Meas. Sci. Technol. 5 (1994) 1071–1077. Printed in the UK

Description of a new Gill titration calorimeter for the study of biochemical reactions. II: operational characterization of the instrument

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Received 19 April 1994, accepted for publication 9 June 1994

Abstract. This paper continues the description of an isothermal titration calorimeter already outlined in the previous paper. We have determined its thermal parameters and report here on the performance of the calorimeter and the method of measuring via electrical compensation of the thermal effect. We have also calculated the effective volume of the reaction vessel and the heat of stirring of the reaction mixture in the vessel, and of injecting water into water. Finally, correct working of the calorimeter has been tested by a dilution experiment of a saccharose solution.

1. Introduction

In the previous paper (El Harrous *et al* 1994) we described the structure of a small-volume (approximately 200 μl), high-sensitivity (approximately 5 μJ), isothermal titration microcalorimeter, composed of twin reaction cells and designed especially for studying biochemical reactions. In this paper we go on to detail how this microcalorimeter functions.

Calorimeters can be divided into two main groups (Spink and Wadso 1976): adiabatic and heat conduction; our microcalorimeter belongs to the latter group. In a perfectly designed adiabatic calorimeter no heat exchange takes place between the physical environment in which the process occurs and its surroundings. In a heat-conduction calorimeter, on the other hand, there is a quantifiable transference of heat between the experimental environment (in our case the reaction vessel) and the space surrounding it (referred to here as the heat sink). Ideally this heat sink has an infinite heat capacity and thus the temperature of the reaction cell is the same at the beginning and end of the process. Generally, in a conduction calorimeter the heat is measured from a magnitude proportional to the flow of heat between the physical environment in which the process takes place and the surrounding sink.

In practice, correct working of a heat-conduction calorimeter depends on the assumption that the calorimeter follows Newton's law of cooling (McGlashan 1979)

$$dQ/dt = -K'(\theta - \theta_s) \qquad (1)$$

which states that the heat flow between the reaction cell and the heat sink is directly proportional to the difference in temperature between them. In the above equation Q stands for heat, t for time, θ for the temperature of the reaction cell and θ_s for that of the heat sink; K' is a proportionality constant, which can be considered as a thermal conductivity, with units energy degree^{-1} time^{-1}. As far as our microcalorimeter in particular is concerned, conduction between the reaction cell and the heat sink takes place via a thermopile and so it can be regarded as a thermopile heat-conduction microcalorimeter. When the difference in temperature between the faces of the thermopile is sufficiently small, the voltage it produces is directly proportional to the difference in the above-mentioned temperature (the Seebeck effect). Thus, for a difference in temperature of $\theta - \theta_s$ between the reaction cell and the heat sink the voltage of the thermopile can be expressed as

$$V = \varepsilon(\theta - \theta_s) \qquad (2)$$

where ε is the proportionality constant. Incorporating this equation into equation (1), we get

$$dQ/dt = -KV \qquad (3)$$

where the proportionality constant K may also be considered as being a thermal conductivity of units energy voltage^{-1} time^{-1}. If we integrate the above equation, we are left with

$$Q = -K \int_{t_1}^{t_2} V\,dt. \qquad (4)$$

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Thus, in thermopile heat-conduction calorimeters, the thermal effect is evaluated by measuring their voltage. If we use the basic equation (3) then we are measuring the power of the process, while, if we use equation (4), we are measuring the heat produced during the process, which is what we do with the calorimeter described here.

To measure the thermal effect with equation (4) it is first necessary to know the thermal conductivity value of the microcalorimeter. Furthermore, the integration time, $t_2 - t_1$, is related to the time constant of the instrument, which is the ratio between its heat capacity and the thermal conductivity. We shall first explain the determination of these two parameters, which are characteristic of the microcalorimeter, and then go on to describe the electrical compensation method for measuring the heat that considerably shortens the time $(t_2 - t_1)$ needed to carry out a measurement. Finally, we shall describe how the microcalorimeter works, controlled by a computer, and test its functioning with a dilution experiment of a saccharose solution.

