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The application of heat conduction microcalorimetry to study the metabolism and pharmaceutical modulation of cultured mammalian cells

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Abstract

The heat produced by animal cells in culture can be used as the primary indicator of the kinetics of their metabolism because the scalar flux of it is a function of the metabolic flux. The validity of the relationship between heat and metabolism was demonstrated theoretically through the concept of thermal advancement and in experiments by the use of continuous cultures. This validation permitted the application of heat flux as a probe of the metabolic state of cells in culture. It consisted of an on-line heat conduction microcalorimeter that measures the instantaneous heat flow and dividing the smoothed signal with one obtained simultaneously using a dielectric spectrometer that records the change in capacitance as an estimate of the amount of viable biomass. In this mini-review, it is shown with Chinese hamster ovary cells (CHO320) genetically engineered to produce interferon-γ (IFN-γ) that heat flux is an early signal of deteriorating metabolism in cultures that produce considerable amounts of toxic lactate under fully aerobic conditions. The early detection favours the use of heat flux as the control variable in fed-batch cultures. This is a particularly useful finding in the context of the pharmaceutical industry because it will help to ensure the high fidelity of the cytokines, antibodies and vaccines produced in large-scale cultures. The monotonic relationship between the fluxes for heat and metabolism means that the enthalpy balance method can be employed to test the validity of the growth reaction for cells in culture. This showed that the crucial ratio between the substrates, glucose and glutamine, in the culture medium was incorrect at 5.5:1 instead of about 3:1, depending on the phase of the culture. Together with other changes to the medium composition, an improved formulation was made that ensured faster cell growth and greater specific rate (flux) of IFN-γ constitutive secretion while decreasing glucose utilisation and, most importantly, halving the excretion of lactate, that is toxic to the cells and harmful to the fidelity of their secondary products. Indirect calorimetry (oxygen uptake rate, OUR) is often favoured over the direct technique, but the former only measures aerobic metabolism. The environmental conditions in cultures favours lactate production even under fully aerobic conditions. Developments in measuring OUR mean that the stationary liquid phase balance can be used successfully to make the calorimetric:respirometric (CR) ratio a valuable tool in optimising cell culture to grow cells that synthesise the maximum amounts of the high fidelity secondary products.

Besides the value of heat flux in improving the cultures of animal cells producing heterologous products, three different techniques are examined that should be valuable in the testing the many compounds that are produced on a speculative basis as potential drugs. They are: (i) a thin-film thermopile transducer as an immunosensor; (ii) infra-red imaging of cells cultured in multi-well microtitre plates and (iii) integrated circuit (IC) calorimetry for small samples and low detection limit. One or more of these methods could well find favour with industry in the near future.

Keywords: Heat flux; Calorimetric:respirometric ratio; Animal cell growth; Cytokine production; Multiwell microtitre plates

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1. Introduction

Cultured animal cells, and particularly those derived from either insect or mammalian tissues, are used increasingly in the pharmaceutical industry for two main purposes beyond the basic research on their properties and functions. First, it is necessary to grow cells in industrial-size quantities in order to harvest their heterologous products that cannot be synthesised in, for instance, bacteria because the latter do not possess the necessary post-translational machinery for glycosylation. The relevant products include cytokines, monoclonal antibodies and vaccines [1–5]. The processes involved in the addition of carbohydrates to polypeptides are highly sensitive to cellular physiological (including metabolic) conditions. When these are adverse, the consequence is poor glycosylation that reduces the efficacy of the product [6,7]. The obvious need for a more precisely controlled environment for animal cell culture was recognised many years ago [8], but even now there is a paucity of on-line biosensors to monitor the cultures [9]. The development of a probe based on the measurement of the heat flow rate, ($\phi = \mathrm{d}Q/\mathrm{d}t$ where Q is heat) by heat conduction calorimetry is described in this paper, together with an expansion of earlier studies on the importance of measuring oxygen uptake rate (OUR) and calculating the ratio of the two sets of data, the calorimetric:respirometric (CR) ratio.

The second reason to use animal cells industrially is that they are the vehicles to test the efficacy of new formulations randomly synthesised in software-driven combinatorial processes and subjected to high input screening. These cells are usually from established cell lines grown as anchorage-dependent cultures and plated on 384- and/or 1536-well microtitre dishes. In many cases, the endpoint for the cytological test is simply cell death for which there is a wide variety of different assays (see [10]). There is often a need, however, for more discerning, sub-lethal tests and Kemp [11] has suggested that the detection of small changes in heat production in response to toxic insult would be a sensitive indicator of altered metabolism. Trials have been conducted using (i) a thin-film thermopile transducer of antimony and bismuth that detects the heat produced by immunocompetent cells immobilised at active thermopile junctions [12]; (ii) infra-red imaging of cells cultured in multi-well microtitre plates and exposed to drugs [13] and (iii) integrated circuit (IC) calorimetry for small samples and low detection limit [14]. These possibilities will be examined in greater detail in this paper.

