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Heat flux and the calorimetric–respirometric ratio as measures of catabolic flux in mammalian cells

R.B. Kemp*, Y. Guan

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

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Abstract

It is advocated that cellular heat flow rate ($\Phi = dQ/dt$, where Q is heat) be expressed as an intensive quantity specific to cell size (X) and termed heat flux ($J_{\Phi/X}$). It has been the practice to cite such data on a 'per cell' basis, but it would be preferable to use biomass (cellular volume or mass). This quantity is shown to be a measure of metabolic activity and, more accurately, catabolic rate coupled to the demand for ATP in anabolic processes and work in the cell. Recent developments in flow microcalorimetry and dielectric spectroscopy reveal that heat flux can be measured on-line, with the potential of industrial use as a control variable in the growth of hybridoma and genetically engineered cells. This is because the enthalpy change of growth can be regarded as a unique kind of stoichiometric coefficient directly related to the mass coefficients in the growth reaction. This can be verified by an enthalpy balance comparing data for material fluxes of catabolites with the value for heat flux. Information revealed by the stoichiometric growth equation can be used to improve medium design.

The ratio of heat flux to oxygen consumption (flux) is known as the calorimetric–respirometric (CR) ratio. It detects anaerobic processes when the value is more negative than −450 (±5%) kJ mol^{-1} O_2. These processes are found in cells growing under fully aerobic conditions, because glycolysis provides biosynthetic precursors with lactate as the by-product. It is suggested that the CR ratio would be a powerful on-line control variable for the growth of animal cells in bioreactors. © 1997 Elsevier Science B.V.

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1. Introduction

Since the introduction, thirty years ago [1], of user-friendly batch and flow heat conduction microcalorimeters, many scientists have investigated the heat dissipation of mammalian cells in vitro (reviewed in [2–5]). Some of the types of cell which have been studied, lived in suspension in the body (e.g. blood tissue and spermatozoa), others required to be dissociated from excised solid tissue (e.g. adipocytes, fibroblasts from muscle tissue and hepatocytes), while many were established cell lines of normal and neoplastic cells; and more latterly, hybridoma and genetically engineered cells used to produce medically important heterologous proteins such as antibodies, enzymes, and immunomodulatory glycoproteins. A good number of these studies prove that it is possible to obtain data for the heat dissipation per cell [4,6–8], and these are listed in Table 1. Of course, the figures

*Corresponding author. Tel.: +44 1970 622333; fax: +44 1970 622350; e-mail: rbk@aber.ac.uk, yyg@aber.ac.uk.

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Table 1
Heat flux $J_{\Phi/N}$ for mammalian cells with ranges or standard deviations for some cell types based on data in Refs. [4,6–8] which gives the original sources

Cell type	$J_{\Phi/N}$/pW per cell
Human erythrocytes	0.01
Human platelets	0.06
Bovine sperm	1.3 ± 0.1
Human neutrophils	2.5 ± 0.3
Human lymphocytes	5
Horse lymphocytes	8
Human T-lymphoma	8 ± 1
3T3 mouse fibroblasts	17
Chinese hamster ovary (CHO) 320 (recombinant)	~23
kB	25
Vero	27 ± 2
Mouse lymphocyte hybridoma	30–50
HeLa-53G	31.2
Mouse macrophage hybridoma, 2C11-12	32 ± 2
LS-L929 fibroblasts	34 ± 3
Chinese hamster ovary (CHO)-K1	38
Human foreskin fibroblast	40 ± 10
Rat white adipocytes	40
Human white adipocytes	49 ± 15
Human melanoma, H1477	80
Human keratinocytes	83 ± 12
Hamster brown adipocytes	110
SV-K14 (transformed) keratinocytes	134 ± 35
Rat hepatocytes	329 ± 13

mean little as they stand – except to show a ten-thousand-fold range, from human erythrocytes (0.01 pW per cell – see [5]) to rat hepatocytes (329 pW per cell – see [4]). Some of the possible reasons for the differences are given in the next section.

2. Reasons for differences in cellular heat production

2.1. Physical environment

2.1.1. Conditions which cause trauma

- Pump rates too high (or too low) in flow calorimetry.
- Inappropriate material for pump and transmission tubing (e.g. high gaseous permeability).
- Damage to cells by pump mechanism.
- Vessels constructed of incompatible materials.
- Batch vessel with stirrers badly designed or turning at the wrong rate of revolution (high or low).
- Batch vessels with too little or no stirring for suspension cells.

2.1.2. Inaccurate/non-existent electrical and chemical calibration

Electrical calibration should be carried out daily and the characteristics for the heat flow from the material under investigation in the measuring vessel to the heat sink through the thermopile(s) must be determined by chemical means [9].

The list of possible physical factors in this section is brief and not exclusive, but Wadsö has given details in many of his reviews (see, for instance, [9,10]; also [11] gives useful information about perfusion vessels).

2.2. Physiological environment

2.2.1. Oxygen

Some scientists do not realize that this gas has a low solubility in water (210.2 μmol dm^{-3} in air-saturated pure water [12]) and even less in physiological solutions, because of the salting-out effect. This is particularly important, of course, in closed vessels (especially without an air-space) but is also the cause of hypoxia (towards anoxia) in unstirred layers of cells, leading to the so-called crowding (Uriah) effect [4,13] in which the physiological conditions are so poor (low oxygen tension, low or high pH, ammonia, etc.) that they cause a decrease in metabolic activity.

