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A calorimetric flow vessel optimised for measuring the metabolic activity of animal cells

Y.H. Guan, P.C. Lloyd, R.B. Kemp*

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

Abstract

The heat flux of animal cells growing in vitro is a direct measurement of their specific metabolic activity because the majority of the substrate Gibbs energy is dissipated as heat with only a small quantity being dissipated as entropy conserved in the form of biomass. Heat flux can be used as a variable to monitor the growth of engineered cells producing heterologous proteins in large-scale, industrial culture. Indeed, since heat flux is a function of metabolism, it can be used as the control variable in fed-batch culture. Until now, monitoring a culture by circulating a cell suspension to a standard thermometric TAM flow calorimeter has been compromised by direction of flow, slow maximum flow rate, narrow bore tubing and small volume of the measuring vessel. A new twin flow module for the TAM calorimeter is described which is optimised for use with cell suspensions as well as for cells growing on microcarrier beads. It consists of a continuous length of stainless steel tubing of 1.5 mm ID which forms the transmission lines, the heat exchangers and the coiled measuring vessel nominally of 1 cm^3 in volume. The heat exchangers are of sufficient volume to permit fast pumping rates (<200 $cm^3\ h^{-1}$) while the increased size of the vessel allows for a lower detection limit. The module is designed for downward flow through this vessel. It also has a calibration heater (nominal 50 Ω resister) between the fine heat exchanger and the measuring vessel. The detector is downstream of this vessel and consists of a thermopile operating on the heat conduction principle. The flow module was chemically calibrated by the exothermic hydrolysis of triacetin in imidazole–acetate buffer. At 100 $cm^3\ h^{-1}$, the effective thermal volume was found to 1.05 cm^3.

For validation, the experiments were on the growth of CHO320 cells producing recombinant interferon-γ in batch culture and under the steady state conditions of continuous culture. The on-line heat flow rate was smoothed by the moving-average technique and showed an increase proportional to cell growth during the batch phase and only small changes after setting up the continuous culture. Repeated experiments gave consistent results without signs of erratic recordings and thus indicated that the specially designed flow module can indeed realise the potential of heat flux to monitor metabolic activity in batch and perfusion cultures together with being a control variable in fed-batch cultures.

Keywords: Flow calorimetry; Animal cells; Metabolic activity; Heat flow rate; Triacetin hydrolysis

1. Introduction

Ever since the calorimetric experiments of Lavoisier on a guinea pig more than 200 years ago, it has been recognised that the heat flowing from living matter is a measure of its chemical activity. This was found to be principally respiration, which was considered to be a very slow combustion, ''to be precise'' to the words of Lavoisier and Laplace. It was only after Gaskell some 80 years later that this combustion was considered to be ''katabolic'' activity.

*Corresponding author. Tel.: +44-1970-622333; fax: +44-1970-633350; e-mail: rbk@aber.ac.uk

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It is now recognised that the rate of heat flow is one of the most appropriate measurements of metabolic activity of the organism or its parts, down to the cellular or even the sub-cellular levels [1]. The earliest thermal studies of microscopic living material involved bacteria and yeast (see the review by Battley [2]) and embrace some astonishing experiments, including the gigantic fermentation of Dubrunfaut that required 12 400 l of molasses! Although Pasteur first recognised that thermogenesis by micro-organisms was a consequence of ''life'', it was left to Rubner at the beginning of this century to quantify the amount of heat produced by organisms using a calorimeter built by him to a published design [3]. From there came the long lineage of calorimetric studies of micro-organisms comprehensively surveyed by Battley [2] and also reviewed over the years by, for instance, Forrest [4], Belaich [5], Miles et al. [6] and Gustafsson [7]. As evidenced by the most recent work [8], a great deal is now known about the thermobiochemistry of particular micro-organisms. This makes it possible to evaluate the performance and efficiency of industrial fermentation processes as a result of experimentation by bench scale, heat compensation calorimetry [9] as well as to probe the mechanism of such delicate metabolic mechanisms as the glycolytic oscillations in yeast using a high resolution microcalorimeter [10].