2. Theoretical

It is well known in thermodynamic terms that, providing there is no change in pressure and volume ($P\Delta V$ work) during a reaction, the heat produced by a reaction should equal the enthalpy change of that reaction. This principle extends to the measurement of heat flow rate by a population of cells in which the temperature is maintained constant and the pressure does not change because usually the cells are in excess physiological solution. This means that the sum of the enthalpies of the specific metabolic reactions in the cells, the enthalpic flux (J_H) should balance the heat produced in size-specific (X) terms (heat flux), $J_H/J_{\phi/X} = 1$ [15]; this ratio is termed the enthalpy recovery. This is the basis for the enthalpy balance approach to study cellular metabolism in which the validity of the description of the reactions is held to account for the amount of heat produced by the cells. It is important to describe all the side reactions that occur in a system as complex as cells bathed in a physiologically buffered aqueous solution with dissolved gases.

The enthalpic flux is a general expression that can be regarded as being equivalent to the biochemical phrase, metabolic flux [16], or to the loose term ''metabolic activity'' [17]. It is defined as

$$J_H = \Delta_\mathrm{r} H_\mathrm{B} J_\mathrm{B,r} \tag{1}$$

where $\Delta_\mathrm{r} H_\mathrm{B}$ is the molar reaction enthalpy and $J_\mathrm{B,r}$ the reaction (r) 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 $|\nu_\mathrm{B}| = 1$ [15].

The first term on the right-hand side of Eq. (1) is the sum of the molar enthalpies of species i under the physiological conditions of the culture,

$$\Delta_\mathrm{r} H_\mathrm{B} = \sum_i \nu_i H_i \tag{2}$$

where H_i is the partial molar enthalpy of species, i. Since this quantity is concentration-dependent, it is not possible to assign to it a value in a dynamic

system such as a living cell [15]. Therefore, it is replaced by the concentration-independent standard molar enthalpy, H_i°, to give the aggregate standard molar enthalpy in Eq. (2).

The second term on the right-hand side of Eq. (1) is

$$J_{\mathrm{B,r}} = \frac{\mathrm{d_r}\xi}{X\,\mathrm{d}t} \tag{3}$$

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

$$\mathrm{d_r}\xi = \frac{\mathrm{d_r}n_i}{\nu_i} \tag{4}$$

where n_i is the amount of species, i. It is Eq. (3) that reveals the kinetic meaning to the term, enthalpy flux. Crucial to understanding this proposition is the need to recognise the central concept of advancement in energy transformation. As seen in Eq. (4), the advancement is stated explicitly in terms of the stoichiometric coefficients, ν_i, of the ith species in the reaction. This concept is extended to the heat flow rate that can be regarded as the rate of thermal (th) advancement, $\mathrm{d_{th}}\xi/\mathrm{d}t$, in the energy transformations [18]. The thermal advancement of energy transformation, $\mathrm{d_{th}}\xi$, is related to $\mathrm{d}\xi_{\mathrm{B}}$ by the expression

$$\mathrm{d_{th}}\xi = \nu_i \Delta_{\mathrm{r}} H_{\mathrm{B},i}\,\mathrm{d}\xi_{\mathrm{B}} \tag{5}$$

where $\Delta_{\mathrm{r}}H_{\mathrm{B},i}$ is the molar enthalpy of the reaction in terms of species i [19]. The change in thermal advancement, $\mathrm{d_{th}}\xi$, is exactly equivalent to the change in heat, $\mathrm{d}Q$.

3. Heat flux as a metabolic probe

Some years ago, Kemp et al. [16] studied the growth and metabolism of genetically engineered Chinese hamster ovary cells (CHO320) that produce constitutively the medically important cytokine, interferon-γ (IFN-γ). They showed that the rate of heat flow measured by heat conduction microcalorimetry is an excellent variable to assay for the state of health of cells because it is the most accurate reflection of overall metabolic rate — the kinetics of the process.