2.2.2. pH

Cells originating in the homeostatic conditions that exist in tissues cannot tolerate a pH outside the 7.2 ± 0.2 range, without deleterious effect. Besides the physical damage, it appears that heat dissipation by the cells is very sensitive to changes in pH [13]. Since this has been found for fresh erythrocytes which have no mitochondria, as well as for several cell lines, it was reasoned that changes in the bulk phase pH affected glycolysis [14], presumably by altering cytosolic pH through the Na^+–H^+ exchange [15,16]. One of the reasons why lactate is harmful to cells [17] could be that it lowers the pH of the medium when excreted from them.

2.2.3. Osmotic pressure

When the data for Table 1 were being collated from original papers, it was noted that some of the results were obtained using cells in very simple, hypo-osmotic media. It is essential that the incubation medium remains iso-osmotic in the 260–280 mOsm range.

2.2.4. Substrates

With the exclusion of endogenous metabolism (see [18] for research on this metabolism in sperm), it is important for correct cell homeostasis to supply cells with substrates. Glucose alone may be sufficient for supplying energy, but mammalian cells do not grow unless glutamine is available to them [19]. In fact, this amino acid appears to be the major energy source for some cell types, for instance HeLa cells [20], though a reciprocal relationship between it and glucose [21] seems a more likely situation. Cultured cells are mostly grown in the presence of serum which contains fatty acids among other compounds. At least some mammalian cells utilize these for energy production in preference to carbohydrates [22]. Serum is replaced by specific nutrients in complex, defined media for hybridoma and genetically engineered cells producing heterologous proteins for pharmaceutical use. This was done originally because serum proteins complicate the task of isolating and purifying the target protein. The benefit, however, is that now a medium of known composition can be optimized rationally for cell growth and protein production.

All the factors in this section will affect metabolic activity and, consequently, heat dissipation.

2.2.5. Waste products

The major catabolic products, which are toxic to cells, are ammonia [23] and lactate, the by-products of glutaminolysis [24] and glycolysis. Ammonia has adverse effects on intracellular organelles, cytosolic and lysosomal enzyme activity and vesicle transport [23]. It freely diffuses across plasma and intracellular membranes in the unprotonated form (NH_3 rather than NH_4^+) and, inter alia, affects the glycosylation process in the synthesis of glycoprotein within the endoplasmic reticulum and Golgi apparatus. It is well known that glutamine decays rather quickly in culture media at 37°C and this adds to the amount of ammonia which can diffuse into the cell, the remainder of which, in some cell types at least, results from glutamine conversion to glutamate at the plasma membrane during the transport of the amino acid [25].

Lactate is freely transported through the plasma membrane. As already stated, it has an effect on metabolic activity as a result of decreasing bulk phase pH. This could be the reason why the dreaded Mycoplasma causes harm when they adhere to cells because they are glycolytic 'engines' [3].

2.3. Size

It is obvious that, at a given level of metabolic activity, heat dissipation varies with average individual cell size. For cells in suspension, size is usually equated with volume (V) and so Kemp [4] has urged calorimetrists to cite heat dissipation as this size-intensive quantity. There are techniques available now to measure the volume of cells, both in the culture vessel (e.g. on-line laser nephalometry and dielectric spectroscopy [26]) and outside it (e.g. off-line Coulter counting and flow cytometry). These assessments of volume are directly related to mass (m) providing, of course, that the mass density is constant, $\rho = m/V$. This may not be the case at different phases of the cell cycle [22] and it is, surely, affected by the osmotic pressure of the medium. It is not known definitively which of the two measures, mass or volume, most closely reflects metabolic activity, but the validity of the relationship is probably dependent on the location of the predominant catabolic pathways – the cytosol or mitochondria – for a particular cell line.

In a famous work, Kleiber [27] showed that the 'specific oxygen flux' of animals decreased with their increasing size. From data for the heat production of cultured Vero cells as their density on Cytodex 1 beads increased with growth [28], it was shown that Kleiber's Rule appeared to apply to cells in terms of protein mass [6]. Thus, cell density (number concentration) may be an important factor in determining the value for heat dissipation. In general, as cells become confluent on the substratum (in this case, the surface of the solid beads), the rate of growth slows down because, after division, the daughters are arrested in early G_1 phase when they are small, and become 'resting' cells (G_0). Kemp and Gnaiger [6] also reworked data for lymphocyte hybridoma cells [29] to show that the increasingly exothermic

calorimetric–respirometric (CR) ratio [30], which corresponded to a greater density of these cells, was due to lactate accumulation. In a more exhaustive study of Uriah crowding in relation to Kleiber's Rule, Singer et al. [13] used human renal carcinoma cells to reinforce the point that "decreasing heat output with increasing cell number in a calorimetric ampoule can be due to a continuous transition from aerobiosis in a 'monolayer-like' suspension to increasingly anaerobic conditions in a 'crowded' ampoule." Carcinoma cells undergo rapid growth and, as first observed by Warburg in 1926 [31], produce much lactate. It seems possible that the apparent manifestation of Kleiber's Rule in cell populations is due to environmental effects rather than to an allometric relationship per se.

