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A MODIFIED CONTINUOUS FLOW MICROCALORIMETER FOR MEASURING HEAT DISSIPATION BY MAMMALIAN CELLS IN BATCH CULTURE

Y. Guan, P. C. Lloyd, P. M. Evans and R. B. Kemp

Institute of Biological Sciences, The University of Wales, Penglais, Aberystwyth, SY23 3DA, Wales, UK

Abstract

Microcalorimeters to monitor the heat dissipation of bench-scale animal cell cultures on line and in real time require a continuous circuit between the vessel measuring heat flow rate and the bioreactor. The modifications to the transmission lines and calorimetric heat exchanger were to: (i) reverse the usual upward direction of the cell suspension in the flow vessel to downwards; (ii) install an in situ washing/cleaning facility; (iii) use low diffusivity PEEK material; and (iv) maintain thermal equilibration by water-jacketing the transmission tubing. Chemical calibration showed that there was more than a 20% difference between the physical volume and the effective thermal volume. An appropriate thermodynamic system was defined in order to permit enthalpy balance studies.

Keywords: animal cell, heat dissipation, heat flux, metabolic activity, microcalorimetry

Introduction

All biological processes are accompanied by heat dissipation and the rate of heat flow from biological organisms is a measure of their metabolic activity [1, 2]. With the advent of microcalorimeters, it has proved possible to measure the heat produced by cells in vitro. In such experiments, the heat flow in terms of unit bulk volume of the measuring vessel (volumic heat flow) represents the total metabolic activity of that cellular system, whereas specific heat flow rate (also termed heat flux, which is defined as heat flow rate in terms of unit amount of cell mass) represents the specific metabolic activity (metabolic flux in precise terms). Cultured cells are increasingly used for biotechnological processes and it would be valuable to monitor their heat flow rate on line and in real time to check the metabolic state and eventually achieve process control. At the low cell density (cell number concentration) typical of a suspension of mammalian cells [3], it is difficult to measure the volumic heat flow using the current generation of bench-scale calorimeters (typically 2-L with respect to a bioreactor) employed in the thermal studies of microbes [1] because the total heat produced is close to or even lower than the present detection limit (ca. 50 mW) of this type of calorimeter. An efficient and economic alter-

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native for measuring cellular heat flow rate is to use a microcalorimeter external to the bioreactor. In this approach there is a continuous circuit between the flow vessel of a heat conduction microcalorimeter and the culture vessel (bioreactor), with the cell suspension driven by a peristaltic pump. In this continuous ex situ calorimetry, the cell suspension is flowed from the bioreactor along a transmission line to the microcalorimeter and then returned, forming a loop. A 4-channel ThermoMetric 2277 thermal activity monitor, TAM (ThermoMetric AB, Järfälla, Sweden) and a 3-L Applikon Bioreactor System (Applikon Ltd., Tewkesbury, Glous., UK) were used to demonstrate the value of this on-line system. The animal cells employed as a model were Chinese Hamster Ovary (CHO) 320 [3], genetically engineered to produce human recombinant interferon-γ and growing in batch culture.

Because the cells were continuously transferred to the microcalorimeter for the heat dissipation measurement, it is important that their metabolic status and their environmental condition were the same in the bioreactor and in the heat measuring vessel. Pre-tests and prior experience [4, 5] showed that appropriate technical consideration should be given to the following issues in establishing a successful continuous flow microcalorimetry system for cells: (i) the viable cell density must be the same as that in the bioreactor and so the cells must not sediment in the circulatory loop, (ii) the dissolved oxygen concentration must be identical in both vessels, (iii) a reduction in temperature of the cell suspension along the transmission line must be prevented by thermal control or compensation, and (iv) the cells should not be injured or metabolically affected by the squeezing action of the pump mechanism.

