ANALYTICAL BIOCHEMISTRY 139, 134–139 (1984)

A Twin Titration Microcalorimeter for the Study of Biochemical Reactions

I. R. McKINNON,[1] L. FALL, A. PARODY-MORREALE,[2] AND S. J. GILL

Department of Chemistry, University of Colorado, Boulder, Colorado 80309

Received October 17, 1983

A small-volume (200 μl) titration calorimeter of high sensitivity (1 μcal) has been developed for the purpose of studying biochemical reactions where the amounts of material are limited to a few nanomoles. High sensitivity is achieved by calorimetric twining, use of glass cells, elimination of vapor space, effective low-energy stirring, and reduction of measurement time. The calorimeter operates using the heat conduction principal with computer-controlled electrical compensation, which reduces the measurement time of each point from 10 to 3 min. This reduction in time is accompanied by a corresponding increase in the precision of measurement. The use of the calorimeter is demonstrated by a measurement of the heat of oxygenation of hemocyanin.

In a previous paper from our laboratory (1) we reported the development of a titration microcalorimeter which enabled the study of systems for which the amount of material was limited to approximately 100 nmol, the heats of reaction were small, and relatively dilute solutions were required in order to avoid problems such as aggregation. Although this instrument met these goals, enabling a study of the lac repressor–IPTG (isopropyl-1-thio-β-D-galactopyranoside) reaction (2), we have found it to be too complicated for routine use. Our experience suggested the possibility of a much simpler instrument that would be easier to construct and operate, have higher sensitivity, and use even smaller amounts of material. We wish to describe this new calorimeter as well as a new, all glass, stirrer injection system.

APPARATUS

In order to simplify construction and operation we have utilized a combination of the heat-conduction calorimeter principal (3), where the reaction cell is thermally connected to a heat sink, and electrical feedback control (1). The effect of the temperature fluctuations of the heat sink are largely cancelled by the use of a twin reference cell. The isolated heat sink makes weak thermal contact with a well-controlled (10^{-4} K) water thermostat through a thermopile with high conductivity. This provides adequate isolation from the bath, yet enables convenient monitoring of differences in temperature between bath and the sink.

The design of the calorimeter allows the use of a variety of cells of different volumes. We have found a 250-μl glass cell constructed from 4-mm NMR tubing to be especially useful.

Two of the most critical aspects of a high-sensitivity titration microcalorimeter have been (i) the design of efficient, low-energy stirrers and (ii) the provision of a low-volume, thermally equilibrated injection system. The solution to these requirements has come with the use of a long glass needle attached to a microsyringe. The tip of the glass needle is bent so that it functions as an effective stirrer when the microsyringe is rotated. Careful axial alignment of this system is achieved by use of adjustable bearings and a special syringe holder. The use of the glass injector–stirrer provides a highly inert, easily filled titration device.

A critical factor of heat-conduction calorimetry is the time required for the system to regain its initial temperature after reaction.

[1] On sabbatical leave from Monash University, Melbourne, Australia.

[2] Postdoctoral Fellow, Granada University, Granada, Spain.

0003-2697/84 $3.00

One wishes to minimize the time in order to perform experiments rapidly and to minimize the effect of baseline drift. In order to achieve rapid time response one can control electric power about some initial steady-state value, increasing or decreasing the power as required to maintain the initial temperature difference between the reaction cell and the heat sink. Where the heat conduction losses are small the heat of a reaction is given by the integration of the change in power with time (1). However, in the present design heat conduction through the thermopile to the sink is significant and one must measure this heat contribution by integration of changes of the thermopile voltage with time. This approach, which is a hybrid of a scanning and a heat-conduction system, gives us the short measurement times necessary for high precision.