2.4. Metabolism

As it appears that heat dissipation by living cells is completely explained by catabolic processes (see later), there is only a need to consider respiration (citric acid cycle and oxidative phosphorylation), the pentose phosphate pathway (PPP), β-oxidation, glycolysis and glutaminolysis. Of these, respiration, β-oxidation and the part of glutaminolysis which involves the citric acid cycle (α-ketoglutarate to malate – see Refs. [19,24,25]) are mitochondrial, together with any oxidation of pyruvate as the product of the PPP and glutaminolysis. Obviously, cells without mitochondria, for instance blood cells such as neutrophils [32] and erythrocytes [33], can only use the cytosolic pathways of PPP and glycolysis, eventually producing lactate from pyruvate to yield NAD^+. In phagocytic cells which kill bacteria with hydrogen peroxide and metabolites of oxygen [32,34–36], the PPP gives the appearance of total glucose combustion with, by Hess' Law, the same enthalpy change (ΔH) as respiration but without side reactions. The molar reaction enthalpy, $\Delta_r H_m$, is -2814 kJ mol^{-1}, where the subscripts 'r' and 'm' represent the complete and molar reactions, respectively. Cells deriving ATP from glycolysis alone consume 15 times more glucose (Glc) than those obtaining it by respiration, but produce slightly less heat per mol ATP. To illustrate the point for cells incubated in a glucose-containing bicarbonate-buffered medium, assume an ATP stoichiometric coupling coefficient ($\nu_{ATP/Glc}$) of 36 (but see the latest estimate of Hinkle et al. [37], who give 30 mol ATP per mol Glc) in respiration and a coefficient ($\nu_{ATP/Glc}$) of 2 in glycolysis with the molar reaction enthalpies ($\Delta_r H_B$, where subscript 'B' indicates a stoichiometric number of unity) calculated from Wilholt [38] and the heats of neutralization from Gnaiger and Kemp [30]. Then, the heat yield for ATP by respiration is $-3044/36 = -84.5$ kJ mol^{-1} ATP and that by glycolysis is $-161/2 = -80.5$ kJ mol^{-1} ATP.

2.5. Growth

In populations of cells in vitro, growth implies an increase in density (number concentration). Many types of fully differentiated cells have lost their ability to divide, and the maintenance of tissue and organ size relies on replacement by stem cells in vivo [22]. This is true of blood cells, including the types of white cell (neutrophils and lymphocytes) listed in Table 1. These only require energy for maintenance and, in most cases, the turnover of macromolecules (principally RNA and protein – mammalian erythrocytes and all platelets have no turnover). Their heat dissipation is small compared with that of actively growing cells, whether established cell lines (e.g. kB, HeLa and Vero cells – see Table 1) or differentiated white cells, hybridized with malignant cells to give 'immortalized' hybridoma cells (e.g. lymphocyte and macrophage hybridomas). Some tissue cells in vivo retain the ability to change from being in the resting phase (G_0) of the cell cycle to undertaking active growth in vitro. These multiply by duplication rather than by being replaced by stem cells and have a high rate of heat dissipation, as for instance hepatocytes (see Table 1).

All the foregoing factors have an influence on the rate of heat dissipation; nevertheless, it is necessary to define its nature under conditions where the physical parameters (e.g. pH, osmolarity, temperature, oxygen tension) are controlled and the physiological conditions appropriate for the cells.

3. Sources for heat dissipation

Metabolism can be split into the catabolic and anabolic half-reactions which, in conservative metabolism, are coupled together by the ATP cycle [39] of synthesis (phosphorylation) and degradation

(dephosphorylation). At steady state, synthesis balances ATP utilization. In a coupled system, the rate of catabolism is determined by the demand for ATP in anabolic processes and cellular work (mostly in active transport pumps, intracellular vesicular transport and cell movement). The latter may be conveniently termed maintenance and, for the present purpose, is considered to be constant. Excluded from maintenance, however, is the turnover of essential macromolecules which, incidentally, have half-lives of different lengths, depending on their nature and function. 'Resting' tissue cells (G_0) in vivo have a continuous requirement for ATP in maintenance and macromolecular turnover. Under normoxic conditions, the energy for maintenance is supplied by oxidative phosphorylation to give ATP. These requirements are relatively small and, therefore, the rate of the catabolic half-reaction is slow. For continually duplicating and stem cells, in vivo or tissue cells, naturally (tumour) or artificially (in the laboratory) transformed into continually dividing cells ('immortalized'), the requirement for ATP in anabolic processes is considerable and is reflected in a high catabolic rate. It is needed in order to rearrange the available electrons of substrates into cellular macromolecules (biomass). Under the homeostatically controlled environment, in vivo, and appropriate conditions, in vitro, these requirements in highly regulated mammalian cells should be met by oxidative phosphorylation. However, cells growing under fully aerobic conditions produce lactate [3–5,19–21,24,25,34–36], a substance normally associated with the anaerobic process of glycolysis. They do so because growing cells require biosynthetic precursors [19,24,40] not available/usable exogenously, which are produced in glutaminolysis, glycolysis and the pentose phosphate pathway. The 'non-biosynthetic' products of the first two of these pathways are lactate and NAD^+ from pyruvate. Incorporation of the de novo precursors into macromolecules requires ATP, of course, and some is provided by these pathways. Besides the glycolysis ATP stoichiometric coupling coefficient of 2, glutaminolysis has a coefficient ($\nu_{ATP/Glc}$) of 8.5 (see [37,41]). It appears highly likely that most of the increased metabolic activity of growing cells is due to the need for (i) – biosynthetic precursors additional to those exogenously supplied in the medium; and (ii) – ATP for building these precursors into macromolecules.

The heat dissipated by the aforementioned combined catabolic pathways can be measured by a calorimeter. The rate of dissipation is therefore a direct measure of the rate of metabolic activity which, in turn, is a direct reflection of the rate of growth in steady state (in practical terms, also in the quasi-steady state). The calorimetric measurement of cellular heat dissipation is carried out at constant temperature and pressure with no net cellular work being performed, because the processes of maintaining life are irreversible; hence, this heat dissipation equals the enthalpy change of the metabolic processes ($\Delta_{met}H$).