A description of the transmission system

The general arrangement of the transmission line between the bioreactor vessel and the flow vessel of the calorimeter is shown in Fig. 1. All the transmission tubing was supplied by ThermoMetric Ltd. (9 Church Meadows, Northwich, Cheshire CW8 4SB, UK) unless otherwise stated. Initially Teflon tubing (1 mm I.D. and 1.4 mm O.D.) was used for the transmission lines. Viton tubing (1.3 mm I.D.) was placed in a variable speed peristaltic pump (Jubile model JU/88W, H.J.Guldener, 8047 Zürich, Switzerland). The Viton tubing and the Teflon tubing were joined together by sleeving the former onto the latter. The flow vessel of the microcalorimeter is made from 24-carat gold with an inner diameter of 1 mm. The joint between lengths of Teflon tubing is made with a silicone sleeve (1 mm I.D. and 2 mm O.D.). Since it is known that the diffusivities of gaseous molecules such as O_2, N_2 and CO_2 in Teflon is relatively high [6], the extensive use of it could lead to a different physiological environment between the bioreactor and the microcalorimetric flow vessel. Therefore it was replaced by PEEK which is known to have a very low gaseous diffusivity. Transmission lines were made of PEEK (1 mm I.D. and 1.56 mm O.D.) together with many as possible of the other accessories (fingertight fittings, unions, tees, valves, tubing elbows, etc.). Most were originally designed primarily for HPLC and so were readily available from P.D. Marketing, Church Lane, Sidlesham, Chichester, W. Sussex PO20 7RH, UK and Phase Separations Ltd, Deeside, Clwyd CH5 2NU, UK. Apart from low gaseous diffusivity, PEEK is bio-

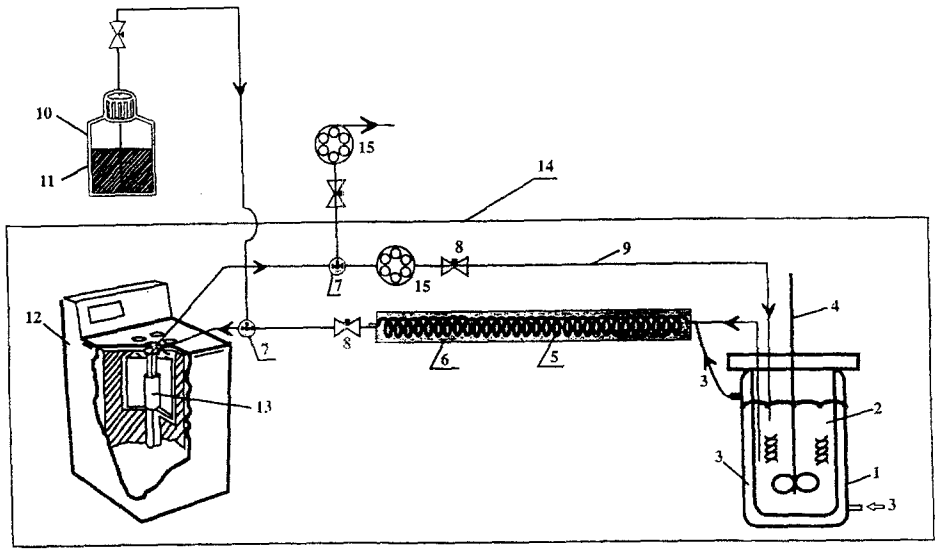

Fig. 1 A schematic diagram for the measurement of heat flow of bioreactor-cultured animal cells using a flow microcalorimeter. 1 – bioreactor; 2 – cultured cells; 3 – jacket water for temperature control in the bioreactor; 4 – agitator; 5 – the outlet tubing of the jacket water is used for warming the cell suspension in the tubing leading to the microcalorimeter; 6 – a non-conductive sponge-plastic pipe is used to reduce heat dissipation along the outlet jacket water attached to the PEEK tubing transmitting the cell suspension to the calorimeter; 7 – PEEK T-piece; 8 – PEEK two-way valve; 9 – PEEK tubing (1 mm I.D.); 10 – glass bottle holding sterilized medium for washing the flow vessel of the microcalorimeter through short-time interruption of the heat flow measurement; 11 – sterile medium for cleaning the flow vessel free of possible accumulated animal cells, which would result in overestimation of the heat flow for the cells in the bioreactor; 12 – representation of the 4-channel Thermometric calorimeter (TAM); 13 – gold vessel assembly; 14 – the dotted boundary, enclosing essentially the bioreactor and the flow vessel of the calorimeter, is an open thermodynamic system for enthalpy balance studies; 15 – peristaltic pump