As earlier stated, an animal was the first subject for a calorimetric experiment. The physician, Mayer, knew of Lavoisier's work and had observed that the colour of the venous blood of Europeans he bled in Java was a brighter red colour than in the cooler climes of Northern Europe [11]. This led him to deduce a figure for the mechanical equivalent of heat from experiments with a working horse in a paper pulp factory and then to propose the First Law (arguably the Second, with Carnot's work in 1924 leading to the chronological First!) of Thermodynamics in 1842. Naturally, he felt that the Law would apply to living systems but it took 60 years until Rubner [12] provided the proof by a 45-day enthalpy balance experiment with a dog in a respiration calorimeter. Of course, it was not possible to isolate cells from warm-blooded animals at that time but the medical imperative of physiology drove scientists to measure the heat produced by human and animal organs and tissues, with muscle being an early candidate for study (see the classical paper by A.V. Hill in 1912 [13]). Over the next 50 years, Hill led the field in discovering that much of the heat was derived from the splitting of creatine phosphate, the means of storing energy in a resting muscle prior to contraction. Using the enthalpy balance method, it was then found that a significant quantity of the heat produced by muscle could not be held to account by chemical reactions, 21% in the case of frog sartorius muscle [14]. The source of the ''unexplained heat'' was the entropy changes taking place during the conformational alterations to muscle proteins and the movement of ions, principally Ca^{2+}, during the contraction cycle [15]. Entropy change during the movement of ions, rather than chemical reactions, was also the probable reason for much of the heat produced by nerves on excitation [16]. While the syncytial muscle fibre and neurons can be regarded as huge cells, the bulk of them in an organism is either in solid tissue or bathed in plasma as blood cells. It was not until the commercial production of microcalorimeters and the advocacy by Wadsö [17] for their analytical use that there was any substantial progress with measuring the heat production of normal-sized animal cells. While they were able to record small changes in heat evolved from cells, these calorimeters were designed to exploit the heat conduction principle and thus measure the instantaneous rate of heat flow ($\Phi = dQ/dt$) from the sample through a detector consisting of thermoelectric elements to the heat sink [18,19].

The fact that the heat flow rate is a reflection of metabolic activity stems from non-equilibrium thermodynamics in which the growth so characteristic of living systems can be described as being dissipative, in most cases, rather than as irreversible because the majority of the high quality Gibbs energy is dissipated as lower quality heat with only a small quantity being dissipated as entropy in terms of biomass [20]. Of course, the early experiments on cells were at a phenomenological level, not concerned with thermodynamics, but treating them as ''guinea pigs'' in the Lavoisier fashion. Although the majority of cells in vivo are adherent to one another and thus, when grown in vitro are maintained adherent to a substratum, many studies on such cells have been prevented from reaching a satisfactory conclusion by technical difficulties (see reviews in [21–23]). With hindsight, the most sensible solution to these problems is to use an appropriate type of microcarrier bead in a stirred

insertion vessel [24–26]. In general, such studies have been confined to the use of HEPES as the buffer to avoid problems of carbon dioxide loss in the traditional carbonate/bicarbonate buffer systems. In many cases, however, cells have less metabolic activity in a low concentration of the bicarbonate ion because there is a mitochondrial requirement for this ion [20,27,28]. It has also been shown that the heat flow rate can vary markedly with variations in extracellular pH probably because the latter has an effect on cytosolic pH and hence glycolysis [29]. This effect is possibly via an Na^+,H^+-ATPase symporter. Nevertheless, there have been some useful studies over the years, particularly on the short-term effects of anti-proliferative agents on cancer cells [25,30].

Many more of the studies on cells isolated from the body have been conducted using flow calorimeters [19–28]. These originated 30 years ago with the commercial exploitation of a design by Wadsö [31] that used a gold flow-through vessel (1 mm ID). The most obvious candidate in the early work was blood and this, together with its various fractions, has received considerable attention over the years as the medical profession strives to understand human disease [32,33]. Extension of this type of research to the use of dissociated cells from solid tissues has proved difficult owing to their inherent stickiness and the consequent tendency to form aggregates that cause blockages in the flow system [21,22]. In more recent years, however, hybridoma and genetically-engineered animal cells have been adapted to grow in suspension so that they can be maintained in industrial bioreactors. The problem that biochemical engineers have with such vessels is to be able to monitor the metabolic processes within them because there is a shortage of suitable biosensors [34]. An answer to this requirement was to pump cells from a bioreactor to a flow microcalorimeter through thermostated, PEEK transmission tubing of low gas diffusivity and then measure on-line but ex situ the heat flow rate and hence the overall metabolic activity [27,35]. In thermodynamic terms, this is an open system with the boundary as an infinitely thin layer in the walls of the bioreactor, transmission tubing and flow measuring vessel.