The overall enthalpy change of metabolism contains the two components – catabolism (cat) and anabolism (ana) – in fully coupled conservative metabolism at steady state, in which the endothermic ATP production completely balances its exothermic hydrolysis – each of which has an enthalpy change, $\Delta_{cat}H$ and $\Delta_{ana}H$. This division can be precisely, and completely made in terms of substrates and end products, provided there is allowance for clearly stated simplifications (see later). Most estimates of the anabolic half-reaction indicate that its value is very close to zero [42,43], and even the highest figure quoted for the aerobic metabolism of glucose $\Delta_{ana}H$ is only 3% $\Delta_{met}H$ [44]. Therefore, the enthalpy change of catabolism approximates that of metabolism, which means that the rate of cellular heat dissipation measures the rate of the catabolic processes.

For fully coupled, aerobic metabolism under steady-state conditions, it is possible to calculate ATP turnover from heat dissipation [45,46], because the Gibbs energy change for glucose consumed aerobically is $-2903\ kJ\ mol^{-1}$; very close to the enthalpy change of $-2814\ kJ\ mol^{-1}$. This is because the entropy change for oxidation of sugars, amino acids and fatty acids is small [47]. Assuming the stoichiometric coupling coefficient (ν_{ATP/O_2}) is 6, the ATP yield from glucose oxidation is $12.8\ \mu mol\ ATP\ J^{-1}$ [46]. Although this approach cannot be extended to anaerobic catabolic pathways in general, it can be applied to the conversion of glucose to lactate, which also has a small entropy change. For lactate excreted into a bicarbonate buffer [30], the enthalpy change is $-153\ kJ\ mol^{-1}$ and the ATP yield is $-15.9\ \mu mol\ ATP\ J^{-1}$ [46]. It should be stressed that the accuracy of

these yields depends on tight coupling [48]. Any slip will result in lower actual values – and how is this to be measured?

4. Heat flux

Heat dissipation from the catabolic processes in the cell is measured as heat flow (rate), $\Phi = dQ/dt$ in heat conduction calorimeters [10]. This is an extensive quantity, which is usually made size-specific simply by division, using cell number (N) in the measuring vessel to give heat flux $J_{\Phi/N}$. However, as already stated, this ignores the actual *size* (biomass, X) of the cell and prevents mechanistic comparisons. From the thermobiochemical standpoint, division of heat flow (rate) by dry weight of biomass ($J_{\Phi/X}$) would be ideal, as it is dry biomass that is used for elemental analyses and measurements of combustion enthalpies [49]. Obtaining dry biomass is a time-consuming and difficult procedure for animal cells, because they are nearly always (and increasingly) in a complex medium containing proteins (often produced by them by design) which must be washed away from delicate cells that easily rupture, causing serious errors. Biomass measured as protein suffers to an extent from the same problem; hence, an assessment of heat flux using numbers of cells as the measurement for size specificity might be preferable at this time.

As mentioned earlier, it is perfectly possible to measure the volume of animal cells off-line using, among other methods, flow cytometry [34] and dielectric spectroscopy [50,51]. Improvements, in recent years, to increase the sensitivity of the latter have allowed these methods to be used on-line in bench-scale bioreactors for growing mammalian cells [52]. In principle, the dielectric spectrometer measures the capacitance (C in farad, F) of the culture. This relies on the cells acting as charged particles. Capacitance has a complex relationship to the volume fraction of viable cells [50], the membrane potential of intact, dead cells being zero (no charge) because the permeability barrier has broken down to allow free passage of ions. However, for cells in bulk average constant volume, it is possible empirically to approximate capacitance [52,53] to,

$$\Delta C = \Delta C_0 + KN_v \quad (1)$$

where ΔC and C_0 are the capacitance increments for the β-dispersion of the medium with, and without cells, respectively, N_v the viable cell number and K a constant mainly determined by the type of cell and, it would appear, also by the physiological conditions. By measuring the change in capacitance due to the presence of cells ($C-C_0$) and combining it with continuous heat flow measurements, it should be possible to measure heat flux on-line. Since this is a relatively novel way to express heat flux, it will be illustrated by reference to a typical set of experiments performed in this laboratory [54].

The probe of a viable cell monitor (Aber, Aberystwyth, UK) was placed on-line in a 3-L bioreactor containing Chinese hamster ovary (CHO) 320 cells which have been genetically engineered to produce interferon-γ (IFN-γ). They grew over a 100 h period in a defined RPMI-based medium, buffered with 20 mM HEPES and 4 mM sodium bicarbonate [54]. The capacitance analogue signal was smoothed, using the moving average technique available on Excel software, and compared to discrete measurements of viable cell volume made using a calibrated Skatron laser-light flow cytometer with a low-angle light scattering detector [34] and employing fluorescein diacetate [55] as the viability stain. As will be seen in Fig. 1, the change in the capacitance signal was comparable to the increase in the biomass (cell volume) over the majority of the growth curve. Only at the lowest cell densities did the capacitance signal

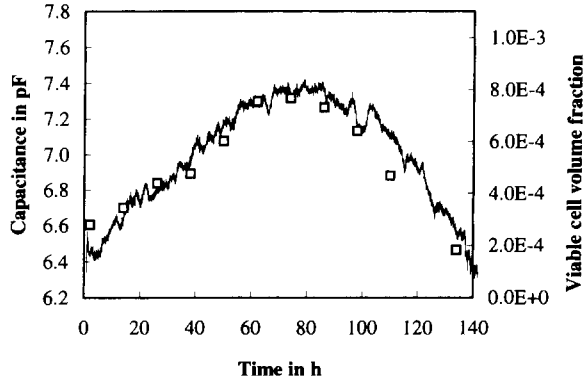

Fig. 1. Comparison of viable cell: (□) volume fraction in bulk medium estimated off-line; (–) with capacitance (pF) continuously measured on-line . These represent the cell density in suspension during the cultivation of CHO 320 cell line in an RPMI-based medium buffered with 20 mM HEPES and 4 mM bicarbonate.

