is initiated externally; and the mixing mode, in which two reactant solutions are led through a simple mixing junction into the calorimetric chamber where the reaction takes place. The theoretical basis for the operation of the LKB type 10700-1 flow calorimeter and its application to zero-order and pseudo-first-order reactions in either mode has been described by Beezer and Tyrrell [4].

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The flow-through mode is suitable for reactions with long half lives ($t_{1/2} > 10^3$ $cm^3\ s^{-1}$) [5], while the mixing cell is used for fast or slow reactions. Further development by Johnson and Biltonen [6] indicates that bimolecular, or higher order reaction rates for liquid phase reactions with half lives in the range of five seconds to several hours may be measured.

The use of flow microcalorimetry for the measurement of reaction rates in the liquid phase has a wide potential in organic, inorganic and biological chemistry, relying only on the change in enthalpy accompanying the reaction as monitor. In addition, full recovery of the experimental material is feasible. In principle the method could be used for gas phase reactions and for reactions of volatile materials, since the instrument lacks any vapour space. A wide range of reaction times can be resolved, providing both thermodynamic and kinetic information. However, there are several practical disadvantages in the basic flow-calorimeter technique that may act as a deterrent to potential users of the system. The conventional procedure for such heat measurement requires a considerable volume of material to continuously fill the flow lines. It also involves successive experiments for the point by point determination of enthalpy change with ligand concentration for the generation of a binding isotherm. An important factor in specialised research is often the stability of the experimental material and its availability. The nature of calorimetric measurement necessitates a well-defined experimental system for meaningful interpretation of results. Purification procedures are often complex, lengthy and usually give a small yield of a product which may well be unstable over prolonged periods. A simple, faster, more eco-

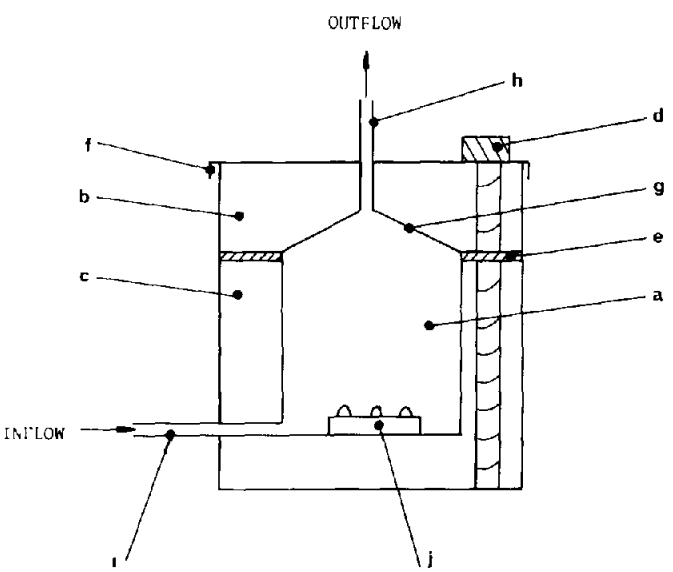

Fig. 1. The closed "constant volume" dilution cell.

nomical method of calorimetric measurement is therefore required in order to improve the quantity and quality of thermodynamic data.

In this context, R.L. Biltonen (personal communication, 1974) and Mountcastle et al. [7] have investigated the generation of continuous ligand binding isotherms by use of experimentally achieved exponential concentration gradients. The method involves the continuous dilution of one reactant by the inflow of solvent at a constant flow rate. The reaction can be monitored directly in the dilution vessel or a property of the effluent may be measured. This paper describes a technique for the generation of a reaction isotherm, which was developed from the initial proposals of Biltonen and applied to the flow microcalorimeter. The method involves continuous monitoring of the heat changes resulting from an exponential concentration gradient created externally to the calorimeter using a dilution device of special design, described in Fig. 1.

THEORETICAL

The experimental arrangement is shown in Fig. 2. Component A is in excess and has a constant flow rate R_A. Component B is diluted in the dilution chamber external to the calorimeter. Solvent is pumped into the chamber at a constant flow rate R_B. The resulting solution of changing concentration then flows from this chamber at rate R_B, into the mixing cell of the calorimeter where it meets reactant A of unchanging concentration and reaction occurs.