a) Description of Calorimeter

The calorimeter along with the syringe–injector–stirrer is shown in Fig. 1. The figure legend describes the various components. Melcor (Trenton, N. J.) thermosensitive modules were used for the thermopiles. These were connected in opposition, and leads were brought out the lid of the submarine through sealed tubes (not shown in Fig. 1). Heaters constructed of 400-Ω m^{-1} resistance wire were wound on the copper reaction and twin cups, and leads were brought out of the submarine top through a sealed tube. NMR tubing (4 mm o.d.) was used for the reaction cells. Bulbs of approximately 200 μl were blown on the ends of the tubes, which were imbedded into the copper cup calorimeter cells with low melting (117°C) metal (Small Parts, Inc., Miami, Fla.). The alloy has a low-volume change upon solidification. The NMR tubes were sealed into 0.250-inch-o.d. stainless-steel tubing with De Khotinsky wax. O-ring seals were used throughout to seal the system from the surrounding water bath. Temperature control of the bath was effected by a PTC-41 Tronac controller (Tronac, Orem, Utah).

The microsyringe stirrer–injector system shown in Fig. 1 is designed so that glass stirrer needles can be axially aligned within the entrance tubes of the reaction cells. The needle is connected to a microsyringe barrel by means of an O-ring seal. The needle is aligned with a stainless-steel plug and close-fitting hypodermic tube extension. The syringe barrel is held centrally within a Lucite sleeve with a compression cap that forces the O-ring seal between the glass needle and the barrel. The Lucite sleeve is axially aligned within locating bearings (ball bearings at the top and a nylon sleeve bearing at the bottom). The initial bearing alignment is achieved by use of a 0.1250-

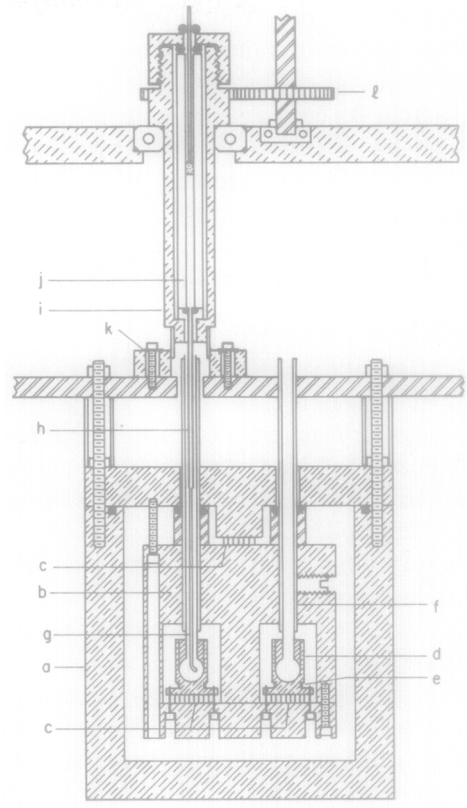

FIG. 1. Twin cell calorimeter with injection stirrer: a) submarine container; b) thermal equilibrator block; c) thermoelectric module; d) calorimeter cell (glass bulb imbedded in copper cylinder with low-melting metal); e) electric heaters; f) close-fitting stainless-steel tube with inside glass tube; g) glass capillary stirrer and injection needle; h) stainless-steel centering sleeve; i) Lucite syringe holder; j) microsyringe; k) sleeve bearing assembly for syringe centering; and l) stirrer drive gear.

inch-diameter drill rod placed within the NMR reaction tube and adjusting the location of the bearings until test plugs slide down the rod and fit the bearings. Rotation of the stirrer and syringe injection is effected by reversible motor control and stepping motor control, respectively (1). The syringe–stirrer assembly permits easy removal from the cell and convenient filling of desired titrant solutions.

b) Principles of Operation

A schematic representation of the twin calorimeter system is shown in Fig. 2. The system consists of a reaction cell (C) and a reference cell (R) surrounded by the block (B). The cells are thermally connected to the block through the thermopiles. The calorimeter is constructed so that the two cells are as similar as possible in respect of the heat capacities ($C_C \approx C_R$) and the thermal conductivities between each cell and the block ($K_C \approx K_R$).