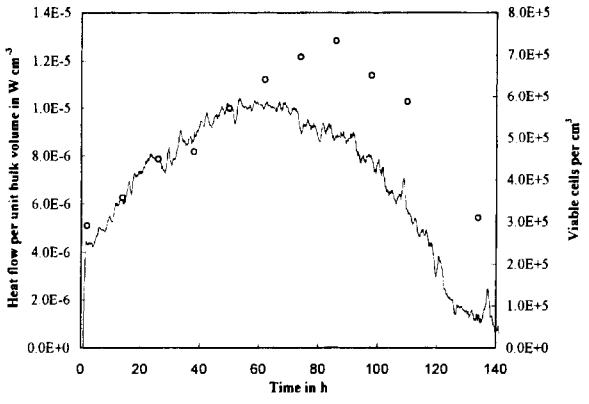

Fig. 2. The heat flow of growing cells measured on-line by the microcalorimeter and scaled to the unit bulk volume of RPMI 1640-based culture medium buffered with 20 mM HEPES and 4 mM bicarbonate (–). Estimates were made for the number of viable cells per cm^3 bulk volume (○) at discrete time intervals.

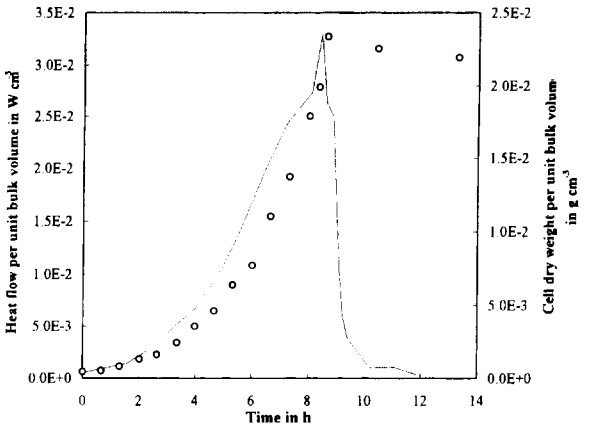

Fig. 3. A comparison of heat flow per unit bulk volume with the concentration of cell mass (as dry weight, ○) of *Kluyveromyces fragilis* NRRL1109 growing aerobically (adapted from [57]).

appear to over-estimate it. A statistical examination of data from several experiments revealed that the effective detection limit is 3.5×10^5 cells cm^{-3} [54]. The VCM probe is 2.5 cm in diameter and intended for measuring biomass in bench-scale and larger bioreactors containing cells which produce medically important, heterologous proteins. Since maximum productivity is important to the pharmaceutical industry, cells are only at such low densities during brief periods of time at cell inoculation. The detection limit is, then, no detriment to the use of the VCM to monitor biomass changes.

It was known that the heat flow of CHO 320 cells in culture only reflected the increase in cell numbers for a relatively small part of the growth curve (see Fig. 2; [53]), implying that heat flux ($J_{\Phi/N}$) in terms of cell number, decreased with time and that metabolic activity – more precisely catabolic flux – declined during the latter stages of growth in batch culture. Heat flow was measured continuously and, thus, had the potential to be used as a variable to monitor cell growth. Others had seen this possibility for yeast [56], and had adopted a protocol to use the extensive quantity, namely heat flow, alone as a control variable in fed-batch culture. This was possible because the organisms, with their relatively simple catabolic process, responded immediately to glucose exhaustion (Fig. 3). Unfortunately from this standpoint, animal cells have many possibilities when deprived of one carbon source to compensate with others and, at the typically low densities in suspension culture, give a weak, noisy heat flow signal which cannot be used as a control variable. For these reasons, it was decided to explore the possibility of size-specific heat flux as an on-line variable. Two techniques [26], laser-light nephalometry and dielectric spectroscopy, come to mind for on-line assessment of biomass. Only the latter was available in this laboratory, but then the software which would allow the two signals to be matched directly to give heat flux in terms of the unit change in capacitance, $J_{\Phi/C}$, is not available here (see Eq. (1)). Therefore, the ratio was calculated off-line as a continuous trace [53] and the data presented in

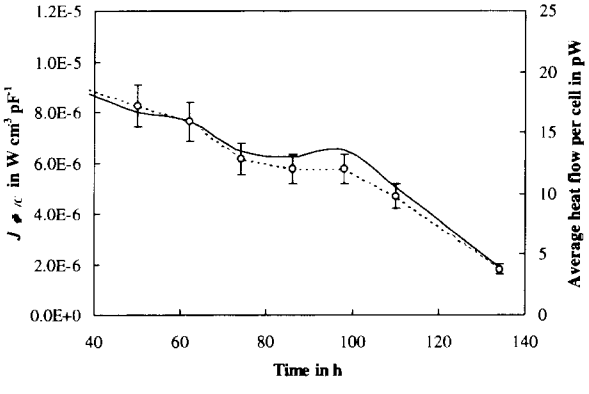

Fig. 4. On-line heat flux measurements (–) adjusted to per cm^3 bulk volume and, (○) heat flow per viable CHO 320 cell over 140 h of a batch culture. In the expression of $J_{\Phi/C}$, the values are given in terms of 1 cm^3 bulk medium volume.

Fig. 4, with heat flow data as a comparison. It will be seen that the two records of heat production gave similar results, providing assurance that heat production could be an on-line control variable.

