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Description of a new Gill titration calorimeter for the study of biochemical reactions. I: assembly and basic response of the instrument

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Abstract. A detailed account of the assembly of a thermopile heat-conduction isothermal titration calorimeter to be used in the study of biomolecular interactions is given. The instrument is built according to the twin-cell principle with a sensitivity of 5 μJ. Its reaction vessel volume is only 200 μl. Stirring heats of the reaction vessel are below 5 μJ.

1. Introduction

From a methodological point of view, calorimetric study of biochemical reactions has mainly been carried out through constant-temperature titration experiments. In this kind of experiment the thermal effects of the mixing of the reagents in different relative proportions are measured; if possible, up to the saturation point. A recent review of the technique has been published by Freire *et al* (1990). Calorimetric titration yields maximum information when the value of the association constant for the reaction in question is such that the free concentration of any of the reagents is never an indeterminably small quantity. This happens when the affinity between reagents is relatively small, and, if the reaction is simple enough from a molecular point of view, calorimetric titration allows one to obtain its complete thermodynamic characterization. For example, for a protein with one binding site for a ligand, one may obtain not only the enthalpy change for the binding but also the equilibrium constant (related to the Gibbs free energy change) and the entropy change. A different situation occurs when the affinity between the reagents is relatively high, that is, the value for the association constant is such that, at any relative proportion between reagents, the one at the lower concentration is completely used up in the reaction, with total association between them. In this case it is only possible to obtain the stoichiometry and enthalpy change of the reaction from calorimetric experiments.

Technical developments in biological calorimetry have pointed to a decrease in the amount of material needed to perform a titration curve. In a review published at the end of the 1970s (Langerman and Biltonen 1979) the operational characteristics of the commercial calorimeters most frequently used at that moment in the study of biochemical reactions were analysed. For an A + B→C reaction with an enthalpy change of 10 kcal mol^{-1} the minimum amount of substance needed for complete calorimetric titration in the best instrument reviewed was 1 μmol. Considering a macromolecule with a molecular weight of 10 kDa, 10 mg was needed to perform one experiment. At the begining of the 1980s the first instrument for isothermal titration calorimetry in which the quantity of substance needed was in the range of 100 nmol was built in the laboratory of S J Gill (Spokane and Gill 1981). This quantity has recently been lowered still more with new instruments, developed once again in the laboratory of S J Gill (McKinnon *et al* 1984) as well as the laboratories of E Freire (Myers *et al* 1987) and J F Brandts (Wiseman *et al* 1989). These instruments are similar in their basic characteristics: for reactions with an enthalpy change of the order of tens of kJ mol^{-1} it is possible to perform a calorimetric titration of tens of nanomoles of one of the reagents by adding succesive amounts of the other, until saturation is reached, if possible. Thus, these instruments can be referred to as nanocalorimeters.

The first of the calorimeters developed by S J Gill and co-workers (Spokane and Gill 1981), although it allowed investigation of the reaction of lactose repressor with isopropil-1-thio-β-galactopiranoside (Donner *et al* 1982) produced some degree of difficulty in realization of the measurements. These difficulties were related both to adiabatic control of the temperature of its only cell and mechanical noise caused by rubbing of the injection

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and stirring needle in the proximity of the reaction vessel. Because of this a new instrument of the heat-conduction type was built (McKinnon *et al* 1984) following the twin-cell principle, in which the injection and stirring needle was centred in the reaction vessel without any rubbing against the walls in the vicinity. Different studies were carried out with this instrument, into biomolecular interactions (Parody-Morreale *et al* 1987) and the dilution heats of small organic molecules (Murphy and Gill 1989). It could be said that this was the first prototype of the second generation of isothermal titration calorimeters developed in the laboratory of S J Gill.