Initial studies using a Thermometric 2277 Thermal Activity Monitor (TAM, Thermometric AB, Järfälla, Sweden) with gold measuring vessel (1 mm ID, capacity 0.6 cm^3) for cultures of CHO320 cells producing recombinant interferon-γ [36], indicated that blockages frequently occurred due to the fact that the cells, although supposedly suspension-adapted, formed conspicuous aggregates. They then became trapped in the flow lines. The system was designed by the manufacturer for upward flow through the measuring vessel to minimise physical disturbance due to air bubbles [37]. At the relatively slow, laminar flow rates ($\leq$35 $cm^3\,h^{-1}$) permissible in this set-up, it appeared that there was an accumulation of cells and aggregates [35]. It was decided, therefore, to change the direction of flow through the measuring vessel to downwards (see Fig. 1). This met with reasonable success for relatively short-term batch cultures of up to $\sim$100 h [27,35].

Industrial needs for bioreactors extend beyond simple, batch cultures to those involving perfusion and fed-batch approaches using both cells in suspension and those growing on microcarriers [38]. For flow calorimetry, the problem of long-term cultures is the appearance of macroscopic debris which can block narrow bore tubing of 1 mm (ID). Microcarriers can be as large as 0.28 mm in diameter and therefore (i) cause blockages, especially at junctions; (ii) are not drawn quantitatively into the transmission tubing from the bioreactor at the maximum permissible pumping rate of 35 $cm^3\,h^{-1}$. This second difficulty is due to effects described by the Stokes–Einstein Law which relates the resisting force offered by a fluid of given dynamic viscosity to the diameter of a sphere, in this case of the microcarrier. The answer to these problems could be a continuous loop of tubing between the bioreactor and the calorimeter with a wider bore than the standard measuring vessel and with sufficient length of heat exchanger to allow a faster pumping rate. In fact, wider bore tubing itself would give an enhanced pumping rate for the same linear flow velocity as used for the standard flow vessel and thus cause similar, minimal damage to the cells in suspension or on microcarriers. Preliminary tests indicated that tubing of 1.5 mm (ID) would serve these requirements and give a pumping rate of 79–97 $cm^3\,h^{-1}$ with an unchanged linear velocity. Since there are no highly reactive chemical species in the cell suspension which would necessitate the retention of gold for the flow vessel, it was decided to use high quality, stainless steel for all the tubing and retain Viton tubing for the