Before determining the scope for using the heat flux variable in a control strategy to monitor cell growth in bioreactors, it is important to explain in the following paragraphs the reasons for the decline in heat flux while the cells are still in growth.

5. Catabolic processes

In reviews of thermochemical data for animals cells growing in vitro in defined media, Kemp [3–5] concluded that, although metabolism could not proceed without micronutrients, hormones and growth factors, the catabolic process was essentially a combination of glycolysis, oxidative phosphorylation, the pentose phosphate pathway and glutaminolysis. This could be summarized by the catabolic half-reaction,

$$\text{Glucose} + \text{Glutamine} + O_2 \rightarrow \text{Lactate} + CO_2 + NH_3 + H_2O + \Delta_{cat}H \quad (2)$$

The net growth of cells is irreversible, which means that the work term, δW, in the First Law is zero. Therefore, at the constant temperature, characteristic of cell cultures, and constant pressure of aeration,

$$Q_{T,P} = \Delta H \quad (3)$$

Hence, the enthalpies of the reactions summarized in Eq. (2) should balance the heat produced by the reaction in size-specific (X) terms (flux), $J_H/J_{\Phi/X} = 1$; this ratio is known as the enthalpy recovery. The enthalpy flux is,

$$J_H = \Delta_r H_B \cdot J_{B,r} \quad (4)$$

where $\Delta_r H_B$ is the molar reaction enthalpy and $J_{B,r}$ the reaction flux in which the subscript 'B' indicates that any given reaction stoichiometry is divided by ν_B such that the stoichiometric form is obtained with $|\nu_B| = 1$. The first part on the right-hand side of Eq. (4) is the sum of the molar enthalpies of species i under the cell culture conditions:

$$\Delta_r H_B = \sum_i (\nu_i H_i) \quad (5)$$

The second part on the right-hand side is:

$$J_{B,r} = \frac{d_r \xi}{X dt} \quad (6)$$

where X is the biomass and ξ the advancement of the reaction,

$$d_r \xi = \frac{d_r n_i}{\nu_i} \quad (7)$$

The enthalpy balance validates the description of the reaction. In cells engineered to produce target proteins (antibodies, enzymes, glycoproteins, etc.) during growth, Guan and Kemp [58] showed that the metabolic (growth) reaction is:

$$\nu_{s_1}[\text{Glucose}] + \nu_{s_2}[\text{Glutamine}] + \nu_o O_2 = [\text{Biomass}] + \nu_p[\text{product}] + \nu_L[\text{Lactate}] + \nu_c CO_2 + \nu_N NH_3 + \nu_H H_2O + \Delta_{met}H_X \quad (8)$$

where ν_i are the stoichiometric coefficients and $\Delta_{met}H_X$ the enthalpy change of the reaction. Under this description, the enthalpy recovery for 2C11-12 mouse macrophage hybridoma cells was found to be 1.05 [7,36,58].

For CHO 320 cells, where recombinant protein IFN-γ is the product in Eq. (8) ('ν_{IFN}[IFN-γ]'), changes in the major substrates and their products are shown in Fig. 5 [53,54]. It can be seen that glutamine was exhausted at 62 h, considerably before the total depletion of the glucose at 86 h. The cells continued to grow and produce IFN-γ in the absence of glutamine, but not glucose. The fluxes for these variables are catalogued in Table 2, where it can be seen that the decline in the crucial catabolites was reflected in decreasing heat flux. This is because the enthalpy change in Eq. (8) can be considered to be an extraordinary form of stoichiometric coefficient and thus in direct relation to the other coefficients. 'Extraordinary' implies that the normal definition of stoichiometry in terms of the relationship between masses has been extended, for this special purpose, to energy [58]. On this basis, heat flux monotonically decreased with the decline in the specific growth rate and the other metabolic fluxes (Fig. 6). This is the basis for believing that heat flux could be a control variable in bench/industrial scales of cell culture. Its decline usually indicates a paucity in the supply of glucose/

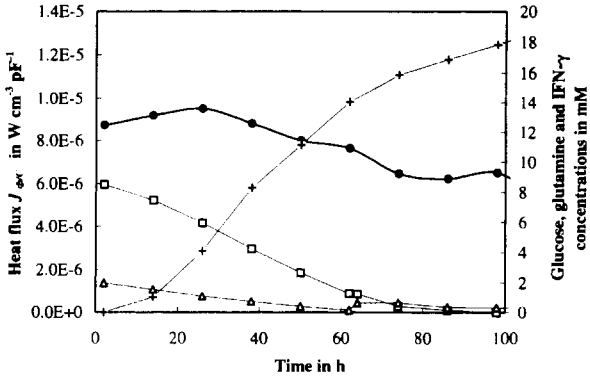

Fig. 5. On-line measurement of heat flux (•) adjusted to cm^3 bulk volume, with off-line assays of changes in the concentrations of (□) glucose, (△) glutamine, and (+) IFN-γ for a batch culture of CHO 320 cells. Note that feeding the cells additional glutamine after its total consumption by the cells failed to stimulate heat flux.