3.1. Heat flux probe for monitoring metabolism in batch cultures

While investigating the pattern of heat flow rate over the course of a batch culture, it became clear that the cellular metabolism began to decline earlier than the reduction in viable cell numbers (see Fig. 1). This

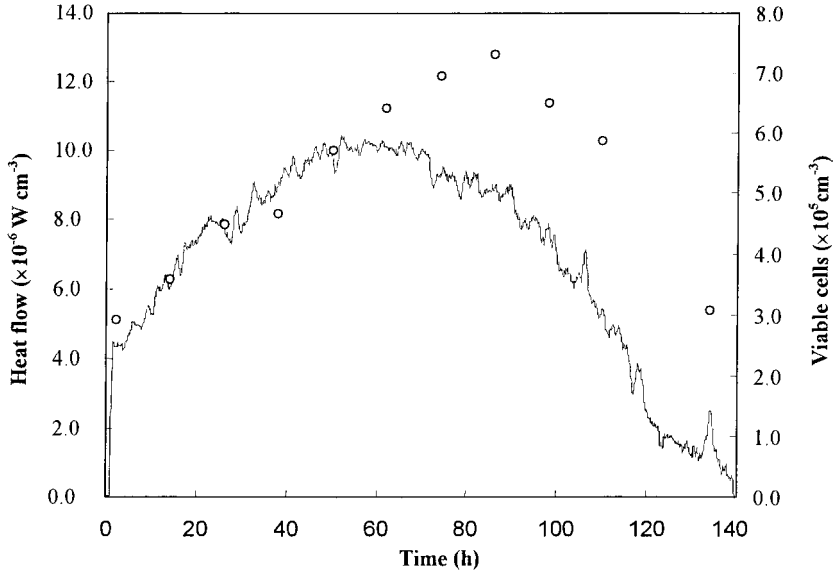

Fig. 1. Comparison of growth in viable numbers of CHO320 cells measured at discrete time intervals (○, off-line) and heat flow (—, on-line, in terms of culture medium volume) with time in batch culture (reproduced from [21] with permission of the publishers).

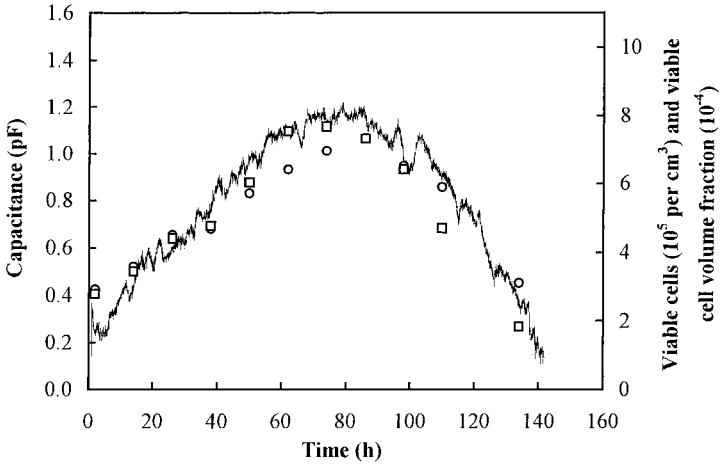

Fig. 2. Comparison of the viable cell volume fraction (□) obtained with the Skatron Argus 100 flow cytometer (off-line), the capacitance (—) measured with the Aber Viable Cell Monitor (on-line) and the cell density (cell number concentration) in suspension (○) during the cultivation of CHO320 cell line in a batch culture. The capacitance signal was smoothed by the moving average technique.

appeared to be due to substrate depletion and/or the accumulation of toxic by-products such as ammonia and lactate [20]. It was reasoned at the time [20,21] that individual cells could still divide for a limited period of time with stressed metabolism, but subsequently could not progress through the major growth phase (G1) of the cell cycle, being arrested prior to it, at the stationary phase (G0). This result meant that the scalar heat flux (also known as specific heat flow rate) in which the heat flow rate is divided by the quantity of biomass (X) in the bioreactor would be a more sensitive indicator of metabolism than the extensive rate. Although there are several methods to measure biomass off-line, there are only two techniques that take full advantage of the potential of the heat flow rate measurement in real time by estimating biomass on-line [20]. These are the optical density using laser light [22] and radio frequency dielectric spectrometry [23]. The latter measures the capacitance of animal cell cultures [24] that was shown to be directly related to the number concentration of viable cells [23]. Subsequently, Guan et al [20] used a Skatron Argus 100 flow cytometer (this manufacturer is now out of business) to calibrate a commercial version of the dielectric spectrometer operating at a frequency of 0.5 MHz and known as the Viable Cell Monitor (Aber Instruments Ltd., Aberystwyth, UK). They showed that in fact the on-line capacitance measurement was directly related to the volume fraction of viable cells, rather than the cell numbers per se (see Fig. 2). By using the Applikon BioXpert software (Applikon Ltd., Tewkesbury, Glous., UK) to smooth both on-line signals by the moving average technique, Guan et al. [20] had effectively developed a combined probe for the apparent metabolic flux based on heat flux.