For a virtually instantaneous reaction in the steady state, the rate of formation of product will be $R_B C_B^0$ and the total heat output rate, corrected for the enthalpy of dilution of components A and B will be

$$dq/dt = -R_B C_B^0 \Delta H_R \qquad (1)$$

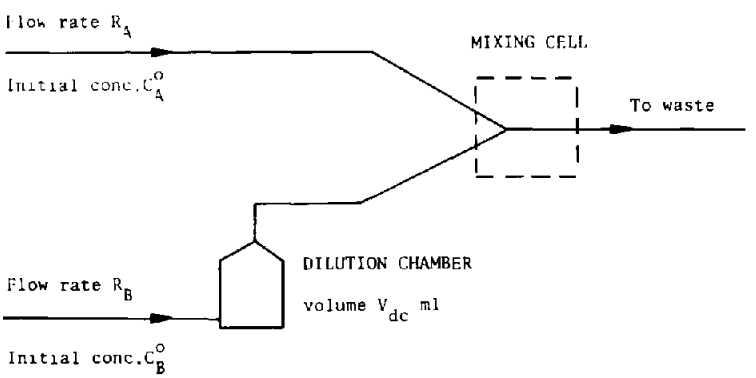

Fig. 2. The continuous titration technique.

where C_B^0 is the initial concentration of component B in mole cm^{-3}, and ΔH_R is the molar heat of reaction (J $mole^{-1}$).

$V_{dc}C_B^0$ moles of reagent B are initially contained in a dilution chamber of internal volume V_{dc} cm^3. When the experiment is initiated a flow of diluent enters the cell at a rate R_B. Assuming perfect mixing, the outflow of solution will be R_BC_B where the concentration of reactant in the dilution chamber is now C_B. In time Δt the concentration of B in the chamber is changed by ΔC_B.

$$V_{dc}C_B^0 = -R_BC_B\,\Delta t \tag{2}$$

Integration of eqn. (2) gives

$$C_B = C_B^0 \exp(-\gamma t) \tag{3}$$

where γ is the constant quantity R_B/V_{dc}.

For a rapid reaction, heat is generated instantaneously in the cell at the point of mixing and is related directly to the actual concentration of reactant at the point of mixing. During dilution the heat output rate is therefore given by

$$dq/dt = -R_B\,\Delta H_R C_B^0 \exp(-\gamma t) \tag{4}$$

By plotting ln dq/dt against time, a linear plot should be obtained with a slope γ, which is predictable from the dimensions of the dilution cell and the constant flow rate. At $t = 0$, the intercept is equal to $-R_BC_B^0\Delta H_R$. The concentration C_B at any time t can be found from eqn. (3).

THE DILUTION CHAMBER

Production of concentration gradients which vary accurately with time requires that the volume of solution inside the dilution chamber remains exactly constant. To this end, a closed dilution cell was designed with main features incorporating the means for easy removal of air bubbles, provision for extremely efficient mixing of the contents and the attainment of a constant volume upon each assembly of the device.

Such a cell is illustrated in Fig. 1. The dilution chamber (a) is enclosed by a lid (b) and base (c). The lid is affixed to the base by means of three screws (d) spaced equally apart around the circular cell, designed so that when tightened the internal volume should be consistently the same. A neoprene seal (e) prevents leakage and stress when the screws are tightened. The metal clamp lid (f) also prevents stress from the screws. The conical roof (g) of the internal chamber is designed to trap any air bubbles which are then easily removed through the outflow tube (h) situated in the centre of the cone. It is also designed to facilitate free flow through the cell, i.e. prevent dead space. The inflow tube (i) is placed at the base of the dilution chamber to facilitate

the efficient mixing of the reactant solution; the incoming solvent is immediately mixed by the circular teflon stirrer (j) which itself has a raised surface for maximum efficiency.

The device requires a single pump with a constant flow rate. The cell is closed and therefore the solution is forced out of the dilution chamber at the same rate as the diluting solvent is pumped in. The system was tested using the standard reaction i.e. the protonation of aqueous Tris by aqueous hydrochloric acid. The enthalpy of protonation was measured as a continuous function of Tris concentration.