compatible and will not adsorb biomolecules. On the other hand, it is relatively taut, is not elastic and will not readily form bends. This is not a problem for the transmission lines, but provides difficulties at joints between it and other materials. The connection of the PEEK tubing to the gold flow vessel assembly was a problem of this type but it was solved as illustrated in the upper right part of Fig. 2: a very short length of Teflon tubing (ca. 2 cm) was sleeved onto the metal tubing of the measuring unit, and then the link between Teflon and PEEK was made by a short piece of Viton tubing matching another sleeve. The joint between the PEEK tubing and the bioreactor insertion metal tube was made by a silicone sleeve.

With this circuit and the pump "pushing" the suspension into the measuring vessel, there was concern that the wheels of the pump might damage the cells. Viability, defined as the fraction of viable cells in the total cell number, was measured using ethidium bromide and fluorescein diacetate [7]. Table 1 shows that even when the pumping rate was increasesd to 60 cm^3 h^{-1}, the loss of cell viability was suffi-

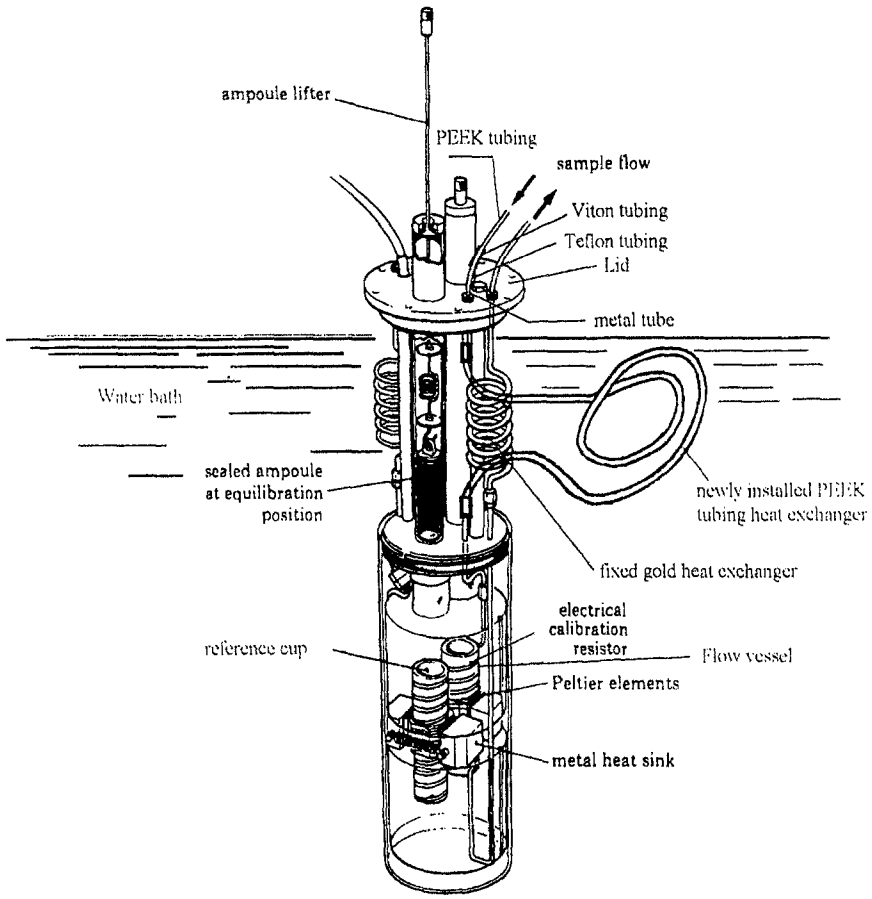

Fig. 2 The standard Thermometric flow microcalorimeter has been modified for downward flow through the measuring vessel which consists of gold tubing wound round the standard cup of the batch microcalorimeter. With the changed flow direction, it was necessary to install a new heat exchanger. This is simply a coil of PEEK tubing (1 mm I.D.) inserted between the lid and the cup. The previous gold heat exchanger is retained downstream of the measuring flow vessel. The reference cup normally contains a sealed ampoule which is not moved when the instrument is in regular use