3.2. Heat flux as a function of material fluxes

When the depletion of glucose and glutamine and the accumulation of ammonia and lactate were measured off-line by standard techniques [20], it was evident that heat flux was an early indicator of the advent of adverse environmental conditions that led to the termination of the batch culture (see Fig. 3). It had been postulated that heat flux, $dQ/dt/1/X$, is a function of the material fluxes and that the specific growth rate is indeed a form of metabolic flux, $dX/dt/1/X$ (see [20]). These assumptions were tested by plotting the average heat flux for each experimental period as a function of the fluxes for the major substrates, important catabolites and growth in terms of biomass (Fig. 4). It will be seen from these results that heat flux was monotonically related to the material fluxes. This was also the case for the specific growth rate and the growth-related production of IFN-γ. Because of this functional relationship, it should be seen that the

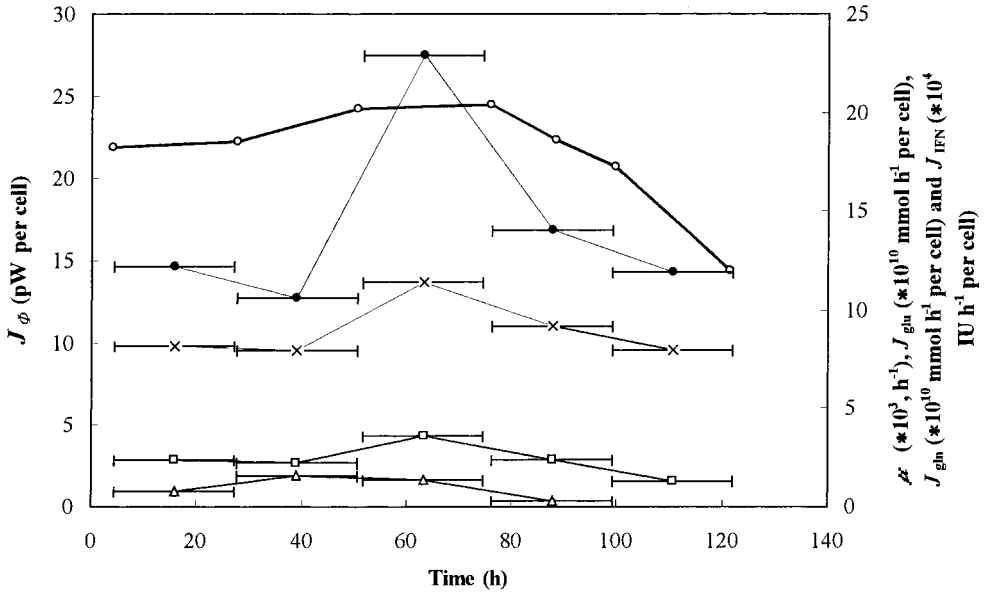

Fig. 3. Comparison of heat flux ($J_{\phi/C}$) with fluxes of glucose (J_{glc}), glutamine (J_{gln}) and IFN-γ (J_{IFN}), and specific growth rate (μ) during the batch cultivation of CHO320 cell line in suspension: (○) heat flux, (□) glucose flux, (△) glutamine flux, (×) IFN-γ flux and (●) specific growth rate. The bars indicate the period over which the discrete off-line measurements were made to give the individual average values for fluxes. In order for the on-line heat flux data to be compared directly with these measurements, it was averaged over each off-line assay period (reproduced from [21] with permission of the publishers).