In this paper, a second model of the second generation is described. The instrument was designed by S J Gill and one of the authors (AP-M) at the University of Colorado and put together and tuned up at the University of Granada. As with the first prototype, it is a twin-cell calorimeter in which the heat effect is measured from the thermopile voltage through which heat is conducted from the reaction vessel to a heat sink in weak thermal contact with a constant-temperature bath. The main differences in this second model compared with the first are the materials used in its construction, a simpler design, which makes the building and assembly easier, the volume of the heat sink and the relative positions of the reaction vessel and the titrant syringe. This latter change, made with the intention of improving the stirring heat of the reaction vessel, is an important modification with respect to the first prototype, as it implies that a small part of the calorimetric block is in contact with air and not directly thermostatized by the water bath.

We present here a complete description of the system (calorimeter and peripheral instrumentation) stressing the hardware aspects. In the following paper we present the dynamic aspects of its characterization, with description of its operation and the determination of the parameters that make it possible. We have chosen a level of detail for the description in this paper that should make it an effective guide to building a similar system. Anyone requiring additional information can write to the corresponding author (AP-M).

2. System overview

Figure 1 shows the general scheme of the calorimetric system. The calorimeter itself is designated by a. It is composed of two parts, the calorimeter block and the tower that supports the motors for injection of the titrant and stirring of the reaction vessel, both to be described below. The calorimeter block is immersed in a thermostatic bath (b), whereas the tower is above the water level. The water bath and the system to control its temperature will also be described in detail later.

As the calorimeter is of the thermopile conduction type, measurement of the thermal effects is made from the change in time of the voltage of the thermopiles connected in opposition (a') placed between the reaction

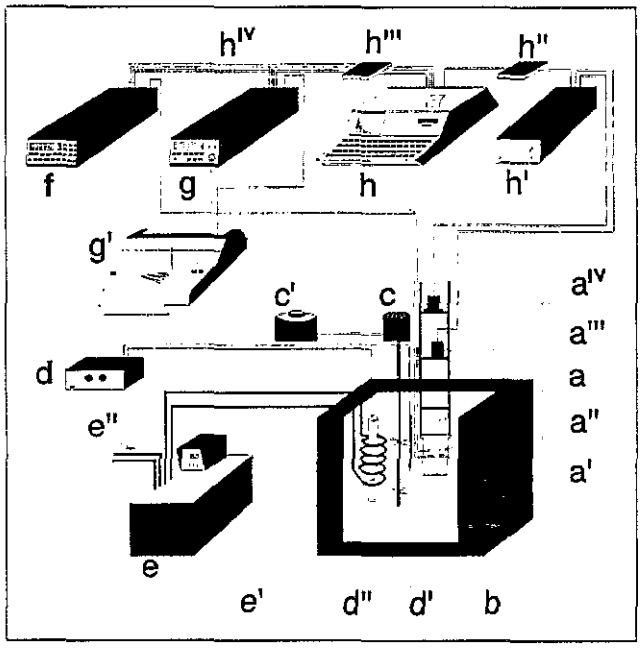

Figure 1. Overview of the components of the system: a, calorimeter; b–e, thermostatic bath and elements to control its temperature; f, power source; g, nanovoltmeter; g', graph recorder; and h, computer and interfaces. For a detailed description, see the text.

and reference cells and the heat sink. This measurement is made by a Keithley 181 nanovoltmeter (g), able to amplify the signal and send it through an analogue output to a graph recorder (g'). To calibrate the calorimeter there is a heating resistance wrapped around a copper cup containing the reaction vessel, through which a known amount of heat is passed. The current intensity through this heater is controlled by a Keithley 224 power source (f).

All the previous elements are the basic ones needed to make the calorimeter work. With this set-up alone it would be a 'passive' instrument in which, on the one hand, one would have to to stir the reacting mixture and inject the titrant manually and, on the other, the measurement of a thermal effect would take 7–8 times the calorimeter time constant. The whole system is, however, controlled by a Hewlett-Packard HP 85 (h) computer with 16K memory extension. This computer permits, firstly, control of the motor (a''') for stirring reaction mixture and of the stepping motor (a^{IV}) for injection of the titrating agent. This control is achieved through a general purpose IO Hewlett-Packard 82940A interface (h'') with its corresponding power amplifier (h'). Secondly, the computer is connected by an IEEE bus (h^{IV}) and its corresponding Hewlett-Packard 82937A interface (h''') both to the nanovoltmeter and to the power source. This allows automatic data acquisition from the nanovoltmeter and the use, by modification of the current to the heater from the power source, of a method of electrical compensation (McKinnon *et al* 1984) that shortens the measurement time to 1–2 times

the calorimeter time constant. The computer thus permits total automatization of the experiments.