2. Method of measurement and control program

2.1. Determination of the thermal parameters

In figure 1(*a*) we show the response of the microcalorimeter when a current of a certain intensity is passed through the heater (resistance $R = 100\ \Omega$) of the reaction cell for a sufficient period of time. It can be seen how the voltage in the thermopile reaches a steady state, which represents a situation in which the heat flowing through it from the cell to the heat sink is equal to the power applied to the reaction cell via the heater. At this moment the first term of equation (3) is given precisely by this power and thus the ratio between this power and the voltage of the thermopile constitutes the thermal conductivity, K, across the thermopile in our system. We repeated the same experiment for powers of between 0.25 and 1600 μW (figure 1(*b*)). In figure 1(*b*) we have shown the logarithms of the power and voltage values, due to the fact that the power applied, and thus the measured voltages, covered a range of various orders of magnitude. The ratio between the two magnitudes was always the same and the value arrived at for K in this series of experiments at 25 °C was $2.80 \pm 0.04\ \mathrm{J\ s^{-1}\ V^{-1}}$ ($0.04\ \mathrm{J\ s^{-1}\ V^{-1}}$ is the standard error). This result confirms that Newton's law of cooling is obeyed in our microcalorimeter within the range of powers studied. It must be borne in mind that our instrument is intended to measure heats corresponding to powers way below the upper limit used in the experiment.

It can be shown (Calvet and Prat 1963) that the rising and decreasing parts of the voltage versus time graph in figure 1(*a*) can be approximated by exponentials in which the time constant is the quotient between the heat capacity of the reaction vessel (or more strictly, the reaction vessel and the copper capsule in which it is embedded (El Harrous *et al* 1994)) and the thermal conductivity across the thermopile. The units of this heat capacity are $\mathrm{J\ V^{-1}}$, where, according to equation (2), the voltage of the thermopile measures a difference in temperature. Linearization of the decreasing part of the curve in figure 1(*a*) is shown in figure 1(*c*). From its slope we can determine a time constant of 94 s and from the product of this latter value and the thermal conductivity already determined we get a value of $263\ \mathrm{J\ V^{-1}}$ for the heat capacity of the reaction cell.

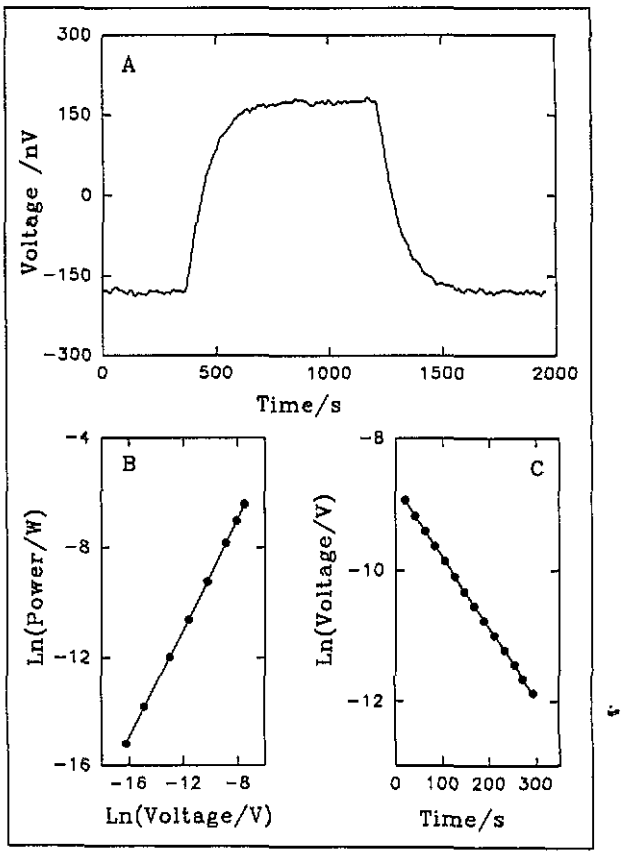

Figure 1. (A) Response of the calorimeter to application of electrical power 1 μW across the heater (100 Ω) of the reaction cell. The power is applied for long enough to reach a stationary voltage. (B) Results obtained for the voltages corresponding to the stationary states after the application of powers in the range 0.25–1600 μW to the heater of the reaction cell. (C) Analysis of the negative slope of the graph in (A). All the experiments described in this figure were carried out at 25 °C.

All these parameters were re-checked in the same way at other temperatures between 15 and 35 °C without any significant variations appearing.