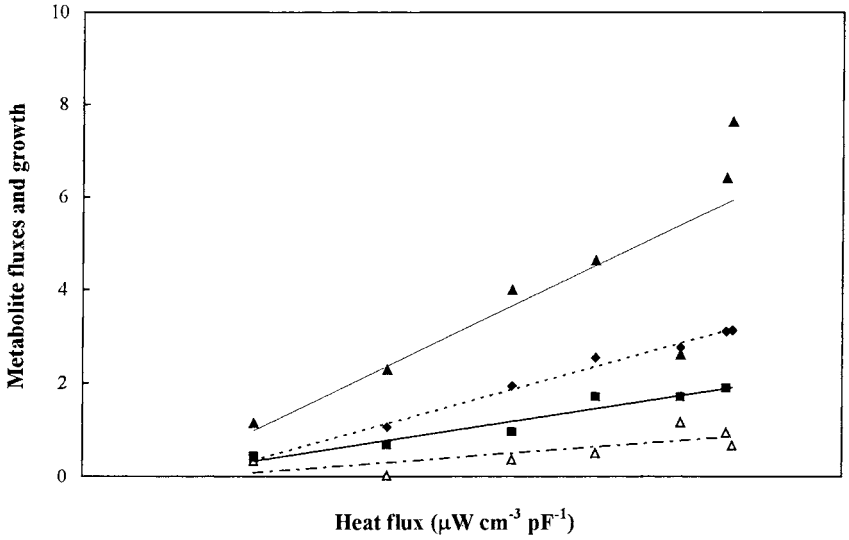

Fig. 4. Heat flux of the cultured CHO320 cells is plotted as a function of the specific growth rate (■) showing the monotonically decreasing relationship. This dependence extends to the fluxes for $10^4 \times$ IFN-γ production flux, IU s^{-1} per cell, (▲) and the major catabolic substrates, $10^7 \times$ glucose consumption flux, mol s^{-1} per cell, (◆) and $10^7 \times$ glutamine consumption flux, mol s^{-1} per cell (△) (reproduced from [32] with permission of the publishers).

quantity for the heat flux measurement was proportional to the constitutive secretion of the cytokine. Thus, it was possible to use the heat flux probe as the indicator of metabolic changes associated with altered substrate requirements during batch culture as well as acting as a guide to improvements in the culture medium for the enhanced growth of the cells that would lead to the improved specific production of heterologous proteins.

3.3. Lactate flux in strictly aerobic environmental conditions

A surprising fact about culturing animal cells has always been the production of lactate by them [25]. At first, this was ascribed to poor conditions for oxygenation that led to anaerobic glycolysis and in many cases this was due to cells in unstirred vessels undergoing sedimentation to become heaped on one another — the so-called crowding effect [21]. It was then found that lactate was produced even in stirred vessels under the most stringent conditions of aeration [20,25–28]. At least part of the reason is that most culture media are not optimised to provide all the biosynthetic precursors required in the anabolic process of growth. This necessitated that glucose and glutamine were utilised to produce amino acids, as well as both being needed for energy production [29–31]. In addition, they are required for nucleic acid synthesis in cell growth, glutamine for incorporation into the nucleic acids and glucose for the production of ribose-5-phosphate by the hexose monophosphate shunt. For the most part, only three carbon units of glucose are utilised for the production of amino acids, leaving the remaining three carbon units to proceed along the glycolytic pathway to pyruvate. It has been shown, at least for the case of CHO320 cells [32], that the mitochondria of cultured cells were not running at capacity. Using carbonyl cyanide *p*-trifluorophenylhydrazone (FCCP) as the uncoupler, it was found that maximum respiratory capacity in terms of OUR was 66% higher than the normal respiratory rate [32]. This means that the pyruvate produced as a by-product of the need for biosynthetic precursors was surplus to energy requirements. It seems reasonable to suggest that it was reduced to lactate primarily to pay back NAD^+ [21]. Even under aerobic conditions, therefore, cells growing in culture undertake glycolysis to produce lactate if the medium does not supply all the required biosynthetic precursors. Because the traditional media were originally designed empirically, all of them are unsatisfactory in this respect. In the pharmaceutical industry, this causes two problems. First, the cellular physiology and, therefore, growth is affected by the toxic properties. This is exemplified by a study of hybridoma cells in which cell growth was retarded in a concentration-dependent fashion by lactate [33]. Secondly, for the case of genetically engineered cells such as the CHO320 line that synthesises glycoproteins, the micro- and macroheterogeneity of the carbohydrate chains was profoundly affected by the changed physiological conditions, particularly pH [7]. A medium optimised for each individual cell type is required to prevent these two types of damage. Xie and Wang [27,28] chose the complete stoichiometric analysis of the metabolism of a specific hybridoma as the means to customise the medium while Bonarius et al. [34] elected to undertake the metabolic flux analysis of another hybridoma cell line and achieved similar success with an optimised medium.