3. The calorimeter

We now describe the calorimeter. As already stated, it is composed of two parts: the calorimeter block, containing the heat sink and the reference and reaction cells, and the tower supporting the systems for titrant injection and stirring of the reaction vessel.

3.1. The calorimeter block

Figure 2 shows a cross section of the calorimeter block. The block is cylindrical and figure 2 displays a section cut through the plane containing both its axis and the axes of the reference and reaction cells. The block has a stainless steel external shield (i), inside which the aluminium heat sink (j) is contained. The external shield is shaped like a cylindrical box with a lip around the upper rim. The heat sink is suspended from the lower surface of the box cover by six screws (not shown). The only contact between the heat sink and the external shield is through these screws and four stainless steel spacers (k), of which only two are shown; the other two are in equivalent positions in the plane perpendicular to that of the cross section shown. The whole block is immersed in a thermostatic bath and, to attenuate temperature fluctuations in the latter, the external shield is surrounded by an sheet of expanded polyurethane approximately 1 cm thick.

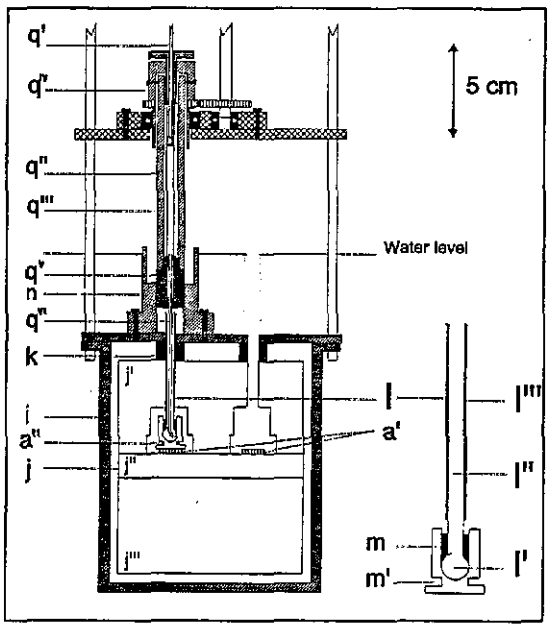

Figure 2. Cross section of the calorimeter block and the titrant syringe, and enlarged cross section of the reaction (or reference) cell: *i*, external shield; i, heat sink; g', syringe; and *l*, reaction (or reference) cell. For a detailed description, see the text.

The heat sink has three parts (j', j'' and j'''). The upper one (j') is trasversed by four orifices. The reaction and reference cells (l) are housed in two of them, which have three different diameters. These two orifices are shown in figure 2: the left-hand one with the reaction cell in place; the one on the right-hand side, in which the reference cell would go, is shown empty for the sake of clarity. The other two orifices (not shown) are placed at the same distance from the axis of the block as the ones shown, and in the plane containing the axis that is perpendicular to the plane of figure 2. These other orifices are of a single diameter, namely the width of the smallest section of the orifices shown. Two stainless steel tubes are housed in them, protruding downwards halfway into part j''. They stick out upwards through the external shield to above the water level in the thermostatic bath (at approximately 4.5 cm from the top surface of the external shield). Through one of the tubes goes the wiring joining the nanovoltmeter to the thermopiles (a'), connected in opposition, and through the other, the wiring from the power source to the heater (a''). The wires are embedded in the tubes with a low-melting bismuth alloy (Small Parts, J-LMA-117). They go from the lower end of the tubes to both the thermopiles and the heater via grooves in the lower surface of part j'' of the heat sink and small holes through it (not shown).