2.2. Method of measurement

In figure 2(*a*) we show the signal produced by the microcalorimeter on injecting HCl for 10 s into a larger quantity of NaOH. The reaction mixture was stirred for 45 s, calculated from the beginning of the injection. The injection is represented by the rectangle of dots, which symbolizes it as a constant communication of chemical power (the height of the rectangle, which will be in this case 12.1 μW) for 10 s. Measurement of the heat involved in the process from the signal V versus t is made by

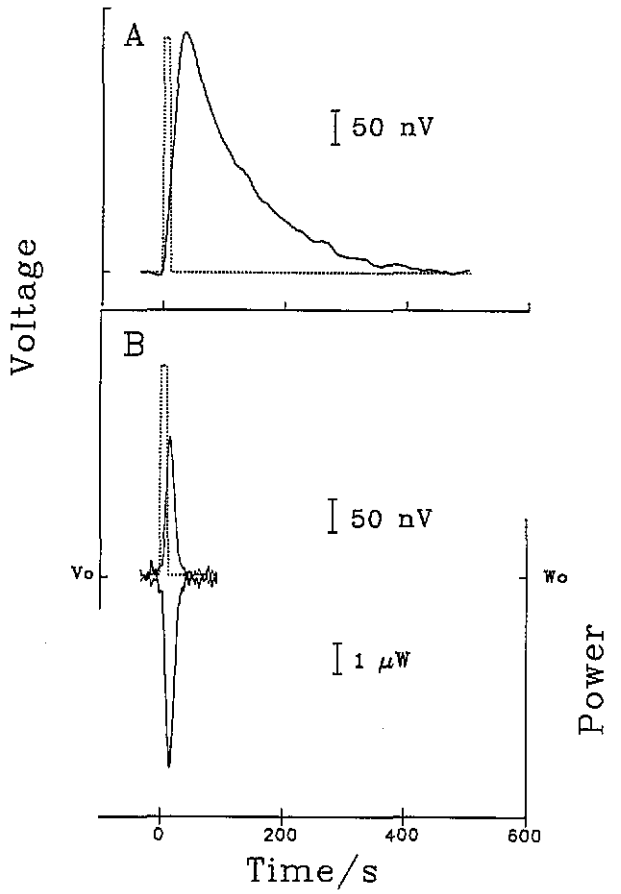

Figure 2. (A) Response of the calorimeter to application of a chemical power (injection of HCl into NaOH) to the reaction cell of 12.1 μW for 10 s. (B) Response of the calorimeter to application of a chemical power of 13.2 μW for 10 s and its electrical compensation. The voltage of the thermopiles is measured in such a way that positive voltages correspond to exothermal effects in the reaction cell. Both experiments described in this figure were performed at 25 °C.

integrating it and multiplying the result by the thermal conductivity, K, in accordance with equation (4). This integration is carried out numerically by the program controlling the experiment. It can be seen from the graph that the time difference $t_2 - t_1$ for the integration is at least 10 min, bearing in mind that it is necessary to have adequately long base lines both before and after the signal for their extrapolation (in order to obtain the base line in the numerical integration) to be correct. In general, in any thermopile heat-conduction calorimeter, it is accepted that the time taken to make a measurement should be about seven or eight times the time constant of the system, in our case around 12 min. During a complete titration, in which at least ten injections would be made, the experiment would take at least 2 h.

This time can be shortened considerably if we use an electrical compensation method of measuring the thermal effect. Using this method (McKinnon *et al* 1984), a determined power W_0 is applied to the heater of the reaction cell in such a way as to create a stationary temperature difference between the cell and the heat sink. In the face of any thermal effect in the reaction vessel the power from the heater changes to keep the difference in temperature constant. If the process is exothermal then the power should fall, whilst if it is endothermal, it will rise. In an ideal situation, in which the difference in temperature remains constant throughout the experiment, the heat is measured by integrating the change in power supplied to the heater with time. In a real situation, however, part of the thermal effect induces heat conduction through the thermopile, which must also be evaluated; in this case by integrating the changes in voltage in the thermopile with time. The way this method works can be seen graphically in figure 2(*b*). The power, W_0, is applied through the heater and we calculate it by dividing the maximum heat to be expected during the injection of a reagent (q_{max}) by the time taken for the injection (approximately 20 s if the quantity injected is 10 μl). This power requires a determined current intensity, I_0. Just as in the experiment shown in figure 1(*a*), the voltage in the thermopile reaches a stationary state, V_0, corresponding to a constant temperature difference between the reaction cell and the heat sink. Let us now presume that a chemical reaction begins in the reaction cell at time 0 (once again an injection of HCl into a larger quantity of NaOH). As in figure 2(*a*), in figure 2(*b*) this reaction is symbolized by a broken rectangle, which represents the communication of a constant chemical power to the reaction cell for 10 s, with stirring for 45 s. In this case the chemical power was 13.2 μW, a value very close to that used in the experiment described in figure 2(*a*), and thus, if the electrical compensation is not applied in the experiment described in figure 2(*b*), then we should expect the graph of V versus t to be quite similar to that in figure 2(*a*). Nevertheless, in this case the heat is measured by applying the electrical compensation in the following way. From time 0 the voltage in the thermopile is measured every second, thus obtaining voltage values, V. Depending on V, the intensity through the heater is modified during the following second according to the equation