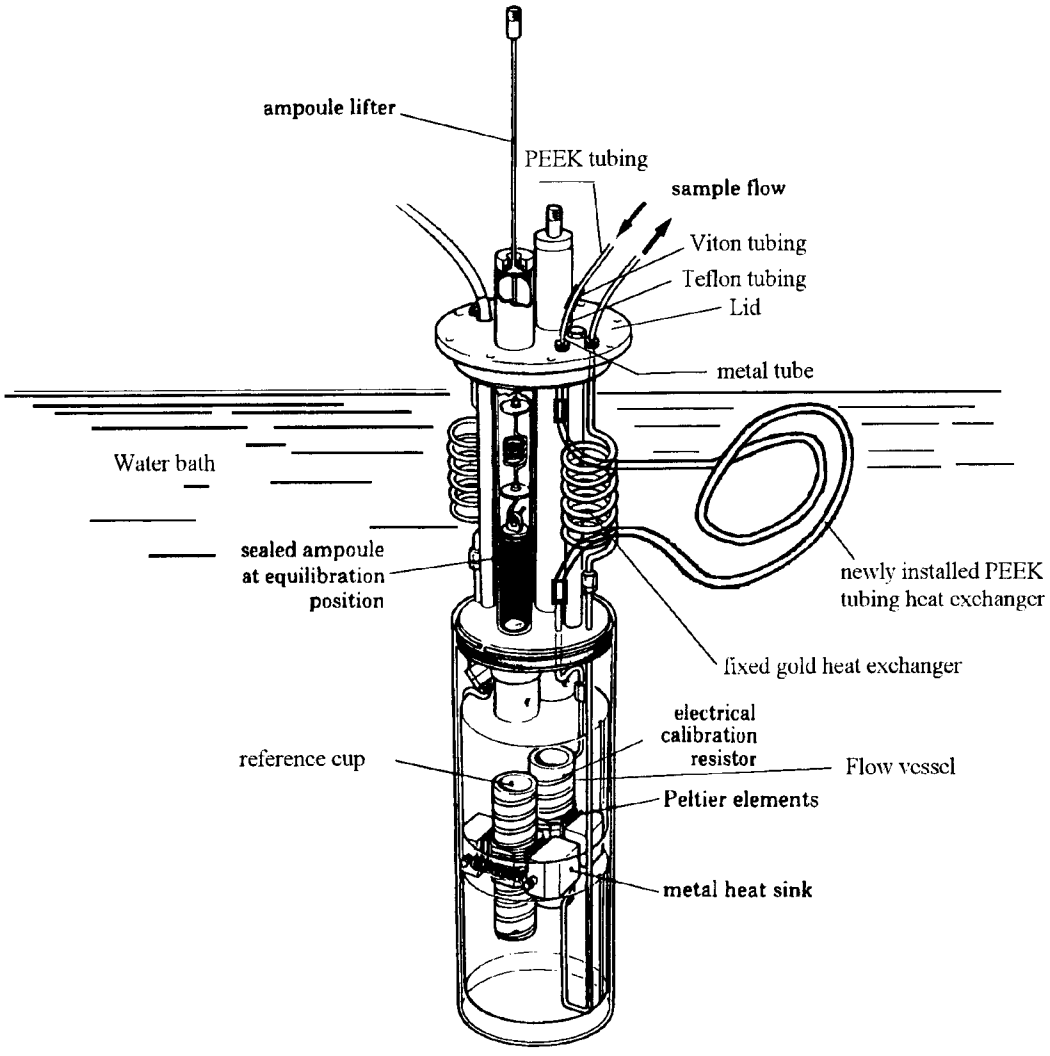

Fig. 1. The standard thermometric flow microcalorimeter has been modified for downward flow through the measuring vessel which consists of gold tubing wound round the standard cup of the batch microcalorimeter. With the changed flow direction, it was necessary to install a new heat exchanger. This is simply a coil of PEEK tubing (1 mm ID) 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 (Reproduced from Ref. [35] with permission of the publisher).

pump. The differences in specification between the standard flow vessel and the new one are summarised in Table 1.

This paper describes a new flow module for the TAM kindly constructed by Thermometric AB. It outlines the chemical calibration of it and shows some early results with it to give the heat flow rate of cells in batch and continuous culture. The possible future applications of the new flow vessel will be discussed with respect to its importance as a biosensor for industrial bioreactors.

2. Design of new TAM flow calorimeter module

The general plan of the bioreactor/microcalorimeter combination has been shown previously [20,35] but a simplified version of it is depicted in Fig. 2. In it, the

Table 1
A comparison of the standard flow vessel with one built specifically to measure the heat flow rate of animal cells suspended in medium and flowing through it

Parts	Standard	New
Transmission line		
Material	PEEK	Stainless steel
Internal diameter	1.0 mm	1.5 mm
Number of joints	>3	Nil
Heat exchanger material	Gold	Stainless steel
Measuring vessel		
Material	Gold	Stainless steel
Nominal volume	0.6 cm^3	1 cm^3
Flow direction	Upward	Downward

stainless steel tubing acts as the transmission line, as well as the flow line through the new module. It originates at an upstream joint proximal to the bioreactor and ends close to the Jubile peristaltic pump (Model JU/88 W. H.J. Guldener, 8047 Zürich, Switzerland) which is downstream from the flow module acting to ''pull'' the suspension through the tubing and return it to the bioreactor. This position of the pump is a precaution to minimise any possible damage to the cells by the pump rollers. This is a ''safety first'' approach because previous studies have shown that repeated cycles through the pump at 35 $cm^3\ h^{-1}$ does not cause any loss of viability [35].