ciently small to permit neglect of it. Nevertheless, the pump was placed in the efflux of the calorimeter (Fig. 1), i.e. the cells were "dragged" through the calorimeter so that there was no possibility of mechanical damage to the cells before the heat flow rate measurement. It was important to keep the temperature of the cell suspension in the tubing from the bioreactor to the calorimeter as close to 37°C as possible. Thus, the transmission tubing was coiled round the outlet tubing of the bioreactor water jacket (as shown in Fig. 1). This combination was then covered by a nonconductive, sponge-plastic pipe, but the temperature on the outside wall of the outlet jacket water tubing was ca. 35–36°C as measured by a thermopile thermometer.

Direction of flow in the measuring vessel of the calorimeter – downward or upward?

The gold flow vessel assembly (Fig. 2) installed in the ThermoMetric microcalorimeter has two components: (i) a heat exchanger of sufficient length to raise the temperature of the flowing liquid from normal room temperature (~20 °C) accurately to the measurement temperature (37.0± 0.02 °C for animal cells), and (ii) a measuring vessel between the thermopiles, nominally of 0.6 cm^3. The system is designed for an upward path of the liquid in the flow vessel so that any bubbles formed for whatever reason cannot stay longer in the vessel than the residence time of the liquid. This minimizes the physical disturbance which can produce significant heat effects in chemical determinations [8]. The manufacturer advises that the maximum flow rate to maintain the temperature in the measuring vessel exactly the same as that in the calorimeter water bath is 35 $cm^3\ h^{-1}$.

Table 1 The influence of the flow rate of a peristaltic pump on the recovery and viability of CHO 320 cells in the effluent.
The peristaltic pump uses Viton tubing (I.D. 1.0 mm). The PEEK tubing was adopted in the flowing line except the joints between the Viton and the PEEK. The CHO 320 cells were maintained in suspension with a suitable agitation speed. The internal volume of the flow line was ca. 5 cm^3. Each test for flow rate was performed within 15–20 min

Flow rate/ $cm^3\ h^{-1}$	CHO 320 cells in the effluent	
	Recovery/%	Viability/%
23.5	83.0	95.7
29.3	84.7	95.2
41.4	85.1	95.0
54.0	86.6	92.9
60.0	90.7	92.1

However, it was observed that the use of this flow mode often led to overestimation of the heat dissipation with respect to the cell density in the bioreactor vessel. Results given in Table 1 indicated that cells tended to accumulate in the flow vessel or within the tubing of the calorimetric cylinder. The slower the flow rate, the greater the cell number concentration in the system. Since cells are slightly heavier than most culture media, they sediment in a liquid of low turbulence. With a flow rate within the range of 23.5 to 60.0 $cm^3\ h^{-1}$, Reynolds Number $Re_D = 6–17 << 1800$. This clearly shows that the moving liquid in the transmission line is in laminar flow. It is very likely that in this hydraulic condition the weight of many individual cells can be balanced by the hydraulic pressure of laminar flow, resulting in the accumulation of the cells in the flow vessel. To overcome this problem, the following two possibilities have been tested: (a) increase the flow rate and (b) use an air/liquid mixture as described by Larsson *et al.* for a yeast suspension [9]. As shown in Table 1, even with a flow rate up to 60 $cm^3\ h^{-1}$ which is much higher than the rate suggested by the manufacturer, it is still not possible to "wash away" the cells ac-

cumulated in the flow lines. Adding air into the flow of the suspension also caused a difficulty since, at the necessary amplification (30 μW) for dilute suspensions of cells, the intentionally created bubbles give rise to intolerable noise.