The analysis of the system is based on examining the rates of energy change in each cell due to various thermal processes. In a given time increment (dt) a change of energy of the reaction cell due to electrical power to the heater (P_C), chemical reaction (dZ_C), and heat conduction through the thermopile ($K_C(\theta_C - \theta_B)$), is equal to the change in temperature of the cell ($d\theta_c$) times the heat capacity of the cell (C_C):

$$C_C d\theta_C = P_C dt + dZ_C - K_C(\theta_C - \theta_B)dt. \quad [1]$$

At the same time the change in temperature ($d\theta_R$) of the reference cell is governed by a similar equation with the appropriate properties referenced by a subscript R:

$$C_R d\theta_R = P_R dt - K_R(\theta_R - \theta_B)dt. \quad [2]$$

Here the chemical reaction term is assumed zero. In a twin calorimeter we assume $C_R = C_C = C$ and $K_R = K_C = K$ so that subtraction of these equations gives

$$Cd(\theta_C - \theta_R) = (P_C - P_R)dt + dZ_C - K(\theta_C - \theta_R)dt. \quad [3]$$

Integration of this equation over a time period τ for the experiment, where the initial and final values of $\theta_C - \theta_R$ are equal, gives the fundamental operating equation

$$Z_C = \int_0^\tau K(\theta_C - \theta_R)dt - \int_0^\tau (P_C - P_R)dt. \quad [4]$$

The temperature difference between reaction and reference cell ($\theta_C - \theta_R$) is measured by the voltage V of the thermopiles, connected in opposition, times a calibration constant α. The power is given by the square of the current (I) times the resistance of each cell heater (R). Thus, the practical operating equation is

$$Z_C = \int_0^\tau \alpha KV dt - \int_0^\tau (P_C - P_R)dt \quad [5]$$

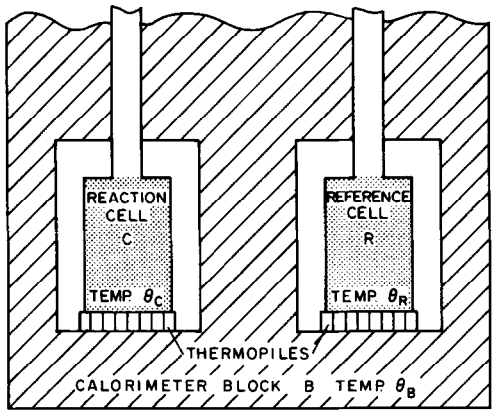

FIG. 2. Schematic representation of twin microcalorimeter region showing reaction and reference cells with measuring thermopiles in contact with calorimeter block.

In the pure heat-conduction calorimeter mode there is no electrical power input, and only the first integral of Eq. [5] is considered. The response time of the calorimeter, τ, is given typically by six or seven times the half-life (C/K) of the system. In a power-controlled system the time τ can be considerably shortened by controlling the power to minimize the value of this first integral. This requires simultaneous measurement of the second integral. This is done conveniently through the use of a computer-controlled, constant-current source.

In order to use power control one must supply power at all times to the reaction cell. The reference cell power is fixed at P_R, which may be zero for the simplest case. One sets

an initial starting condition by applying initial power P'_C and P_R and allowing the system to reach an initial steady state temperature difference $\theta'_C - \theta'_R$ with thermopile voltage V'. From Eq. [5], where $Z_c = 0$, the initial steady-state voltage is given by $\alpha K V' = P'_C - P_R$. Thus, Eq. [5] may be written as

$$Z_C = \alpha K \int_0^\tau (V - V')dt - \int_0^\tau (P_C - P'_C)dt. \quad [6]$$

The calorimeter constant αK is obtained in the absence of chemical reaction either (i) by supplying a large precise power for a given time to the reaction cell heater and measuring the voltage time integral, or (ii) by measuring the voltage output for a steady-state power value.

The control of the power to the reaction cell is facilitated by use of a Keithley 220 programmable current source. A variety of control options are possible. We have found that a simple control strategy based on the idea that changes in the control power ($P_C - P'_C$) should be negatively proportional to the output voltage ($V - V'$) is an effective way to shorten the recovery time. Thus, when heat is generated chemically within the reaction cell and $V - V'$ becomes positive, power (P_C) is reduced to the reaction cell so that the second integral makes a significant positive contribution to the measurement of the chemical heat effect Z_C. In practice, the current source is controlled at a chosen value of power for given time period (5 s). The second integral is given by the sum of these finite energy quantities. The first integral evaluation is determined by the sum of mean voltage readings, averaged over a period of 1.070 s.