Connection of the bioreactor to the flow calorimeter can only occur once the cell suspension is in place and, because of possible contamination, it must involve quick action in the presence of 70% alcohol. A simple, but effective design, based on the tips for autoclavable pipettes is shown in Fig. 3. The Viton tubing links from the bioreactor stainless steel outlet, via the ''quick-fix'' connector, to the stainless steel tubing of the flow module. This then enters the module within the TAM (see Fig. 2). The core of the module is the continuing stainless steel tubing that carries the cell suspension through it (Fig. 4), and firstly, acts as the preheat exchanger in contact with the water of the thermostated bath at 37°C (±0.01). It then passes into the stainless steel container where it constitutes the fine heat exchanger. Downstream, the tubing makes contact with a 50 Ω resister acting as the calibration heater, before being tightly coiled to become the measuring vessel with a nominal capacity of 1 cm^3. It is then directed through the detector before leaving the container and the module to terminate adjacent to the pump, for which the tubing is Viton (1.3 mm ID). The detector is a thermopile consisting of semi-conducting thermoelectric plates [18] positioned between the heat source (measuring vessel) and the solid, stainless steel heat sink (see Fig. 5). It can be seen that the thermopile is rather more remote from the measuring vessel than in conventional heat conduction

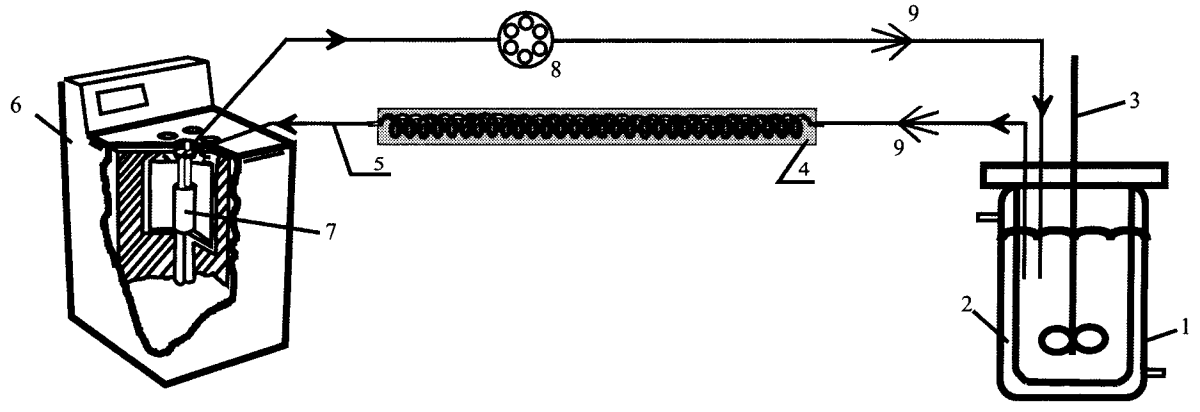

Fig. 2. A schematic drawing of the relationship between the bioreactor for growing animal cells and the Thermometric calorimeter with custom-designed flow module to monitor on-line and ex situ the heat flow rate of the cells as an index of their metabolic activity. (1) Bioreactor; (2) jacket water for temperature control in the bioreactor; (3) agitator; (4) Nichrome wire wrapped around insulated stainless steel tubing to reduce heat loss in transit of cell suspension from bioreactor to calorimeter; (5) Viton tubing (1.3 mm ID); (6) 4-channel Thermometric microcalorimeter (TAM); (7) new stainless steel flow vessel assembly (see Figs. 4 and 5 for details); (8) peristaltic pump; (9) quick fix connector (see Fig. 3 for details).

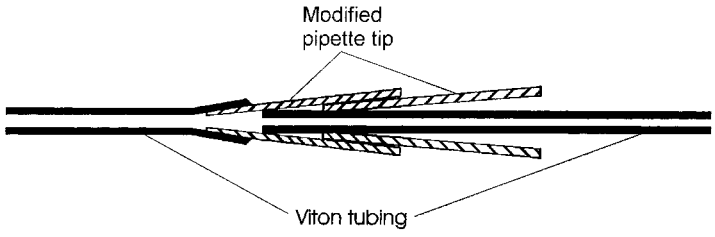

Fig. 3. An illustration of the quick fix joint for connecting tubing from and to the bioreactor.