EXPERIMENTAL

Solid Trizma base [tris(hydroxymethyl)aminomethane] 99.9% pure, was obtained from the Sigma Chemical Company. Poole, Gt. Britain. HCl "Aristar" grade solution was obtained from B.D.H., Poole, Gt. Britain. Freshly boiled, de-ionised water was used as the solvent.

The instrument available for this work was an LKB model 10700-1 flow microcalorimeter, based on the design of Monk and Wadsö [8]. The flow-through and mixing cells were both of approximately 0.45 ml volume and were constructed of radial gold spirals. The calorimeter was operated in the mixing mode at 298 K and housed in a room maintained at 298 ± 0.5 K. This arrangement allowed precise calorimetric measurements to be obtained at the sensitivity level of 10 μV full scale deflection or above. Integration of the calorimetric data was directly achieved using a printer driver unit (LKB 10758). Activation of the printer was controlled by an accurate timing unit which, at set intervals of 5 s, relayed the amplified calorimetric signal to the printout.

The experimental apparatus was assembled as shown in Fig. 2. Solution A of fixed concentration was pumped through the calorimeter using a motor driven syringe pump (Hamilton gas-tight syringe, 20 cm^3 capacity; Braun perfusor) operated at 1.8×10^{-3} cm^3 s^{-1}. Solution B to be diluted was pumped through the closed dilution cell and into the calorimeter by a peristaltic pump operated at either 6.1×10^{-3} or 2.6×10^{-3} cm^3 s^{-1}. These flow rates were selected to provide satisfactory experimental operation. As the test reaction was virtually instantaneous, very fast flow rates with correspondingly short calorimeter residence times could be used. However, slower flow rates would produce more stable base lines and ensure efficient mixing in the dilution cell. The flow rate was determined by weighing the amount of water pumped through the calorimeter over a defined time interval.

Water used in the preparation of the test solutions was glass-distilled, de-ionised and boiled to remove carbon dioxide. Solutions were prepared fresh and brought to 298 K before use. Successive experiments were per-

formed for solutions of initial concentration in the range 0.01–0.05 mole dm^{-3} HCl (solution A), 0.003–0.012 mole dm^{-3} Tris (solution B).

Details are given here for a typical experiment. Water was pumped through both flow lines of the calorimeter and a base-line established with the recorder on the most sensitive range to be used (i.e. 10 μV fsd, equivalent to 147.14 μW). The dilution cell was filled with Tris solution which was then pumped into the calorimeter against an inflow of HCl from the Hamilton syringe. An appropriate recorder sensitivity (e.g. 100 μV fsd, equivalent to 1.497 mW) was employed to monitor the extremely rapid heat change that occurred on entry of the reactants into the mixing cell. After the reaction steady state was established, the solution being pumped into the dilution cell was changed from Tris to water: the silicone tubing leading from the Tris solution to the peristaltic pump was looped under the surface of the water and cut. This procedure avoided the creation of air bubbles in the flow line during solution changeover.

A period of 30–40 min elapsed before the diluted Tris entered the mixing cell. The reaction peak deflection was thus re-established by the inflow of undiluted Tris during this period and the subsequent onset of dilution could be clearly monitored. As the Tris was diluted, the heat of reaction decayed exponentially. The recorder sensitivity was changed accordingly until eventually a constant baseline deflection was obtained which signified that the Tris was now at infinite dilution. This final deflection therefore corresponded to the signal for the dilution of HCl. The heat of dilution of the acid solution could then be measured by subsequently filling the Hamilton syringe with water and following the voltage decay to a baseline signal corresponding to the flow of only water through the calorimeter. In an ideal experiment the final baseline was the same as the baseline established at the start of the experiment. At all times the calorimeter signal was measured relative to the