$$I = I_0 - (V - V_0)G \qquad (5)$$

which indicates that the current through the heater (I, or the corresponding power, W) is modified by a quantity proportional to the difference between the measured (V) and base line (V_0) voltages, G being the proportionality factor. This proportionality factor changes for each titration, depending upon the maximum heat expected for the injection, q_{max}, according to the empirical formula

$$G = -150(1 + \log q_{max}) \qquad (6)$$

with q_{max} in joules.

The result of applying this electrical compensation method can be seen in figure 2(*b*). The heat is now calculated by evaluating two integrals (that of the signal $V - V_0$ versus t and that of the signal $W - W_0$ versus t) and adding the two results. The most important effect of application of this compensation method lies in the shortening of the measurement time. Having applied practically the same chemical power for the same length

of time in the experiments in figures 2(*a*) and (*b*), it can be seen that the time needed for measuring in the experiment in figure 2(*b*) is almost ten times less than in the previous experiment. This leads to a considerable shortening in time spent on a complete calorimetric titration. As a normal operating routine, 90 s is devoted to each measurement and 3 min from the beginning of the injection of a reagent into the reaction cell to the beginning of the following measurement. Thus a titration involving ten injections will take some 30 min.

The heat measured via the graph of V versus t in the experiment in figure 2(*b*) is 10.5 μJ, approximately 8% of the total heat produced by the chemical reaction being studied. In general this method fails to compensate for between 5% and 10% of the total thermal effect, whatever its magnitude.

Finally, it should be said that this electrical compensation methodology has been adjusted in a totally empirical fashion. The determination of the initial power (W_0) on the basis of the maximum heat expected during an injection, the modification of the intensity of the current across the heater according to equation (5), and the evaluation of factor G in equation (5) via formula (6) have all been arrived at by a process of approximation. We have found no alternative way that might allow us to compensate electrically for more than 95% of the thermal effect without the system making buffered oscillations.

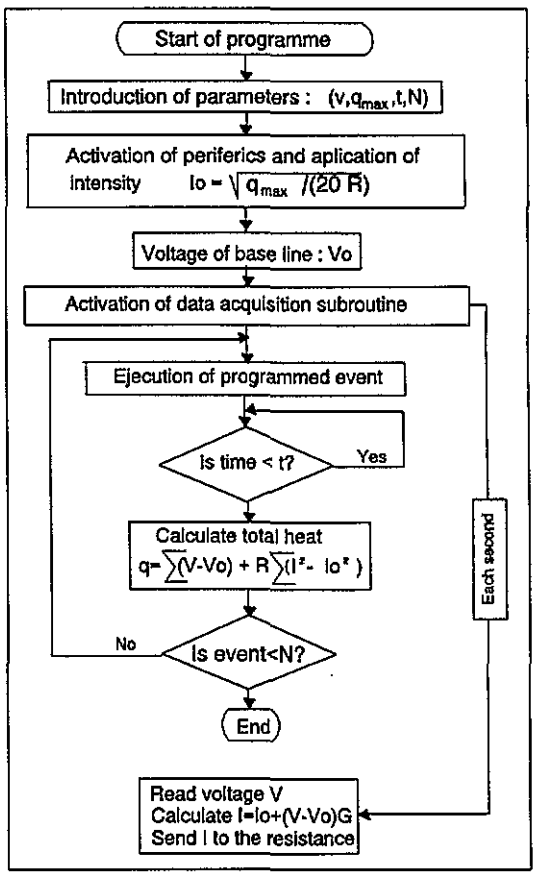

Figure 3. Flow chart of the control program of the microcalorimeter.

2.3. Description of the control program

The whole process of the electrical compensation system that we have described above, together with integration of the signals to obtain the magnitude of the heat produced, are carried out automatically by a Hewlett-Packard HP85 computer, which controls the experiment (El Harrous *et al* 1994).