Finally the conventional upward flow was changed to downwards and this led to success. The modified flow vessel assembly is shown in Fig. 2. Since the soft 24-carat gold heat exchanger in this unit cannot be touched without damage, it could not be utilized as an upstream heat exchanger in the new configuration. To overcome this restriction, a small section of the original stainless steel return tubing

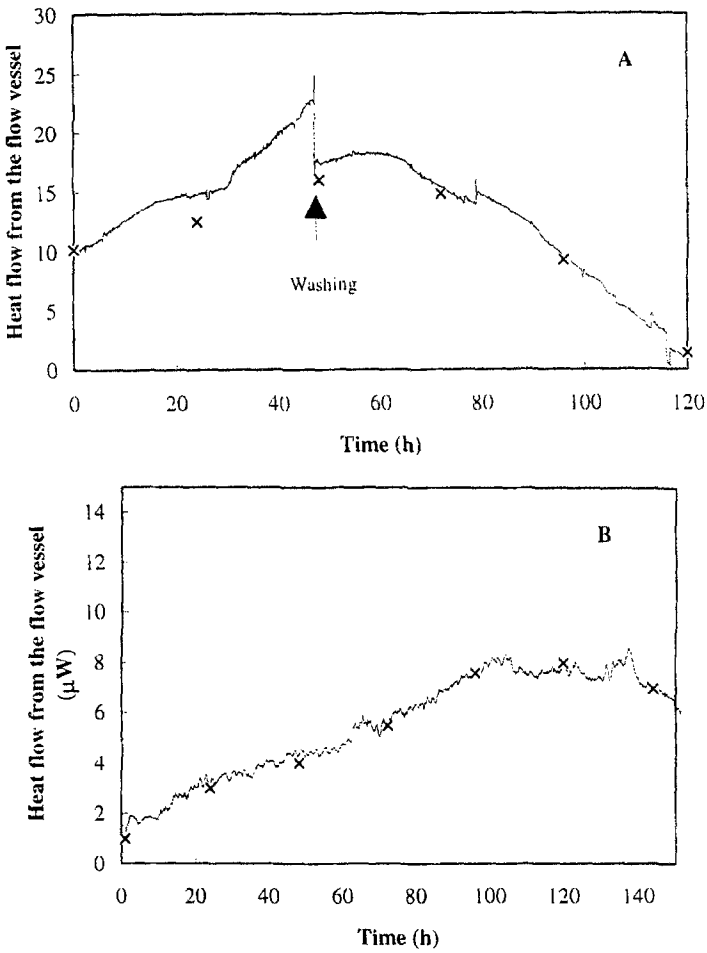

Fig. 3 A comparison of on-line heat flow rate measurements using upward flow and downward flow in the flow vessel of the microcalorimeter. (A) In an experiment the on-line heat flow rate measurement using conventional upward flow was compared with off-line measurement using an insertion vessel. (B) In another experiment the on-line heat flow rate measurement using downward flow was compared with off-line measurement using an insertion vessel. On-line heat flow rate with flow vessel (—), off-line heat flow rate with an insertion vessel (×)

(distal to the measuring vessel) was removed and replaced by a long piece (ca. 0.5 m) of PEEK tubing to form the new heat exchanger. This was connected directly with the spiral flow vessel giving the flow a downward direction. These changes essentially solved the cell accumulation problem. A comparison of the two flow directions in terms of the analogue signal is given in Fig. 3. The signal noise did not appear to increase when switching from upward to downward flow. Even so, a continuous internal washing system, in parallel to the continuous flow tubing, has been installed as a further protection system. The intention was that, if cell accumulation occurs, then this facility can be used to "sweep" the sedimented cells in a downward direction (Fig. 1). It was utilized to check that there was no overestimation. To run this facility, the designed external loop for the cell suspension from the bioreactor to the calorimeter was interrupted by suitable PEEK valves. Sterilized fresh medium was sucked from a source into the flow vessel assembly at 130 cm^3 h^{-1} using a second peristaltic pump (LKB-Produkter AB, Sweden). The layout for this arrangement has been pictured in Fig. 1. This "cleaning" procedure lasts for 10–15 min and