We have called the structures housed in part j' of the heat sink the reaction and reference cells (l). The cross section of these structures is also shown on the right-hand side of figure 2. The reaction takes place in a glass bulb (l'), of approximately 200 μl capacity, blown at the end of a NMR tube (l'') of 4 mm external diameter. We will call this bulb the reaction vessel. The total length from the top of the glass tube to the lower part of the bulb is approximately 7 cm. The upper 5 cm of the glass tube is in turn housed in a stainless steel tube (l'''), which makes contact with part j' in the heat sink and protrudes upwards through the external shield cover. The glass tube is fixed to the stainless steel one by a thin layer of wax. The reaction vessel is embedded with the same low-melting bismuth alloy used in the wiring tubes into a small, cylindrical copper cup (m). The heater (a''), made with a manganine wire approximately 15 cm long and of 100 Ω resistance, is coiled in a small groove (m') in its lower part. The lower surface of the copper cup makes thermal contact with the upper surface of a thermopile (Melcor FCO-6-66-05L); the lower side of the thermopile is also in thermal contact with part j'' of the heat sink. Heat thus flows from the reaction vessel to the heat sink through the copper cup and the thermopile.

Assembly of the calorimer block is quite easy. First, the reaction and reference cells and the wiring tubes are placed in the corresponding orifices in part j' of the heat sink. Then, with the thermopiles in position on its upper surface, part j'' is screwed to part j'. After making the electrical connections the whole thing is screwed to the lower surface of the external shield lid. Finally, the heat sink is completed by screwing part j''' to part j''. Part j'''

is to increase the heat capacity. Once this is done the external shield can be closed. Thermal grease is used in any surface contact in the assembly of the heat sink.

In order to keep the scheme in figure 2 clear, the holes and their relative positions for screwing the different parts are not shown. Nor do we show the o-rings in the lip of the external shield vase and in the spacers between the external shield lid and the heat sink, which are needed to keep the calorimeter block water-tight.

Finally, on the upper surface of the external shield lid, concentric with the reaction cell, there is a cylindrical piece of brass (n) to centre the syringe end used for injection of the titrant.

3.2. The tower

The scheme of the tower, which supports the injection and stirring systems, is shown in Figure 3. The tower is made up of five columns (of which only two are shown) screwed to the upper lip and the calorimeter block lid in the relative positions of five of the six vortices of a regular hexagon, and three circular 0.6 cm thick aluminium plate levels. On the upper level a stepping motor (a^{IV}) for titrant injection is fixed. The intermediate level holds a micrometer (o) and the stirring motor (a''). On the lower level is screwed a rectangular aluminium plate (p) containing two bearings. One of the bearings holds the axis of the stirring motor with a driving gear, which meshes with the driving gear of the titrant syringe (q), which in turn is housed in the other bearing.

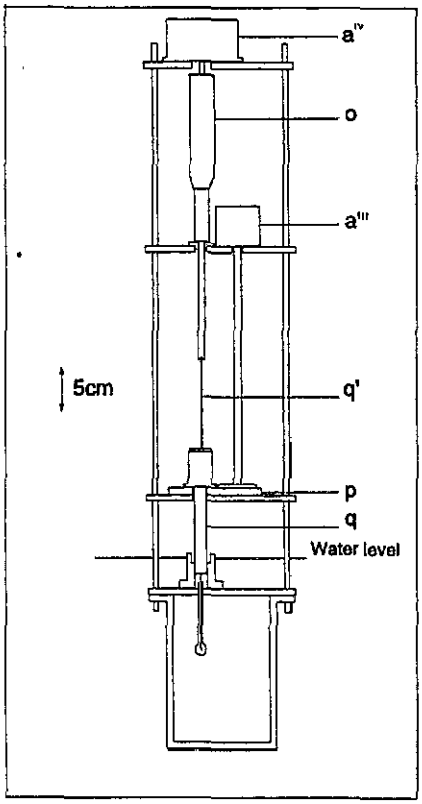

Figure 3. Tower to support the systems for titrant injection and stirring of the reaction vessel.