The approach taken by Guan and Kemp [35,36] is illustrated in this paper by a study of CHO320 cells that produce IFN-γ in batch culture. The results shown in Fig. 2 were described by a generalised growth reaction (r) that relates the fluxes of the major substrates and products to the enthalpy flux:

$$\begin{aligned} &v_{S_1}\text{glucose} + v_{S_2}\text{glutamine} + v_O O_2 \\ &\quad \rightarrow v_X\text{biomass} + v_p\text{IFN-}\gamma + v_L\text{lactate} + v_C CO_2 \\ &\quad + v_N NH_3 + v_H H_2O + v_X \int_{t_2}^{t_1} J_{H,r}\,dt \end{aligned} \tag{6}$$

in which the stoichiometric coefficients, v_i, are used for the *i*th species. It is important to note that, although the fluxes depicted in Fig. 2 change with time, the stoichiometric coefficients constitute a set of constant values only for a given metabolic state and of course they will change for different states [36], i.e. a given stoichiometric reaction is only tenable if there is no change in yield, including that of heat, $Y'_{Q/X}$. Growth reactions can be validated by the enthalpy balance approach. For this purpose, it is assumed from the work of Battley [37,38] that the enthalpy change for anabolism is negligible. Thus, the growth reaction depicted in Eq. (6) does not include the contribution of amino acids other than glutamine. As mentioned

earlier, some of the glutamine is partly and/or completely oxidised to provide energy [29–31], as well as being incorporated into nucleic acids. Guan and Kemp [35] took the data for each time period shown in Fig. 3 and constructed stoichiometric half-reactions for catabolism. As an example, the half-reaction at 52–76 h for the redox reaction of glucose and glutamine to form lactate, ammonia and carbon dioxide was

$$C_6H_{12}O_6 + 0.084C_5H_{10}N_2O_3 + 0.64O_2 = 1.91C_3H_6O_3 + 0.68CO_2 + 0.17NH_3 + 0.43H_2O \quad (7)$$

The calculated enthalpy flux was determined for each time period using standard enthalpies of formation ($\Delta_f H^o$) and compared against the enthalpy flux measured by flow calorimetry. The ratio of the two, the so-called enthalpy recovery, was 1.02. This gave confidence that all the reactions had been held to account and made it possible to construct the stoichiometric growth reaction for each time period [35]. For the example shown in Eq. (7), this was

$$C_6H_{12}O_6 + 0.377C_5H_{10}N_2O_3 + 0.44O_2 = 3.52CH_{2.0}O_{0.82}N_{0.18} + 1.3C_3H_6O_3 + 0.46CO_2 + 0.12NH_3 + 0.29H_2O \quad (8)$$

The first term on the right hand side of Eq. (8) is the elemental composition of the combined biomass and IFN-γ glycoprotein calculated from the material and enthalpy balances (see [35]). It clearly emerges from Eq. (8) that the cellular demand for glucose and glutamine during the culture period from 52 to 76 h was markedly different at a ratio of 2.65:1 (glc:gln) to that supplied in the medium (5.5:1). This finding explains the observation in Fig. 3 that glutamine was exhausted before glucose. It was the impetus to design a new medium optimised for the CHO320 cells to produce IFN-γ. Guan and Kemp [36] have tabled the changes, given the rationale for the choice of substrates new to the otherwise commercial medium that is based on a formulation by the Roswell Park Memorial Institute (RPMI) and articulated the reasons for altering the concentrations of many of the pre-existing components, especially the amino acids. They compared data obtained for the various consecutive periods in batch cultures (see Fig. 3 for an illustration of the discrete measurements) with those during a similar period of time for a cell culture in the original medium described by Hayter et al. [39]. As an example of the improvement achieved with the new medium, the results for the period of 36–60 h showed that the glucose consumption flux in the improved medium was 30% lower (4.1 $mol^{-17}\ s^{-1}$ per cell) than originally (5.8 $mol^{-17}\ s^{-1}$ per cell), while the specific cell growth (μ) was 67% higher and, most encouraging, there was a 27% improvement in the IFN-γ flux to 6.1 IU h^{-1} per cell. There was very little difference in the glutamine flux at 1.7 $mol^{-17}\ s^{-1}$ per cell, compared with 1.8 $mol^{-17}\ s^{-1}$ per cell in the original medium. Therefore, the improved yield of IFN-γ, $Y_{IFN/glc}$, was due to the more efficient utilisation of glucose, (1.49 IU mol^{-1} glc versus 0.83 IU mol^{-1}) by cells growing in the improved medium [36]. Much of this enhancement probably was due to the fact that, judging from the decreased lactate flux (5.3 $mol^{-17}\ s^{-1}$ per cell in the new medium compared to 11.0 $mol^{-17}\ s^{-1}$ per cell originally), the improved medium supplied the cells with more of the necessary biosynthetic precursors, particularly amino acids. If the similar contributions of glutaminolysis to lactate production in the two batch cultures are neglected, then the lactate yield from glucose ($Y_{Lac/glc}$) at 1.29 in the new medium is a marked improvement to the yield of 1.90 in the original one. The reduced accumulation of the toxic lactate (see [33]) to maintain a ''better'' physiological environment in the medium probably contributed to stronger cell growth and IFN-γ production. It is also likely that the product would have a higher fidelity in terms of micro- and macroheterogeneity of the oligosaccharide chains [7].