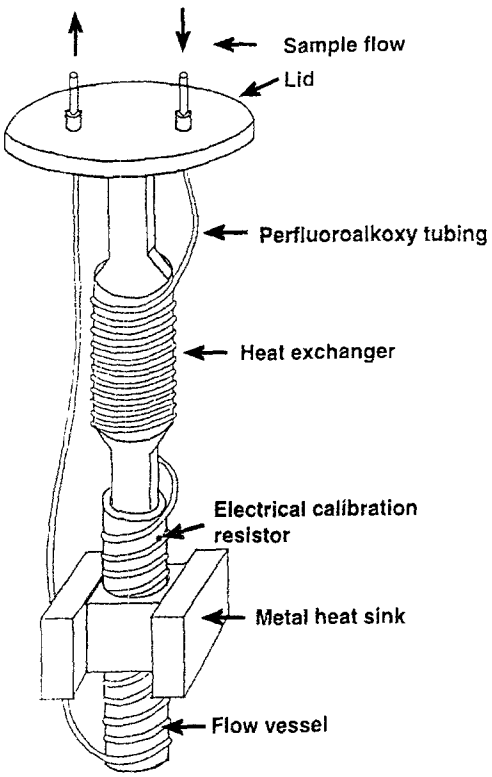

Fig. 4 A mock-up of a newly designed microcalorimetric flow vessel. The flow line is a continuous length of Perfluoroalkoxy tubing (0.96 mm I.D.) which has a very low gaseous diffusivity. The heat exchanger consists of 26 turns of the tubing (volume 2.3 cm^3) and below it is the measurement vessel (0.38 cm^3) wound round the outside of the cup. The calibration heater sits alongside in the same groove

afterwards the flow system was switched back to cell suspension. In comparison with a previous heat flow curve, a lower balanced heat flow would indicate that there was cell accumulation.

Apart from the on-line heat flow measurement using the gold flow vessel, on-line estimations were made using a 4-cm^3 perfusion unit [10]. This essentially acts as an insertion vessel (ThermoMetric 2250-series micro reaction systems) with a modified turbine stirrer [11]. To perform this measurement, a 3 cm^3 sample of cell suspension was taken directly from the bioreactor at the required time and immediately transferred into the pre-warmed, 4-cm^3 stainless steel vessel with the 1 cm^3 head space acting as a reservoir of oxygen for the cells. The interior of the turbine shaft was saturated with distilled water daily to avoid the large endothermic enthalpy of vaporization of the test liquid. After sealing the vessel, the unit was inserted into the chimney of the calorimeter (with amplification range 100 μW) step by step as advised by the manufacturer. Cell-free medium was used to obtain the baseline of this calorimeter channel. It was observed that although the value for this line could vary from day to day, the full scale of the heat flow rate obtained during electrical calibration was stable for weeks.

As a further step to avoid the limitation of the present flow vessel assembly, a new flow vessel has been designed and a mock-up for a microcalorimeter is shown in Fig. 4. The coiled heat exchanger is designed for downward flow. The transmission tubing is made from a continuous length of Perfluoroalkoxy tubing (0.96 mm I.D.) and thus there are no joints. This feature is not only beneficial to reduce cell accumulation from conventional suspensions, but is also suitable for circulation of suspensions of macroporous microcarrier beads containing cells. The value of microcarriers is that they allow cell cultures to achieve high densities.

Chemical calibration

The importance of a chemical calibration has been stressed by Wadsö for many years [12]. This need is particularly acute for flow vessels because their geometry and their relationship to the calibration heater makes electric calibration highly suspect. The measurement of on-line heat flow rate of CHO 320 cells in continuous circulation offers, therefore, a good example to illustrate the necessity for chemical calibration, since an accurate measurement of heat flow is directly associated with the thermal volume of the flow vessel.