Both the stepping and the stirring motors are controlled by the computer. The stepping motor moves the micrometer, which in turn pushes the syringe plunger (q'). The stirring motor rotates the syringe via the driving gears. The reaction vessel is stirred by the bent tip of the glass tube through which the titrant is injected.

For a detailed description of the syringe we must go back to figure 2. Essentially it is composed of two parts: a brass tube (q''), which houses the glass barrel (q''') from a 250 μl commercial syringe (Unimetrics), and an upper part (q^{IV}) that is screwed onto the tube, also made of brass, which holds the driving gear and constitutes the part of the syringe that is placed in the bearing. Screwed to the lower end of the tube is a stainless steel piece (q^{V}) through which a glass tube of 1.3 mm external diameter and 25 μl approximate volume is centred (held in place by a couple of small Teflon grummets). As mentioned above, this is the tube through which the titrant is injected.

4. Bath temperature control and calorimeter base line

The elements to control the thermostatic bath (b) temperature are detailed in figure 1. The bath is made out of a camping ice-box placed inside a wooden box; the boxes' walls are separated by approximately 3 cm of expanded polystyrene. The approximate water volume in the bath is 30 l. The water surface is covered by small pieces of expanded polystyrene in order to increase its thermal isolation. The bath is stirred by two propellers in one axis that enters the bath vertically, as close as possible to the middle of the rear. The stirring motor (c), the speed of which is controlled by means of a variable resistance (c'), is fixed to the room wall in order to avoid its vibration affecting the system. Bath temperature control is achieved by means of a Tronac PTC-41 bath temperature controller (d), which heats the bath at the same speed at which it loses heat to the cooling sink (e') and the surroundings. To measure the bath temperature a thermistor (d') is immersed next to the calorimeter block. A 200 W light bulb (d'') supplies the heat. The cooling sink is a copper coil around the light bulb, through which water, coming from an auxiliary bath (e), circulates at a temperature 1 °C below the bath temperature. Tap water (e'') is used as the cooling sink in the auxiliary bath. Finally, the calorimeter block is immersed in the bath slightly towards one side and equidistant from three of the four sides.

Trace C of Figure 4 is the recording of the water bath temperature fluctuations. They are in the range ± 1–2 m°C. This control is not as good as the specifications given by the maker of the temperature controller, who claims that a temperature control up to five times better than ours could be achieved. Our results did not improve after slight modifications of the bath geometry.

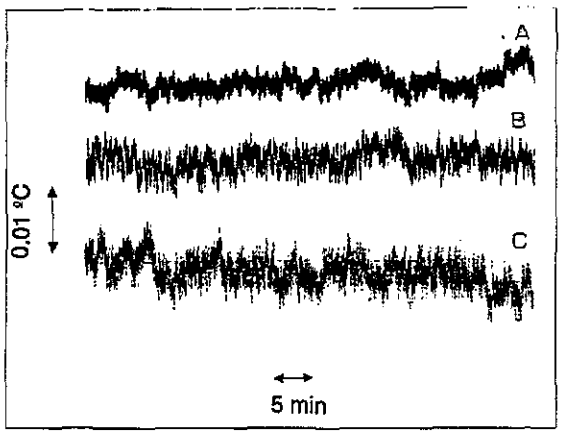

Figure 4. Bath temperature control at 25 °C (trace C). Trace B shows the temperature fluctuations of an LKB Bioactivity Monitor bath at 25 °C. Trace A is the analogue output of the thermometer used in measurements after shortening the input.

Nevertheless, the water temperature control in our bath is equivalent to that of one of the better commercially available baths for calorimetry, the temperature fluctuations of which we have also measured and are shown in trace B.

One does not have to worry much about bath temperature fluctuations if the calorimeter base line is good, as it is in our case. In Figure 5, traces B, C and D are three base lines at three different temperatures (15, 25 and 35 °C). The base line is the oppositionally connected thermopiles' voltage recorded with time when no event is taking place in the reaction vessel. It can be seen that the base line noise is almost the same as specified by the maker for the nanovoltmeter used when its input is shortened (15 nV), shown in trace A. Base lines have been registered with temperature differences between the room and the thermostatic bath of up to 5 °C, and these differences seem to have no significant effect. No systematic study has been made to see what effect higher temperature differences might have on the base line.