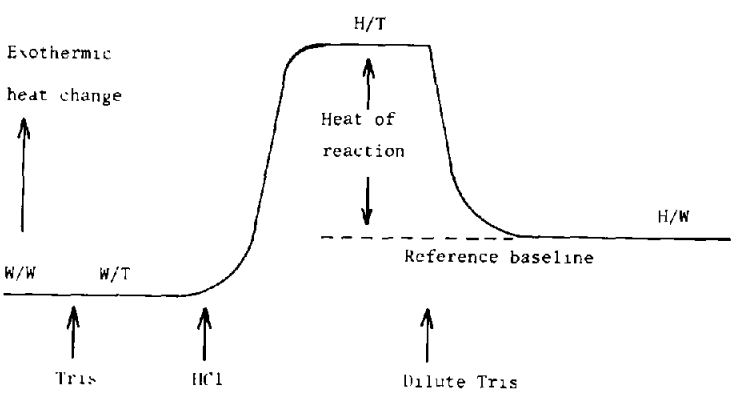

Fig. 3. The experimental sequence for the measurement of the enthalpy of protonation of Tris as a continuous function of Tris concentration. Solutions entered the mixing cell in the order. 1, W/W = water vs. water; 2, W/T = water vs. Tris (at the initial concentration C^0); 3, H/T = HCl vs. Tris; 4, H/W = HCl vs. water (i.e. the Tris is at infinite dilution).

signal observed when solvent, in this case water, was pumped through both flow lines.

The enthalpy of dilution of solution A is a constant factor throughout any such experiment and therefore easily corrected for. The enthalpy of dilution of solution B is a function of the exponential concentration gradient and is therefore determined separately, by repeating the above procedure but using water in place of solution A. For the concentration range of Tris employed in these experiments, no significant enthalpy of dilution was observed. The experimental sequence is depicted in Fig. 3, and the entire operation normally took up to three hours.

RESULTS

The protonation of Tris was chosen as a test reaction to demonstrate the application of the continuous titration procedure to the generation of a reaction isotherm. The heat of protonation of Tris was measured as a continuous function of Tris concentration. Voltage/time readings were obtained and the signal values converted to units of heat.

At the same time a correction was made for the effects of thermal disequilibrium which has been shown to be endogenous to the flow calorimetric system [8,9]. It was established that heat is lost through the air gap between parts of the mixing cell surface not in contact with the thermopile and also leaves the cell with the effluent liquid. A detailed analysis of the characteristics of this heat loss was performed by Poore and Beezer [9] who made the important observation that at any given flow rate the rate of heat dissipation from the calorimetric mixing cell when heat is generated in the whole chamber (i.e. a chemical reaction in solution) is not the same as when heat is generated in the small heater located in the base of the cell (i.e. during electrical calibration). Thus, even at very low flow rates the heat loss affected the electrical calibration constant and the time constant of the calorimeter. The general assumption that by performing all electrical calibration under the same experimental conditions as the reaction, any effects of thermal disequilibrium are therefore nullified, was shown to be invalid and the conventional procedure of obtaining the heat output simply by multiplication of the measured signal with the electrical calibration constant is therefore inaccurate.

Poore and Beezer [9] have obtained an absolute measure of the effluent heat loss as a function of flow rate and heat input, and the concomitant dynamic correction factors were established and tabulated. The analysis given in a subsequent paper [9] essentially allows a correction for heat loss incurred through the nature of the flow technique. It also distinguishes electrical heat from a heat of reaction so that the effects of thermal disequilibrium on each type of heat measurement can be separately accounted

for. This is essential for the accurate evaluation of reaction enthalpy and kinetics. This analysis was therefore employed in converting the voltage/time measurements from the continuous titration experiment into values of total heat ouput rate ($Q^{\cdot}$).

Values of ln $Q^{\cdot}$ were plotted as a function of time in Fig. 4. The slope (γ) was measured from the linear portion of the graph and the volume of the dilution cell calculated from the relation $\gamma = R_B/V_{dc}$, where R_B is the flow rate through the dilution cell. The mean value of the dilution cell volume,