The essential aspects of the program are shown in the flow chart in figure 3. The first values to be introduced into the program are the four basic parameters of the experiment: the volume of the titrant to be injected, v; the maximum heat expected during the injection, q_{max}; the time during which heat measurement will take place, t, which will normally be 90 s; and the number of events, N, to be carried out (stirring only, stirring plus injection, base line noise). Following this the nanovoltmeter, power source and injection and stirring motors are switched on and the current intensity I_0 is transmitted from the source to the heater in the reaction cell.

After waiting for the base line of the microcalorimeter to attain a stable value, V_0, the data acquisition subroutine, which measures every second the voltage V and modifies the intensity I through the heater, is set in motion. We are now in a position to begin what we call an 'event'. If this consists, for example, of the injection of a reagent, then the injection and stirring motors will be switched on; with a 10 μl injection the former will work for 20 s for the injection and the latter 45 s for stirring (30 s clockwise and 15 s anti-clockwise). From the first moment of the event until reaching the measurement time, t, the values of $V - V_0$ and I are stored and used to evaluate the thermal effect when the measurement time finishes. The values of V_0 and I_0 that are taken into consideration are the average values of the voltage and current intensity during the 30 s immediately preceding the beginning of the event. This process is repeated for each of the planned events. The measurement time for an event is 90 s and the next event could be started immediately thereafter. We usually wait another 90 s before starting the next event.

3. The working of the microcalorimeter

With the above explanation of the automatic programming of the experiment the description of the working of the microcalorimeter is almost complete. Let us take, for example, titration of a macromolecule with a ligand. The procedure would be the following. Firstly, 0.5 ml of a solution of the macromolecule is put into the reaction cell and we wait until the system reaches equilibrium, that is, the base line of the instrument returns to its original position. The time needed to return to equilibrium depends on the difference between the temperature of the solution put into the reaction cell and the working temperature of the microcalorimeter. If we try to ensure that both temperatures are similar then the time required

to reach equilibrium should be around 30–45 min. The syringe is then filled with the solution of the ligand and put in place, waiting another 20 min for the system to return to equilibrium once more. The reaction vessel, which is the physical space in which the titration takes place, has a volume of some 200 μl; nevertheless, we need 0.5 ml of macromolecule solution in the reaction cell so as to make sure that the ligand solution in the injection tube coming from the syringe balances thermally with the solution in the reaction cell. Furthermore, in this way we avoid causing excessive noise in the base line as the extra 0.3 ml covers the glass tube above the reaction vessel, avoiding its exposure to air. The system goes on to carry out the programmed successive injections of the ligand solution into the macromolecule solution. As we have already said, one injection usually takes place every 3 min and the time taken to inject 10 μl is 20 s. At the same time as injection begins, so too does stirring of the mixture in the reaction vessel (170 rpm) by the slightly bent end of the glass tube through which the ligand is injected from the syringe. As has also been mentioned above, this stirring takes place for 45 s, 30 s clockwise and 15 s anti-clockwise, or vice versa. Heat measurement is usually made over a period of 90 s. The experiment ends when all of the injections programmed have been completed. Some stirring is often programmed at the beginning or end of the titration to measure their heat values.

3.1. Stirring and injection heats of water into water

Heat produced by stirring the reaction vessel and by injection of one solution into another always forms part of any process followed in the microcalorimeter. The average values for ten stirrings of the reaction vessel and ten injections of water into water with simultaneous stirring are set out in the third and fourth columns of table 1. Both are exothermic processes; for the first we obtain an absolute mean value of around 7 μJ and for the second, 12 μJ. In our previous paper (El Harrous *et al* 1994) we established from a simple observation of the base line a stirring heat below the sensitivity of the instrument itself (5 μJ) and we see that the heat measured (7 μJ) is only marginally above that value. The value of 12 μJ for injection of 10 μl of water into water is slightly higher than expected but we still regard it as a good result.

In the first and second columns of table 1 we show the values obtained by applying the measurement methodology explained above to the base line of the instrument when no process is taking place inside the reaction vessel. As is to be expected, the average value of ten measurements is close to zero. The standard error of around 3 μJ can be taken as being due to base line noise and is perfectly compatible with the sensitivity of 5 μJ that we have assigned to the instrument.