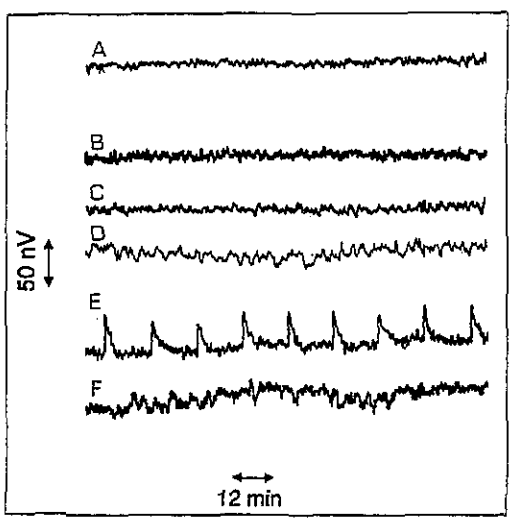

Figure 5. Calorimeter output recordings. Traces B, C and D are the calorimeter base lines at 15, 25 and 35 °C at room temperatures of 17, 22 and 30 °C, respectively. Trace E is the calorimeter signal when a current of 100 μA intensity is passed through the heater for 10 s every 12 min. Trace F is the calorimeter signal when the reaction vessel is stirred at 170 rpm for 45 s every 12 min. Trace A is the nanovoltmeter analogue output with its input shortened.

The calorimeter's sensitivity is related to its capacity to detect thermal effects above the base line noise clearly. In trace E of figure 5 we show calorimeter signals after heat inputs of 10 μJ through the heater to the reaction cell. These thermal effects are clearly distinguishable from base line noise. As peaks with half the area would be within the limits of perception with respect to the base line, we might say that our calorimeter has a sensitivity of approximately 5 μJ. Given the reaction volume of 200 μl the specific sensitivity (sensitivity per unit volume) of the instrument is approximately 25 μJ ml^{-1}. After reviewing the literature we have found only one titration calorimeter, namely the one developed by Dr Brandts (Wiseman *et al* 1989), with a specific sensitivity better than ours.

Trace F in figure 5 is the calorimeter signal when the reaction vessel is stirred at 170 rpm for 45 s every 12 min. 170 rpm and 45 s are the real stirring speed and duration in a standard experiment, in which stirring and titrant injection are started simultaneously, the latter lasting for 20 s when it is of 10 μl. The stirring heats of the instrument can be seen to be below its sensitivity, namely below 5 μJ.

Finally, it is worthwhile making a comment about the building of the calorimeter following the twin-cell construction principle. According to this principle, the reaction and reference cells are built to be identical. As the thermopiles put both cells in contact with the same heat sink, measurement of the differential voltage of the thermopiles is an indirect measurement of the temperature difference between the cells (see for example McKinnon *et al* (1984)). The peaks in trace E of figure 5 correspond to heats of 10 μJ passed in the reaction cell through the heater and their height is 30–40 nV. Assuming that the heat supplied is used just for heating the 200 μl of water in the reaction vessel, ignoring heating of the copper cup, we could say that the increment in temperature of the reaction vessel is approximately 10 μ°C. Assuming again that the 30–40 nV peak height is due to those 10 μ°C, we may conclude that the base line noise shows temperature fluctuations in the μ°C range. That is, there is a factor of 1000 improvement over the temperature fluctuations of the water bath. Obviously, this improvement is not only due to building according to the twin-cell principle. It has to be taken into account that the insulation of the external shield attenuates the bath fluctuations. In addition, the high capacity of the heat sink and the low thermal contact between it and the external shield cause a new attenuation in the fluctuations in the heat sink. When these fluctuations are equally detected by both thermopiles, these being connected in opposition, they

are subtracted, precisely because of the sameness of the reaction and reference cells.

Acknowledgments

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