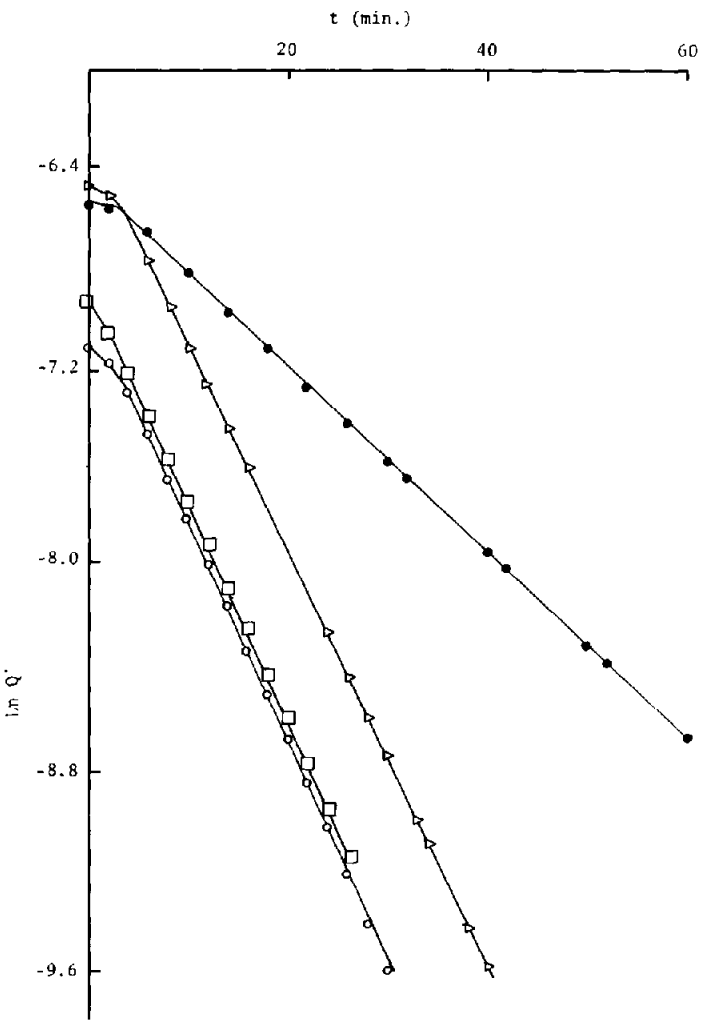

Fig. 4. The enthalpy of protonation of aqueous Tris by aqueous HCl. The measured heat output, $Q^{\cdot}$, as a function of time for the experimentally achieved exponential concentration gradient. Experiments were performed using Tris concentrations (C^0) and total flow rates of: ●, 0.012 mole dm^{-3}, 4.39×10^{-3} cm^3 s^{-1}; △, 0.006 mole dm^{-3}, 7.93×10^{-3} cm^3 s^{-1}; □, 0.004 mole dm^{-3}, 7.93×10^{-3} cm^3 s^{-1}; ○, 0.0034 mole dm^{-3}, 7.93×10^{-3} cm^3 s^{-1}.

TABLE 1

Parameters derived from the experimental calibration of the technique for continuous titration: the protonation of aqueous Tris by aqueous HCl

R_{Total} ($cm^3\ s^{-1}$)	C^0_{HCl} (mole dm^{-3})	C^0_{Tris} (mole dm^{-3})	$\Delta H^0_{st\ st}$ (kJ $mole^{-1}$)	t_N* (s)	C_{t_N} [a] (mole dm^{-3})	ΔH_{t_N} [a] (kJ $mole^{-1}$)	γ [a] (s^{-1})
7.93×10^{-3}	0.0143	0.0034	−39.96	1302	0.000503	−39.09	1.4750×10^{-3}
7.93×10^{-3}	0.0143	0.0040	−41.05	1182	0.000738	−41.54	1.4297×10^{-3}
7.93×10^{-3}	0.0250	0.0060	−42.57	1302	0.001005	−42.72	1.3726×10^{-3}
4.39×10^{-3}	0.0500	0.0120	-47.04 ± 0.06	1062	0.006131	−47.44	6.3233×10^{-4}

[a] Values obtained from the graph ln Q^* vs. time, t (Fig. 4).

$\Delta H^0_{st\ st}$ = the enthalpy measured for the steady state reaction prior to the onset of Tris dilution.

t_N = the time measured from the start of the Tris dilution ($t = 0$), corrected for the time lag between steady state reaction and the onset of the true exponential concentration gradient. The time lag was estimated from the graph, ln Q^* against t.

C^0 = initial concentration.

C_{t_N} = concentration of Tris at time t_N, and is derived from eqn. 3.