The exothermic hydrolysis of triacetin in an imidazole-acetic buffer was used for the chemical calibration of the flow vessel [13]. The data for the heat flow rate was retrieved on-line by a PC using the software DatLab AQ (Cyclobios, Innsbruck, Austria). With the aid of a data processing program, Oroboros Datagraf 2.1 (Oroboros, Innsbruck, Austria), the data was then transformed into ASCII file for further treatment. It could take more than 1 h to achieve a stable value. In the initial period of time the trace was noisy and thus it was not included in the subsequent analysis using the least-square method. All the data for a stabilized reaction was correlated as the following kinetic reaction,

$$\hat{\Phi} = \hat{a} - \hat{b}t + \hat{c}t^2 \tag{1a}$$

For the flow vessel used in this work for a particular condition, the following relationship was obtained (with correlation coefficient $r^2=0.9529$),

$$\hat{\Phi} = 27.31 - 1.233\times10^{-4}t + 9.232\times10^{-10}t^2 \tag{1b}$$

The known kinetics of the same reaction can be generally expressed by,

$$\Phi = a - bt + ct^2 \tag{2a}$$

and for the corresponding experimental condition this was given [13] previously as

$$\Phi = 21.80 - 7.9\times10^{-5}t + 3.5\times10^{-10}t^2 \tag{2b}$$

where Φ is heat flow (μW) per gram of triacetin solution, $\hat{\Phi}$ is the heat flow rate (μW) from the triacetin solution contained in the measurement vessel, t is time (unit: s), a, b, c, $\hat{a}$, $\hat{b}$ and $\hat{c}$ are the corresponding coefficients for these kinetic equations.

Theoretically all of the three pairs of coefficients can be used to calculate the effective thermal volume of the flow vessel. Practically the coefficients c and $\hat{c}$ is most sensitive to both experimental and correlation errors and the coefficients a, $\hat{a}$ are least subject to these errors. For this reason, the effective thermal volume should be calculated by:

$$V_{\text{eff}} = \frac{\hat{a}}{a\rho} \tag{3}$$

where ρ is the density of the triacetin solution at 37°C as documented in [13]. For the flow vessel at 35 cm^3 h^{-1} and 37°C the measured effective thermal volume is 0.77 cm^3 compared with actual physical volume given by the manufacturer of 0.6 cm^3. Note that the difference between them is more than 20%. This example thus highlights the significance of chemical calibration towards absolute values of heat flow in calorimetry. In many cases such as the calculation of calorimetric respirometric ratio [14], it is necessary to obtain the absolute heat flow rate.

A thermodynamic system

To quantify an energy (enthalpy) balance for a cultured living system, an important issue is to choose correctly a suitable thermodynamic system. By doing so properly, both conceptual understanding and mathematical treatments could be simplified to some extent. Von Stockar *et al.* [15] considered this question in depth and proposed the boundary of a system based primarily on the consideration of a bench-scale calorimeter. In that case, the calorimeter in fact was an intact bioreactor and so it is very natural to draw the system boundary around the bioreactor entity. It is important to decide upon the boundary of the working system reported in this work and shown in Fig. 1. Since the calorimeter used is physically separated from the bioreactor, there exist at least two possibilities for thermodynamic systems relevant

to the requirement for an enthalpy balance: (i) the system boundary encloses both the bioreactor and the flow vessel assembly of the calorimeter (not the calorimeter itself, and (ii) the system just surrounds the flow vessel of the microcalorimeter. In either case the boundary must be infinitely thin at the wall of the flow vessel itself. When only this vessel is considered as a thermodynamic system, the advantage is that this system is virtually a closed one since the medium composition in the input and the output can be regarded as negligibly different; but the disadvantage is that it cannot involve the exchange of gases between the liquid and gaseous phases. For this reason, the working system for enthalpy balance has been chosen to enclose both the bioreactor and the flow vessel of the calorimeter. This is shown in Fig. 1 as a dotted boundary.

For the liquid, if we ignore the irreversibly lost energy accompanying the suspension flow and the various motions of the cells, and express heat flux in terms of unit amount of biomass, the enthalpy balance leads to:

$$V_{sys} X \Delta_t H_X J_{X,r} = \Phi \tag{4}$$

where V_{sys} is the system volume and so the effective thermal volume of the flow vessel of the calorimeter, X is a form of cell mass concentration, $J_{X,r}$ is the reaction flux in terms of unit amount of biomass and $\Delta_r H_X$ is the molar enthalpy of the growth reaction in terms of unit amount of cell mass, Φ is the volumic heat flow, dQ/dt .

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