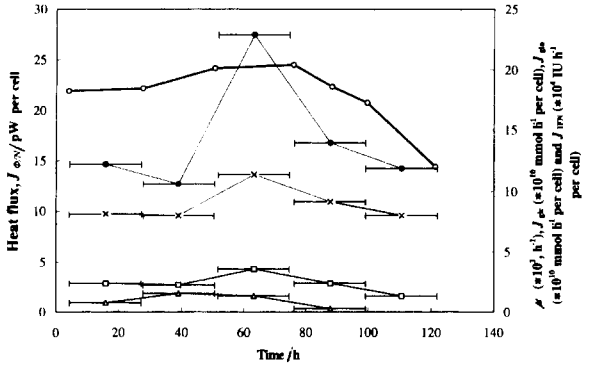

Fig. 6. Comparison of heat flux ($J_{\Phi/N}$) with fluxes of glucose (J_{glc}), glutamine (J_{gln}) and IFN-γ (J_{IFN}), as well as specific growth rate (μ) during the batch cultivation of CHO 320 cell line in suspension: (○) – heat flux; (□) – glucose flux; (△) – glutamine flux; (×) – IFN-γ flux; and (•) —specific growth rate. The bars indicate the period over which the discrete off-line measurements are made to give the individual average values for fluxes.

glutamine, which could form part of a control strategy in which the response would be to feed substrate(s) (fed-batch culture).

6. Heat flux and medium design

The exhaustion of one substrate before the others seen in Fig. 5 is a typical occurrence in cell culture because, in general, an empirical approach has been adopted in formulating culture medium, rather than a rational one based on demand by the cell. As already shown, the growth reaction for many cell types in culture is relatively simple. The stoichiometric catabolic half-reaction can be written from the data used in Table 2 at 51–76 h (see [58]),

$$C_6H_{12}O_6 + 0.084C_5H_{10}N_2O_3 + 0.64O_2 = 1.91C_3H_6O_3 + 0.68CO_2 + 0.17NH_3 + 0.43H_2O \quad (9)$$

Since heat flux is totally a reflection of catabolic flux, this reaction can be validated by a type of enthalpy balance different to that explained in Eqs. (4)–(7). In essence, the enthalpy flux of the half-reaction is determined from the difference between the standard enthalpies of formation ($\Delta_f H^\circ$) of the products and the reactants, together with the reaction flux. The figure is then compared with the heat flux. For the time period used to construct Eq. (9), this was 24 pW per cell and very similar to the calculated enthalpy flux of 24.2 pW per cell, an enthalpy recovery of 1.01. On this firm basis, from the available data it was possible to construct the growth reaction as follows:

$$C_6H_{12}O_6 + 0.377C_5H_{10}N_2O_3 + 0.44O_2 = 3.52CH_{2.0}O_{0.82}N_{0.18} + 1.2C_3H_6O_3 + 0.46CO_2 + 0.12NH_3 + 0.29H_2O \quad (10)$$

Table 2
Fluxes for the batch cultivation of CHO 320 cells producing IFN-γ in RPMI 1640-based medium buffered with 20 mM HEPES and 4 mM bicarbonate. Data given have 95% confidence.

Periods (h)	Fluxes			
	μ ($\times 10^{-2}$) (h^{-1})	J_{glc} ($\times 10^{-7}$) (mol h^{-1} per cell)	J_{gln} ($\times 10^{-7}$) (mol h^{-1} per cell)	J_{IFN} ($\times 10^{-4}$) (IU h^{-1} per cell)
26–38	1.9±0.2	3.1±0.3	0.7±0.1	7.6±0.7
38–50	1.7±0.2	2.5±0.3	0.5±0.05	4.6±0.5
50–62	0.7±0.1	1.9±0.3	0.4±0.03	4.0±0.3

The most obvious aspect, revealed by studying this reaction, is that the cellular requirement for glucose and glutamine at this particular stage in exponential cell growth (51–76 h) is 2.65 : 1, whereas the medium contains 5.5 : 1. Although other periods of time during growth shows slightly different ratios (see Ref. [58]), at no time did they actually approach the ratio in the medium. This explains why glutamine was depleted before glucose (see Fig. 5), and indicates a future strategy that the culture should be fed small amounts of the two substrates in the ratio appropriate for that period of growth. The emphasis is on the word 'small' – this is to allow optimal utilization of the substrates and avoid any risk of catabolite repression (the Crabtree effect). The instruction for feeding would arise from the decline in heat flux triggering the control action.

Once the growth reaction and catabolic half-reaction is known, the anabolic half-reaction can be calculated by subtraction. This reaction is not exclusive because it does not include exogenous amino acids or the breakdown products of hormones and growth factors, all of which become incorporated in cellular macromolecules. Nevertheless, the information contained in the expression for anabolic half-reaction could be valuable in considerations of efficiency.

7. Calorimetric–respirometric ratio

Lavoisier's theory [59] states that respiration is a slow combustion. Thornton [60] showed that the heat of combustion of a wide range of organic compounds averaged −111 kJ per equivalent of available electrons, a value which is now known as Thornton's Rule. It was obtained using bomb calorimetry ($\Delta_c H'$), where no work can be performed, so it is the maximum enthalpy change for complete oxidation of carbon compounds. Gnaiger and Kemp [30], among others, took a different approach, arriving at values for carbohydrates, amino acids and fatty acids from known standard molar enthalpies of formation ($\Delta_f H^\circ$). By these calculations, the oxycaloric equivalent ($\Delta_k H_{O_2}$), as it is known, was shown to be −450 (±5%) kJ mol^{-1} O_2 for all biologically useful organic substrates [30]. Bearing in mind that this value holds when work (and efficiency) is zero, any experimental values more exothermic than the equivalent must be due to *additional* processes not involving oxidation. The combination of heat dissipation (by calorimetry) and oxygen consumption (usually polarographically; see Ref. [61]) has been termed the calorimetric–respirometric (CR) ratio [30]. In the normal, completely aerobic environment of animals, this ratio is always close to the theoretical oxycaloric equivalent (for animals living under hypoxic conditions, see Ref. [62]), though with one exception (brown adipocytes; see Ref. [63]); when tissue cells are studied in vitro they always have CR ratios more negative than the oxycaloric equivalent [3–6]. For many years, it was suspected that such values were due to poor aeration (Pasteur effect) and, because this results in anaerobic glycolysis, lactate analyses were performed on the culture media. By the enthalpy balance method, the values can be reconciled with the measured CR ratio as follows [4,6]:

$$\Delta_k H_{O_2(ox+anox)} = \Delta_k H_{O_2} + (Lac/O_2)\Delta_k H_{Lac} \quad (11)$$

where $\Delta_k H_{O_2(ox+anox)}$ is the observed CR ratio, Lac/O_2 the molar ratio of lactate production and oxygen uptake and $\Delta_k H_{Lac}$ the molar enthalpy change for lactate (−153 kJ mol^{-1} for lactate excreted into a bicarbonate buffered medium [30]). In most cases such calculations verified the explanation for highly exothermic CR ratios, namely glycolysis.