ΔH_{t_N} = the enthalpy change at time t, corresponding to the Tris concentration C_{t_N}, and is derived from eqn. 4.

calculated using the values of γ given in Table 1 is 4.186 ± 0.043 cm^3. This can be compared with the measured value of 4.299 ± 0.005 cm^3. For a given flow rate, the values of the slope agree within one per cent for a total flow rate of 4.30×10^{-3} or 7.93×10^{-3} cm^3 s^{-1}, respectively. The volume of the cell obtained from the values of γ is within three per cent of the value obtained by direct measurement of the cell.

The graphs depicted in fig. 4 are linear in accordance with the exponential dilution function predicted by the equations given in the theoretical section. However, in each experiment a small time lag was exhibited in the observed changeover from the steady state signal output prior to dilution and the true exponential concentration gradient which was subsequently established. This was a property associated with the entry of the gradient front into the mixing cell. This was corrected for as follows: in each experiment the zero time, t_0, for the start of dilution was measured from the graph at the point of intersection between the steady state signal and the extrapolated exponential heat decay. The time factor is very important when the concentration of Tris and the associated enthalpy value are calculated from the heat output at a given instant after the onset of dilution [eqn. (4)]. This value of ΔH was compared to the enthalpy of protonation of Tris which was calculated for the steady state condition established when Tris of constant concentration was pumped through the calorimeter prior to dilution. These values are given in Table 1.

DISCUSSION

Several conclusions can be drawn from the data in Table 1. Referring to the enthalpy values obtained under the steady state conditions, the results seem to fall into two groups: measurements performed at low concentrations of Tris (0.003–0.006 mole dm^{-3}) give lower enthalpy values ($\Delta H = -40$ to -42.6 kJ mole^{-1}). For experiments using a higher concentration of Tris, the value of ΔH was -47.04 ± 0.06 kJ mole^{-1}. A similar difference was observed by Wadsö [10]: for concentrations of the order of 10^{-4} to 10^{-3} mole dm^{-3}, $\Delta H = -45.6$ to -46.4 kJ mole^{-1}; for concentrations of the order of 10^{-2} mole dm^{-3}, $\Delta H = -47.07$ kJ mole^{-1}. No explanation was found for this discrepancy between the two groups of results. It was suggested that systematic errors were unlikely to be involved as entirely different calorimeters were used to obtain the heat measurements within both groups of results. In the present work, it is possible that the reaction measured under steady state conditions for the lower Tris concentrations of the order of 10^{-3} mole dm^{-3} was incomplete, as the HCl was then present in only a slight excess, i.e. in these experiments the Tris was pumped at a faster rate than the HCl (6.1×10^{-3} as opposed to 1.8×10^{-3} cm^3 s^{-1}), so that the actual reactant concentrations in the mixing cell were almost equal. Also CO_2-free solutions

were used. On dilution, the reaction could be expected to be complete as the acid came to be in considerable excess. However, for each experiment, the enthalpy change calculated from the heat output at a given time during the dilution of Tris is no more than the value calculated for the steady state condition. In addition, all these values are still lower than the enthalpy measurement of -47.04 kJ mole^{-1} derived from the heat of reaction observed at the higher concentration of Tris (10^{-2} mole dm^{-3}) when HCl was present in a significant excess. The latter value is in very good agreement with that obtained by Wadsö at a similar concentration level (-47.07 kJ mole^{-1}) and also agrees with other values cited by Wadsö which were derived from accurate potentiometric measurements: -47.61 [11] and -47.41 kJ mole^{-1} [12].

Consideration must be given to the mean standard error involved in each type of enthalpy measurement. For the steady state reaction ΔH is calculated from the equation

$$Q^{\cdot} = -R_T C_T^0 \Delta H$$

where $Q^{\cdot}$ is the measured heat output corrected for effects of thermal disequilibrium [9]. In this case the mean standard error of ΔH is estimated to be $\pm 2.1\%$. Enthalpy measurements derived from the data after the onset of dilution are calculated from eqn. (4)

$$Q^{\cdot} = -R_T \Delta H C_B^0 \exp(-\gamma t)$$

and are subject to a standard error of $+3.1\%$. The steady state and late time dilution enthalpy values would therefore seem to differ only as a reflection of the acceptable experimental error involved in their separate estimation.