A Keithley programmable current source, a Fluke 8502A digital voltmeter, and a Keithley 148 nanovoltmeter are used as the principal control units for the calorimeter.

RESULTS AND DISCUSSION

An indication of the range, sensitivity, and electrical precision of this system is given by a number of test runs. The proportionality of voltage output with steady-state cell power input is maintained over three decades of power. Sensitivity for finite measurements is determined largely by baseline voltage stability and the calorimeter recovery time period. Baselines at the highest practical sensitivity, along with the uncontrolled time response for a step change of power, are shown in Fig. 3. Noise fluctuations over a 2-min period are in the range of 0.1 μW. The response time of the calorimeter is greatly shortened by computer power control, as shown in Fig. 4. More significant are the voltage–time integral and the power–control integral over a 2-min time period. These integrals will provide the limits of any chemical reaction initiated in the calorimeter. The results of a series of different tests are shown in Table 1. First of all, the baseline integral shows typical fluctuations of 1 μjoule. Stirring heats generated by stirring for 30 s at 150 rpm are reproducible in the range of 8 $\pm$ 2 μjoule. This amount and rate of stirring provides complete mixing upon introduction of 0.2 M sucrose into water. The heat of injection of 10 μl of water into water within the cell produces a reproducible heat effect of 2 $\pm$ 2 μjoule. The effect of both stirring and injection is the sum of the separate experiments. Overall, the operational sensitivity appears to be in the range of 3 μjoule. This is factor of five times better than our previous more elaborate system (1).

The ability of this system to perform precise titration experiments is indicated by a simple acid–base experiment shown in Fig. 5, where the heat measurement associated with each

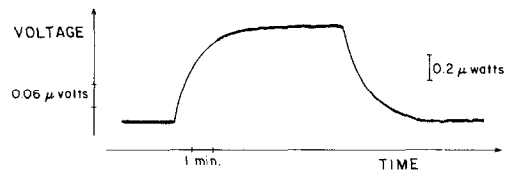

FIG. 3. Time response of thermopile output for an applied power of 0.763 μW. Calculated system parameters given by this result are heat conductivity (2.80 W V^{-1}) and heat capacity (150 joule V^{-1}). The half-life time response is 37.2 s.

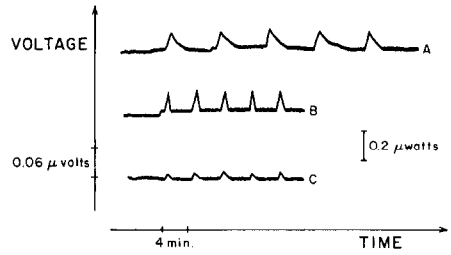

FIG. 4. Thermopile voltage output versus time for three experimental situations: (A) 10 μjoule of heat applied over 30 s with no feedback control; (B) 10 μjoule applied over 30 s, followed with feedback control (note rapid return to baseline); and (C) repeated stirring heat effects (11 μjoule) with calorimeter operating under feedback control at all times.

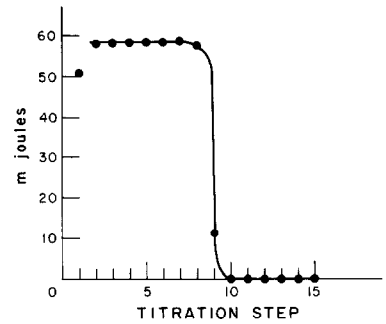

FIG. 5. Calorimeter results of acid–base titration of 0.041 M KOH (in cell) with 0.100 M HCl in titration steps of 10.2 μl. Note low value of first is due to necessary equilibration time after syringe needle is inserted into the calorimeter.

titration step is marked on the figure. The lower value of the ninth step shows the titration is complete. Additional titrant increments show negligible heat effects. By knowing the concentrations of acid and base in this experiment, the effective volume of the reaction cell can be determined (1) as 243 μl.