From the exercise of constructing the thermodynamic growth reaction for cultured mammalian cells using non-equilibrium thermodynamics [35], it is possible to define a variable to assess growth efficiency, namely the enthalpy efficiency (η_H). This is the ratio of the amount of energy conserved as biomass to the energy used for complete catabolism and anabolism [40,41]. However, the method of making energy balances at relatively frequent intervals during the batch culture in order to calculate enthalpy efficiency is very time-consuming (see [35]) whereas the use of the CR ratio (see Section 4) to achieve the same end only requires one reading at each time interval of the two on-line probes for the fluxes of heat and oxygen.

3.4. Fed-batch cultures

One of the major problems in the large-scale culture of mammalian cells is to prolong the culture and, thus, maximise the amount of target protein produced by the cells. The classical way to do this is to feed nutrients to the cells when they require it for growth — a fed-batch culture — rather than supplying them all when the culture is set-up at zero time [42]. The main problem preventing the wider use of fed-batch cultures in industry is the lack of a suitably sensitive on-line probe to detect early enough that metabolism has slowed, but not to a degree that has caused irreparable harm to the cellular physiology. Because of this deficiency, Xie et al. [43] had to count the numbers of viable cells after staining with a viability dye. It is well documented that loss of permeability to colloidal dyes is a relatively late event in the process of cell death [44]. Guan and Kemp [45] proposed that heat flux could be an excellent on-line detector of nutrient depletion because it directly measured metabolic flux. They did so on their earlier assumption [20] that in one sense the heat flux shown in Eq. (6) could be considered as being equivalent to a stoichiometric coefficient. This idea was given mathematical form by them in [36]. They reasoned from Eq. (6) that growth could be characterised at any given time in the batch culture by a set of stoichiometric coefficients with biomass being taken to include IFN-γ as an indistinguishable part of anabolism:

$$\vec{v} = (\, v_{S_1} \quad v_{S_2} \quad v_O \quad 1(\text{cell}) \quad v_L \quad v_C \quad v_N \quad v_H \quad Q\,)$$

where Q is the integral of the heat flow rate. This set reinforces the earlier claim [20] that there is a one-to-one corresponding relationship between the metabolic flux and the stoichiometry of the growth reaction. It follows that this is related to the rate of advancement ($\mathrm{d}\xi/\mathrm{d}t$) [36], the rate at which the reaction proceeds

$$\frac{1}{X}\frac{\mathrm{d}\xi}{\mathrm{d}t} \leftrightarrow \vec{v}$$

Since heat flux, $J_{\phi/X}$, is a form of metabolic flux, this relationship can be shown by the following equation as

$$v_i = f(J_{\phi/X}) \tag{9}$$

In order to demonstrate the validity of the foregoing theoretical considerations, Guan and Kemp [45] set up continuous cultures at a series of different dilution rates. The results [45] gave complete confidence in using heat flux as the control variable in the fed-batch experiments. The averaged decrease in the on-line heat flux monitored by the Applikon BioXpert software over a 1 h period was the biosensor signal to trigger the feeding of a nutrient cocktail (glucose, 50 mM; glutamine, 16 mM) to the cells. As seen in Fig. 5, the biosensor-controlled nutrient feeding

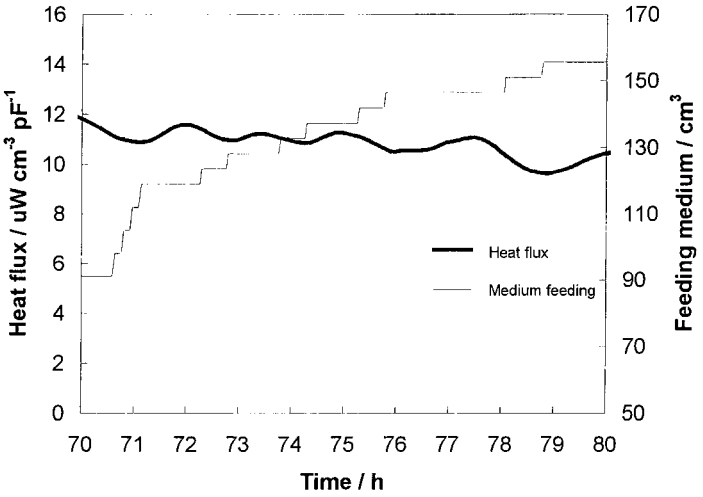

Fig. 5. This shows a small section of the heat profile for a fed-batch culture (from 70 to 80 h) to illustrate that the medium feeding was triggered by the declining heat flux values over the 1 h assessment period. The heat flux was restored, to a varied extent, by this feeding strategy (reproduced from [19] with permission of the publishers).

effectively restored the metabolic activity and thereby the growth of the cells at concentrations below ca. 10^6 cm^{-3}. Above this level, feeding slowed the deterioration in metabolism to a degree dependent on the time in culture [45].