The results presented here demonstrate the practical use of the Tian correction factors [9]. Thus, if the heat output data were not corrected for the effects of thermal disequilibrium, the graphs of ln $Q^{\cdot}$ vs. time were found to be non-linear. Taking as an example the experiment which gave an enthalpy change of -47.04 kJ mole^{-1} for the protonation of Tris, the corrected heat output rate $Q^{\cdot}$ is approximately three per cent larger than the heat output rate obtained by conventional calibration. Although this adjustment is quite small, the necessity for the correction to be performed in order for the exponential function of $Q^{\cdot}$ to be exhibited on the graph illustrates the significance of these correction factors. At higher flow rates the need for correction would be even more imperative.

In their report on the use of exponential concentration gradients for the generation of ligand binding isotherms, Mountcastle et al. [7] suggested the use of calorimetry as a monitor system and a theoretical analysis was presented. An attempt was made to correct the measured heat output for non-steady-state conditions using the equation

$$S_t = S + \tau \frac{dS}{dt}$$

where S_t is the true heat-effect signal, and τ is the response time of the instrument. This correction derives from the Tian equation [13] and can be seen to form part of the formula for the calculation of $Q^{\cdot}$ as presented by Poore and Beezer [9]. However, no account was given for effluent heat loss and its effects on the calibration constant and response time of the calorimeter. It is, therefore, an imperfect correction and its use would incorporate significant error even at fairly slow rates. One important effect would be error in the estimates of t_0, the time of the start of dilution. Thus, the definition of t_0 is crucial in the calculations of concentration and heat output during reaction dilution [eqs. (3) and (4)], although it is to be noted that t_0 does not affect the value of the constant γ, characteristic of the exponential dilution curve.

In any experimentation it is desirable to achieve the maximum accuracy possible. Many calorimetric applications require extremely precise measurement and indeed the whole process of development in the field of microcalorimetry is directed towards achieving a high level of precision and sensitivity. Thus, in order for the quality of thermodynamic data to be improved, the potential sources of error in the calorimetric technique must be recognised and corrected for by procedures such as those described here.

The results demonstrate the accuracy and reproducibility of exponential concentration gradients generated by the dilution cell depicted in Fig. 1. In each case the value of γ as determined by analysis of the data is within three per cent of that calculated from the ratio of R_B/V_{dc}. The logarithm of the gradient was shown to be a linear function of time over a concentration range of 10^{-3}–10^{-4} mole dm^{-3}, and the technique is thus applicable to the study of equilibrium reactions such as ligand binding. It is feasible that the technique could be applied to kinetic studies under conditions where the heat output rate is proportional to the rate of reaction. The initial velocity could then be directly measured as a function of the logarithm of reactant concentration. Such an extension has been proposed by Mountcastle et al. [7].

The technique developed and presented here for the generation of a reaction isotherm by continuous titration, has several advantages over other possible titration-type procedures. One method, for example, would be to change the flow rate of one reactant. It has been shown elsewhere [9] that such a procedure would induce significant errors in heat measurement related to the flow rate and would necessitate a complex correction process. Another method would be to interchange one flow-line between solutions of different reactant concentration. This would simplify the correction procedure as the flow rate would be constant. However, the experimentation would be tedious, expensive in sample volume and slow in operation. By contrast, the dilution cell that was designed and tested in this work would seem to be an ideal instrument for the generation of an exponential concentration gradient. The cell design ensures that the volume of the cell

contents remains constant not only for the duration of an experiment, but also between successive runs. The requirement of concentrated sample is kept to a minimum; the solution capacity is maintained by the inflow of solvent into the dilution chamber. After the dilution has been initiated no further manipulation is required and a complete set of data is obtained in the minimum experimental time. Successive experiments can be performed at a rate which is impossible to achieve by conventional techniques. This represents an important advantage in the calorimetric study of unstable, expensive preparations and allows an evaluation of the thermodynamic parameters to be obtained from a greater number of experiments than would otherwise be possible.

The results provide additional evidence for the growing opinion that systems involving Tris do not provide a good chemical standard for titration calorimetry (e.g. ref. [14]). Thus, the apparent dependence of the enthalpy change on Tris concentration plus the possible effects of absorbed carbon dioxide can cause uncertainties in the system. This emphasises the need to find a system which is free of analytical limitations and therefore suitable as a chemical standard in flow calorimetry.

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