Finally, an application of this system to a biochemical system of interest to us is shown in Fig. 6 for the reaction of oxygen with *Homarus americanus* hemocyanin (4). Here the reaction is only partially complete with each addition of titrant. The heat of each titration step is thus determined by the amount of reaction that takes place and the enthalpy change per mole of reaction for a given step. The full analysis of these results requires the use of precise ligand binding curves and will be considered in another paper. We wish to point out that the high sensitivity of this calorimeter allows the use of low concentrations of he-

TABLE 1

TEST MEASUREMENTS OF HEAT EFFECTS OVER A 2-MIN INTEGRATION PERIOD

Heat (μjoules)[c,d]			
Baseline	Stirring[a]	Injection[b]	Stirring[a] + injection[b]
−1.25	7.1	−0.4	10.9
1.46	6.3	0.0	9.6
−0.71	7.1	2.1	12.1
−0.96	5.0	1.4	11.3
−1.46	8.8	0.4	7.1
−0.42	10.4	−2.1	7.1
−1.00	8.8	1.3	7.9
0.71	7.5	5.4	7.1
0.04	9.6	3.8	7.9
0.13	9.6	5.0	6.6
Mean −0.35	8.0	1.7	8.8
σ_M 0.29	0.5	0.8	0.6

[a] Stirring for 30 s.

[b] Injection of 10 μl water into water at 0.6 μl/s.

[c] Positive values indicate a heat effect that flows through the thermopile from the cell to the sink.

[d] Order in which the experiments where done: 1–3, stirring; 4–8, stirring + injection; 9–12, stirring; 13–17, stirring + injection; 18–20, stirring; 21–30, injection; 31–40, baseline. All the experiments were done with the same setting without moving the syringe or refilling syringe.

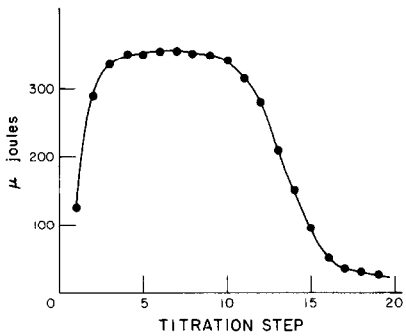

FIG. 6. Calorimetric titration of *Homarus americanus* hemocyanin (0.28 mM deoxygenated hemocyanin in 0.1 M Tris, 20 mM $CaCl_2$, pH 8.0) with successive 10.2-μl injections of dissolved oxygen (1.02 mM in the same buffer).

mocyanin and thus avoids troublesome viscous stirring heats. These are the first direct calorimetric measurements that have been obtained on the heat of oxygenation of hemocyanin. The versatility of this calorimetric system should have broad application for the study of heat effects in a variety of biochemical systems.

ACKNOWLEDGMENTS

We wish to acknowledge the support of grants from the National Institutes of Health (HL22385) and from the National Science Foundation (CHE-8018630) for various aspects of this work. One of us (S.J.G.) also wishes to acknowledge many helpful discussions on calorimetric details with Ingemar Wadsö of the University of Lund, Sweden. A.P.-M. acknowledges the fellowship from the U. S.–Spanish Joint Committee for Scientific and Technological Cooperation, Madrid, Spain.

REFERENCES

1. Spokane, R. B., and Gill, S. J. (1981) *Rev. Sci. Instrum.* **52,** 1728–1733.
2. Donner, J., Caruthers, M. H., and Gill, S. J. (1982) *J. Biol. Chem.* **257,** 14826–14829.
3. Spink, C., and Wadsö, I. (1976) *Methods Biochem. Anal.* **23,** 1–159.
4. Markl, J., Hofer, A., Bauer, G., Markl, A., Kempter, B., Brenzinger, M., and Linzen, B. (1979) *J. Comp. Physiol.* **133,** 167–175.