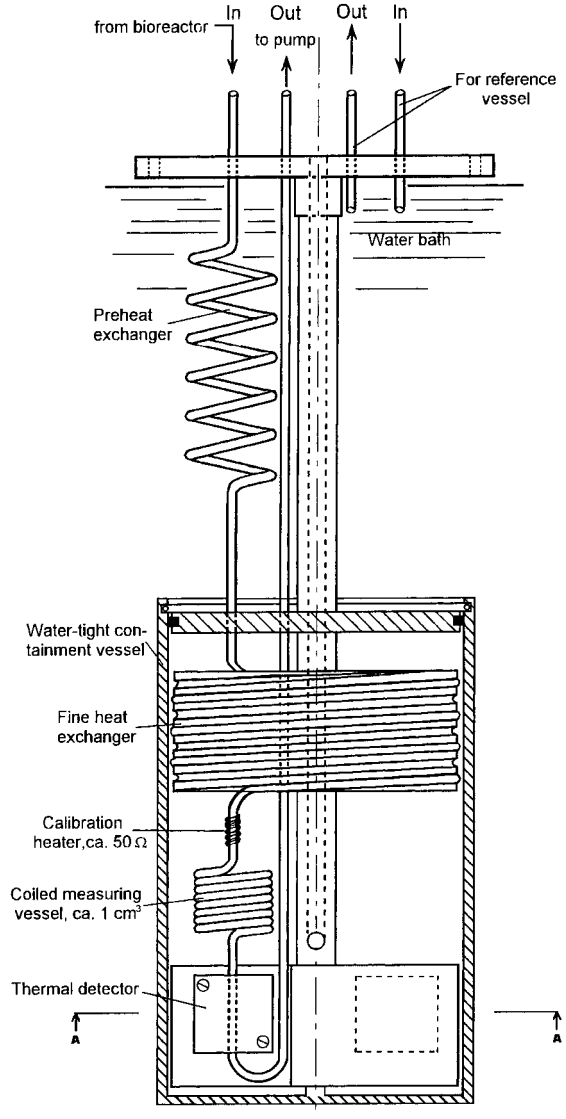

Fig. 4. A pictorial description of new flow module for Thermometric 2277 Thermal Activity Monitor. The section labelled by A–A is illustrated in Fig. 5.

calorimeters but this is to make it possible to accommodate the bulky, coiled vessel and the detector in the available air space (see Figs. 4 and 5). The overall dimensions of the cylinder module are circumscribed by the physical size of the TAM. In line with other instruments of its type, the new flow calorimeter measures a property proportional to the total heat flow between the measuring vessel and the heat sink. The proportionality is found by calculation but it is still necessary to calibrate the calorimeter both electrically and chemically under the exact operating conditions of the experiment (e.g. temperature, rate of pumping, type of medium, etc.). Although not shown on the drawing, the module also contains an identical reference channel. For ideal calorimetric experiments, medium without cells must be circulated round this

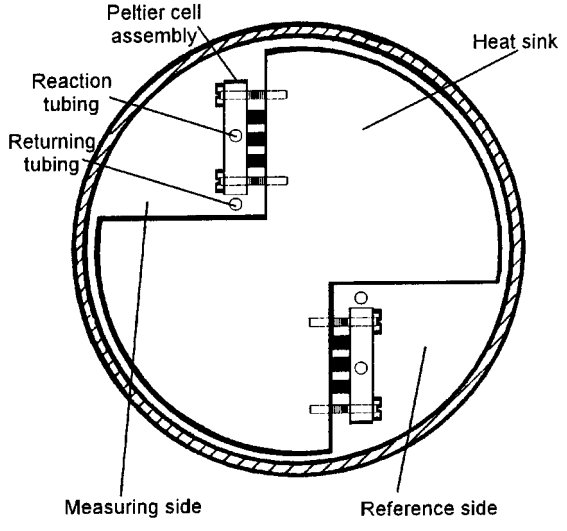

Fig. 5. A sectional look into the layout of the thermal detector in the new flow module for Thermometric 2277 Thermal Activity Monitor. The vertical position of this section is labelled in Fig. 4 as A–A.

assemblage at exactly the same temperature and flow rate as for the test system. Temperature equilibration of the cell suspension and medium in the flow lines is aided by wrapping Nichrome wire as heater elements around the transmission tubing upstream from the flow module cylinder.

3. Calibration

It is important to highlight that, unlike many flow calorimeters [18,19,31], the calibration heater in the new device is positioned prior to the coiled, measuring vessel (see Fig. 4) and considerably distal to the detector. Thus, it is vital that calibration is performed with the appropriate culture medium being circulated round the system under the exactly same conditions as in the experiment. The standard electrical calibration is carried out with flowing medium at the beginning and the end of each experiment.