3.2. The basic mixing process

A scheme of the basic mixing process that occurs in the microcalorimeter after an injection, *i*, can be seen in figure 4. The effective volume of the reaction vessel, V_e, remains constant throughout an experiment, as the injection of a volume into it means that a similar volume must be ejected. The basic supposition made in the solving of any calculation is that the volume of solution ejected from the reaction cell plays no part in the process being studied. For example, if the reaction of a macromolecule with a ligand is being studied, then on injecting volume v of ligand solution the volume of macromolecule ejected from the solution in the reaction vessel does not react with the ligand. The validity of this supposition is verified by the agreement between experimental results and predictions. As an example we shall see this later on for the known process of dilution of saccharose solution in water. The volumes injected never exceeded 10 μl, at a flow rate of 0.5 μl s^{-1}, in any of the experiments undertaken.

To calculate the concentrations of reagents in the reaction vessel during any titration it is useful to define the dilution factor, D:

$$D = 1 - v/V_e \qquad (7)$$

Table 1. Solvent–solvent mixing and stirring heats (μJ). The heats shown in the first column were measured for 10 min without applying the electrical compensation method. The rest of the heats were measured for 90 s, applying the electrical compensation method, with a current, I_0, of 0.46 mA across the heater. A negative sign corresponds to an exothermal effect. All measurements were made at 25 °C. Injection was of 10 μl of water for 20 s. The reaction vessel was stirred for 30 s clockwise and 15 s anti-clockwise.

	Base line		Stirring	Injection plus stirring
	2.1	0.2	−10.0	−5.0
	4.8	5.3	−8.4	−13.6
	−2.3	−1.9	−3.6	−10.2
	0.4	−1.4	−1.7	−15.3
	2.4	−0.6	−7.9	−14.9
	−1.7	3.2	−6.2	−9.1
	4.4	−0.8	−10.2	−11.7
	−1.1	−0.6	−4.9	−9.3
	−6.4	1.8	−5.9	−13.0
	−3.4	0.8	−8.8	−14.8
Average	0.6	0.6	−6.8	−11.7
Standard error	3.5	2.2	2.8	3.3

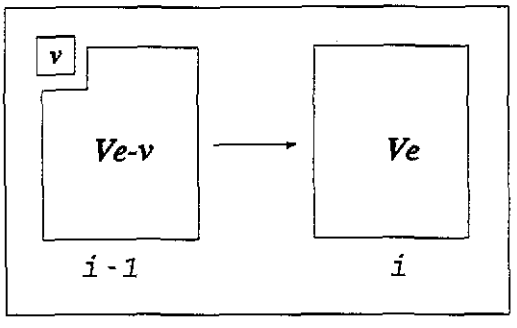

Figure 4. Basic mixing process after injection *i*. V_e is the effective volume of the reaction vessel and v the volume injected from the syringe.

where, as already stated, v is the volume injected into the microcalorimeter and V_e the effective volume of the reaction cell. If the reagent injected via the syringe is called L and M is the reagent to be titrated in the reaction vessel, then their concentrations in the latter after injection i will be

$$[L]_i = [L]^0(1 - D^i) \qquad [M]_i = [M]^0 D^i \quad (8)$$

where $[L]^0$ is the concentration of the reagent in the syringe and $[M]^0$ is the starting concentration of M in the reaction vessel.

3.3. Calculation of the effective volume of the reaction vessel

When a reaction with a high association constant is studied in the microcalorimeter all the titrant reagent that enters into the reaction vessel reacts completely with the reagent to be titrated until this latter is completely used up, that is until the equivalence point is reached. A calorimetric titration curve in this case, in which the heat measured for each injection is represented, will be composed of a horizontal sector followed by a sharp fall to zero. It can be shown that the ratio between the heat measured upon injection, when the point of equivalence is reached, q_k, and the heat measured at the prior injection, q_{k-1}, is related to the initial reagent concentrations and the dilution factor via the equation

$$\frac{q_k}{q_{k-1}} = \frac{([M]^0 + [L]^0)D^{k-1} - [L]^0}{[L]^0\{(1/D) - 1\}}. \quad (9)$$

This equation allows us to calculate the reaction volume as long as the first term, the injection volume, v, the concentration of the titrant in the syringe, and the initial concentration of the reagent to be titrated in the reaction vessel are known precisely.