The only published CR ratio for cells that shows no anaerobic processes is that for hamster brown adipocytes, −490 kJ mol^{-1} O_2, [63]. This, most emphatically, happens not because only these were placed in fully aerobic conditions. Rather, it occurs because they are a special case of cells which have evolved solely to produce heat by completely uncoupled respiration using UCP (uncoupling protein). They do not, and cannot grow because the majority of the mitochondria do not produce ATP. A few of them do so, singly for maintenance purposes (mostly plasma membrane ion pumps) and not for synthesis.

The real reason for these 'anaerobic' processes is the requirement for biosynthetic precursors not available in the medium (often, not in the correct amounts). In the glycolytic pathway, amino sugars arise from fructose-6-phosphate and glutamine (the amino donor); serine (for fatty acids) and alanine from 3-phosphoglycerate, phenylalanine; tyrosine and tryptophan from phosphoenolpyruvate; and oxaloacetate from pyruvate and carbon dioxide (anaplerotic

Table 3
Representative data for the fluxes of heat and oxygen to give the calorimetric–respirometric ratio for CHO 320 cells grown in a batch culture for 140 h.

Culturing time/(h)	Physiological quantities			
	Total cell density $\times 10^{-5}$/ (cm^{-3})	Heat flux, $J_{\Phi/C} \times 10^{-6}$/ (W cm^{-3} pF^{-1})	Oxygen flux, $J_{O_2} \times 10^{-17}$/ (μmol O_2 per cell)	CR ratio [a]/ (kJ mol^{-1} O_2)
2	3.00	8.72	2.40	−617
14	3.69	9.18	2.57	−658
26	4.71	9.51	2.58	−668
38	5.01	8.80	2.76	−677
50	6.07	8.00	2.49	−693
62	7.00	7.64	2.65	−599
74	7.63	6.46	2.75	−428
86	8.02	6.23	2.66	−450
98	7.69	6.51	2.67	−451
110	8.00	5.04	2.12	−461
134	8.63	1.88	1.17	−327

[a] CR ratio was calculated using the ratio of O_2 consumption rate per unit bulk volume to heat flow per unit bulk volume.

reaction). It will be noted that these reactions mostly require three-carbon units to be produced at a fast rate from glucose. As a result, much pyruvate is produced which is surplus to energy requirements and is converted to lactate to pay back NAD^+. The enthalpy change of this reaction (Glc→Lac), as already stated, is additional to the oxidative process and gives the more exothermic CR ratio.

The pentose phosphate pathway is important in the formation of ribose-5-phosphate and provides reducing power (NADPH) in its oxidative branch, which would therefore come within the 'umbrella' of the oxycaloric equivalent. The same applies to glutamine which, besides incorporation into purines and pyrimidines and acting as the amino donor for amino sugars, is partly oxidized to lactate using a portion of the citric acid cycle. This enables other amino acids to be formed from α-ketoglutarate and oxaloacetate. The lactate formed from pyruvate in this oxidation causes a complication in verifying Eq. (11) for any particular cultured cell. It will always produce an overestimate of the anaerobic processes and could result in a significant discrepancy between the CR ratio and the ratio, $\Delta_k H_{O_2(ox+anox)}$, calculated from enthalpy changes and reaction fluxes by the enthalpy balance method.

From the foregoing appraisal it follows that, under fully aerobic conditions, the CR ratio should be most exothermic in rapidly growing cells and decrease towards the value for the oxycaloric equivalent with a decline in growth rate. There are two pieces of evidence to support this supposition. Schön and Wadsö [28] grew Vero cells on Cytodex 1 beads and, after 3 days, measured their heat production in the perfusion vessel of a Thermometric microcalorimeter. Many tissue cells, including Vero, are inhibited from growth by intercellular contact and, as this point approaches, growth slows down because of the increasing frequency of contacts as the substratum becomes more closely packed with cells. It was shown that the rate of lactate production was 11×10^{-17} mol s^{-1} per cell at 2.9×10^5 cells cm^{-3} with a heat production of 28 pW per cell whereas, with a higher cell density (3.8×10^5) under the same aeration conditions, the lactate excreted was less than half (5×10^{-17} mol s^{-1} per cell) and the heat production was 20% lower at 23 pW per cell. With the slowing down of growth the requirement for biosynthetic precursors was reduced, a fact reflected in the lower lactate production.

More recently, this phenomenon has also been noted for CHO 320 cells producing IFN-γ in a bioreactor, under the conditions detailed in [53,54]. As can be seen in Table 3 [64], the CR ratio declined towards the value for the oxycaloric equivalent (−450 kJ mol^{-1} O_2) as specific growth slowed and there was no longer a requirement for biosynthetic precursors.

With the coming possibility of a satisfactory on-line measuring device for oxygen consumption [65], the

CR ratio would be an excellent control variable in the biotechnological exploitation of mammalian cells.

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