Chemical calibration must be performed before any experiments are initiated so that meaningful data can be obtained from the instrument. As Lavoisier implied, metabolic activity is a relatively slow process, and therefore, the chemical reaction should be similar. This is particularly the case for flow-through vessels for which there is, of course, a finite time between the reaction vessel outside the calorimeter and the measuring vessel inside it. Even at 100 $cm^3\ h^{-1}$, the residence time from the bioreactor to the measuring vessel is 3.6 min. Chen and Wadsö [39] first demonstrated the use of the hydrolysis of triacetin in an imidazole–acetate buffer for this purpose and, although its use for non-stirred ampoules has been exhaustively investigated [40], there have been few reports of its application to flow calorimetry [35]. The triacetin mixture (1 dm^3) was circulated from the 3 dm^3 Applicon Bioreactor (F.T. Applicon, Tewksbury, Gloucs., UK) through the calorimetric flow vessel at 100 $cm^3\ h^{-1}$ for 24 h, the signal for heat flow rate was digitised in the Applicon Biocontroller and the data were retrieved on-line and displayed using Applicon BioXpert software, v.1.10 (Fig. 6). The trace was characterised by initial instability (see [35]) and thus the first part was ignored for the subsequent analysis by the method of least-squares in which the data for the stabilised hydrolysis was correlated as a second-order thermal

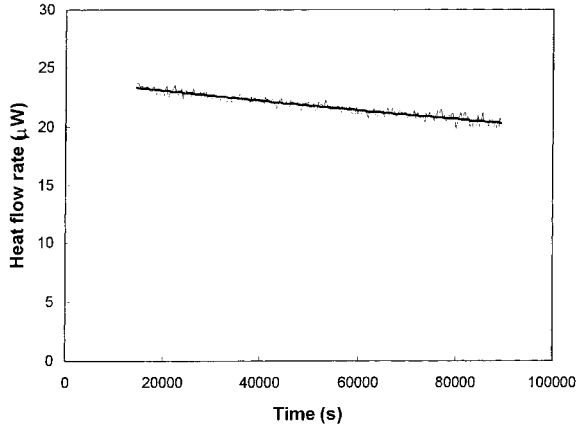

Fig. 6. Chemical calibration of the new flow vessel using triacetin at 37°C and at 100 ml h^{-1} pumping rate.

kinetic reaction:

$$\hat{\Phi} = \hat{a} - \hat{b}t + \hat{c}t^2 = 23.97 - 5 \times 10^{-5}t + 6 \times 10^{-11}, \quad (1)$$

where $\hat{\Phi}$ is the measured heat flow rate (μW), $\hat{a}$, $\hat{b}$ and $\hat{c}$ are the corresponding thermal rate coefficients for the kinetic equation and t is time (s).

In comparison with a reliable kinetic equation measured previously for 1 g of the triacetin solution at the same condition:

$$\Phi = a - bt + ct^2 = 21.81 - 7.9 \times 10^{-5}t + 3.5 \times 10^{-10}, \quad (2)$$

where Φ is heat flow rate for 1 g of a triacetin solution, a, b and c are reference thermal rate coefficients of the reaction.

From Eqs. (1) and (2), the effective thermal volume (V_{eff}) was calculated by

$$V_{\text{eff}} = \frac{\hat{a}}{a\rho} = 1.05\ (cm^3) \quad (3)$$

where ρ is the density of the triacetin solution at 37°C (see [39]). This result means that the thermal and nominal volumes were very similar at the chosen pumping rate.

4. Initial calorimetric experiments

The first objective for research involving the new TAM flow module will be to study the metabolic

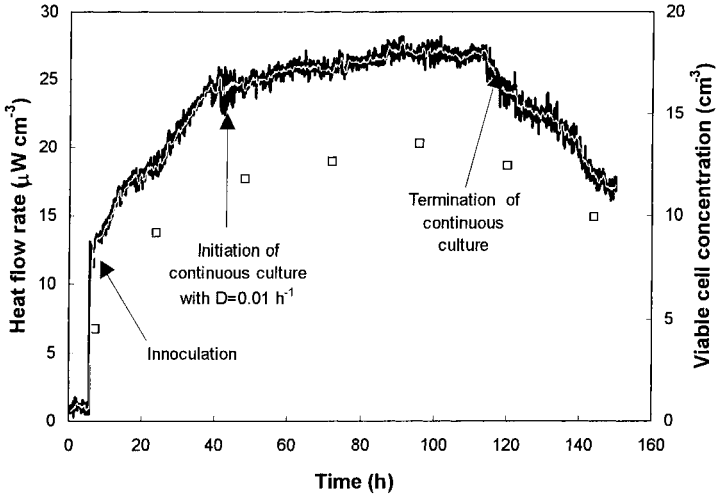

Fig. 7. A comparison of the on-line heat flow rate with the viable cell density (□) in a bioreactor culture initially in a batch culture, and then switched to a continuous one, and finally terminated medium feeding. The black curve (–) is for raw signal of heat flow rate and the white curve is an on-line smoothed heat flow rate by 30 min moving-average treatment.