The result of an experiment designed to titrate HCl with NaOH is shown in figure 5. According to the criteria set out above, we have been able to determine an effective volume of the reaction vessel, V_e, of 203 μl for this experiment. From a propagation-of-errors analysis the standard error for this value is about 4 μl.

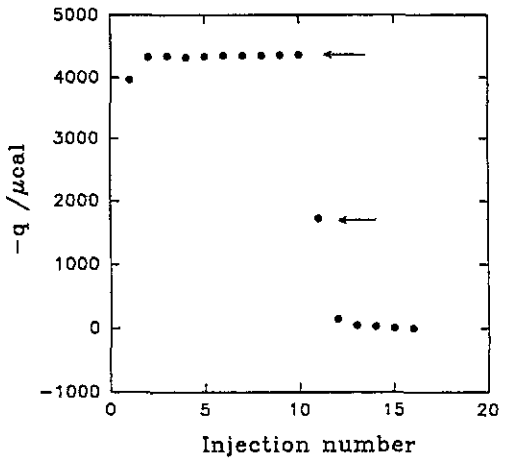

Figure 5. Calorimetric titration of 0.0216 M of HCl with 0.0312 M of NaOH at 15 °C. The volume of the injections of NaOH was 10 μl. To calculate the effective volume of the reaction vessel we used the ratio between the heats indicated by the arrow, as described in the text.

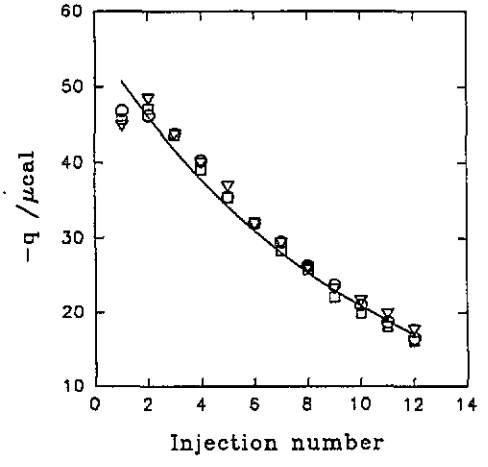

Figure 6. Dilution of a saccharose solution in water at 20 °C. Here are shown the heats measured in three experiments, in which 10 μl injections of 0.20 M saccharose solution were made into initially pure water. The line joins the points calculated from the data provided by Gucker *et al* (1940).

3.4. Saccharose dilution

A study of the dilution of a saccharose solution in water is one of the conventional ways of testing the working of a calorimeter for measuring heats of the order of microjoules. The results of three experiments of this type are set out in figure 6. The reproducibility of the instrument's capacity is immediately evident. A comparison can also be made of the heats measured with those calculated from the apparent relative molar enthalpy, φL_2, for saccharose at 20 °C of Gucker *et al* (1940):

$$\varphi L_2 = 539.3m - 28.94m^2 \quad (10)$$

in joules per mole, where m is the molality. Bearing in mind that Gucker's equation is calculated from results with a dispersion of about 3%, the agreement between our values and his is highly acceptable.

Calculation of the expected values for subsequent injections from the apparent relative molar enthalpy was made taking into account the basic mixing process shown in figure 4 and in accordance with the expression

$$q_i = \varphi L_2(m_i)[L]_i V_e - \varphi L_2(m_{i-1})[L]_{i-1}(V_e - v) - \varphi L_2(m^0)[L]^0 v \quad (11)$$

as the apparent relative molar enthalpy corresponds to infinite dilution of the saccharose solution from the specified concentration. The [L] terms are the molar concentrations of saccharose, which are calculated for each injection from equation (8), and from which the corresponding molalities for calculation of φL_2 are obtained.

Finally, a comment must be made about the values for the first injection, which are below those to be expected, as can be seen for the experiments in figures 5 and 6. This is due to diffusion of the titrating reagent from the end of the injection tube during the time taken to equilibrate the microcalorimeter before beginning the titration. Nevertheless, as there is only a lapse of 3 min between injections during the titration, this diffusion can be regarded as negligible and the rest of the titration points are not affected by it. Consequently, in analyses of the experiments we ignore the values for the first injection.

Acknowledgments

This work was supported by grant BIO90-0592 from the Comisión Interministerial de Ciencia y Tecnología, Ministerio de Educación y Ciencia, Spain. MEH acknowledges fellowships from the Ministère de l'Education Nationale, Morocco and the Ministerio de Asuntos Exteriores, Spain. We thank Dr J Trout for revising the English text.

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