activity of CHO320 cells in steady state achieved by the use of continuous cultures. The initial experiments described here are to validate the new flow module and the opportunity was taken to obtain preliminary data for continuous cultures. In order to obtain the relatively high cell density required for this type of culture, at first the cells must be grown as a batch from an initial inoculum of 4.5×10^5 cm^{-3} (see Fig. 7) in an RPMI medium improved from that used in earlier work [27,36], essentially by the addition of more amino acids, including glutamine, and a lipid supplement [41]. Note that the high initial cell concentration at inoculation was for shortening the batch culture time so that a continuous culture can be started earlier. After 42 h, the cells had grown to ca. 1.2×10^6 cm^{-3}, whereupon the continuous culture was set at an initial dilution rate of 0.01 h^{-1}. The metabolism of animal cells is slow compared to that of micro-organisms and thus establishment of the steady state took another 40 h during which the cell density had slowly increased to 1.35×10^6 cm^{-3}. This viable cell number was then maintained for the following 30 h, at which time medium dilution was discontinued by switching off the bioreactor pump. This resulted in a sharp decline in the number of viable cells, probably owing to the fact that one or more of the substrates became exhausted and limited cellular metabolic activity to an extent that caused cell death. The heat flow rate was recorded on-line throughout this time (Fig. 7). The current version of the Applicon BioXpert software (v.1.10.030) includes a facility for smoothing by the moving-average technique and, when set at 30 min, this reduced scatter without introducing systematic errors (see "white space" in Fig. 7). It will be noted that heat flow rate increased during the batch phase proportionally to the increase in cell numbers. The rate was even higher during the initial part of the continuous culture because a metabolic steady state was not achieved until 80 h. The aim of these experiments was not to do the definitive continuous culture tests but to ensure that the flow module gave reliable results. The representative recording of heat flow rate seen in Fig. 7 gave no sign of blockages or other irregularities while, at the same time, pointed the way to the potential of the flow module in studying the metabolic activity of cells by calorimetry.

5. Conclusions

A flow calorimeter has been designed and constructed to a specification that makes it suitable for studies of animal cell metabolism in all forms of culture, but particularly for the long-term fed-batch and perfusion cultures associated with cells engineered to produce such heterologous proteins as

monoclonal antibodies and glycoproteins. Debris tends to build-up in these cultures but it does not block the wide-bore tubing of the new device. It should also prove its worth in obtaining data for the metabolic activity of cells grown on the comparatively large microcarrier beads. The heat exchangers have been designed to equilibrate liquid flowing through the tubing at 100 $cm^3 h^{-1}$, meaning that the cells are rapidly returned to the carefully controlled, bulk conditions of the bioreactor within 4.5 min. This is a distinct advantage in biothermochemical studies of metabolism for which it has to be assumed that the environmental conditions are the same in the flow vessel as in the bioreactor. An earlier investigation with the standard TAM flow vessel [35] showed that cells had a viability of >95% at 35–40 $cm^3 h^{-1}$. For the same linear velocity, the wider bore tubing of the new flow module would allow a pumping rate of ca. 100 $cm^3 h^{-1}$. The nominal volume of the measuring vessel for the dedicated flow module is 67% larger than that of the standard vessel, meaning that the practical detection limit is lowered in terms of cell number concentration. The effective (thermal) volume was shown to be very close to the nominal volume, giving credibility to the soundness of the design. This is particularly useful for the early stages of batch cultures and for fast pumping rates which tend to increase background noise.

To our knowledge, this is the first report of heat flow rate data for animal cells in continuous culture. The findings are being repeated and analysed at the present time, with the aid of off-line data for the major metabolites. They will be valuable in setting the most appropriate conditions for the demonstration of fed-batch culture of CHO320 cells with on-line heat flux data being used to control the feeding of the major nutrients, glucose and glutamine, in the appropriate quantities. The use of on-line heat flux as the controlling variable is particularly valuable for biotechnology because it gives a continuous measure of overall metabolic activity in a rigorous physicochemical relationship.

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