4. Calorimetric:respirometric ratio

The operation of anaerobic catabolic pathways under fully aerobic conditions can be detected by the ratio of heat produced to oxygen consumed in the relatively slow process of substrate combustion by the cell. It is known as the calorimetric:respirometric (CR) ratio [46]. Many years ago, Thornton [47] showed that the heat of combustion for a wide range of organic compounds was an average of −115 kJ per equivalent of available electrons, a value now known as Thornton's rule. The data were obtained using bomb calorimetry ($\Delta_c H'$) in which no work can be done and the efficiency is zero, so it is the maximum enthalpy change for the complete combustion of carbon compounds. Another approach taken by Gnaiger and Kemp [46] was to calculate the enthalpies of combustion for carbohydrates, amino acids and fatty acids from the known molar enthalpies of formation ($\Delta_f H^o$). These standard values are often known as the oxycaloric equivalents ($\Delta_k H_{O_2}$) and the average for all biologically useful organic substrates was found to range from −430 to −480 kJ mol^{-1} O_2; $\Delta_k H_{O_2} = -450$ kJ mol^{-1} ± 15% [46]. Because this average value is correct when there is no work and efficiency is zero, any experimental results for the CR ratio more negative than that for the given oxycaloric equivalent must be due to additional energetic processes that do not involve oxidation. As stated earlier, the most common anaerobic product for cells growing in culture under regulated, totally aerobic, oxygen supply is lactate [33]. Net production of lactate from glucose is accompanied by a dissipative enthalpy change, $\Delta_k H_{Lac}$, of −80 kJ mol^{-1} when the acid is buffered in the cytosol. The plasma membrane is very permeable to lactate (and pyruvate) and then the enthalpy change depends on the nature of the buffer in the medium [46]. It is −63 kJ mol^{-1} when lactate is excreted into a bicarbonate buffer, −59 kJ mol^{-1} into a phosphate buffer and −77 kJ mol^{-1} into 20 mM HEPES buffer. The molar amount of lactate produced per unit amount of oxygen consumed (Lac/O_2) indicates the relative extent of aerobic glycolysis. The catabolic (k) heat change per mol O_2, $\Delta_k H_{(ox+anox)}$ (CR ratio), is then calculated as

$$\Delta_k H_{(ox+anox)} = \Delta_k H_{O_2} + \mathrm{Lac}/O_2 \times \Delta_k H_{Lac} \quad (10)$$

In studies of CHO320 cells in batch culture [20], the oxygen flux was close to being constant through the classical three phases of lag, log and senescence (Fig. 6) whereas the heat flux mirrored the cell growth profile. As a result of the growth-related increase in heat flux during growth, the CR ratio was more negative than the values for the oxycaloric equivalent of the catabolic substrates, reaching −700 kJ mol^{-1} O_2 compared with the oxycaloric equivalent for fully aerobic metabolism of −450 kJ mol^{-1} ± 15% [46]. It is most likely that this was due to lactate production and it would be possible to construct an enthalpy balance to test this hypothesis using Eq. (10), but for the probability that some of the glutamine in the medium was partly oxidised to produce lactate and adenosine triphosphate (ATP). This process was hidden in the value for the combustion of the organic compounds and led to an overestimate of aerobic glycolysis. The reconciliation between the CR ratio and the lactate flux in aerobic glycolysis can only be resolved by using radioactively labelled glutamine. When this type of experiment was performed for 2C11–12 mouse macrophage hybridoma cells, the contribution of glutaminolysis to the total lactate flux was 38% [48].

If the on-line information obtained by using heat flux as the metabolic probe for batch cultures could be augmented by measuring the OUR continually, then data for the CR ratio would be available for use in a control strategy that gives separate information on respiration and the total catabolic process. The problem has been that the available methods of measuring OUR do not have the required sensitivity for the relatively low cell number concentration in batch culture unless the oxygen concentration was measured by mass spectrometry — an expensive solution when each bioreactor would need a dedicated instrument. Eyer et al. [49] and Oeggerli et al. [50] among others have written the required oxygen mass balance for the whole culture vessel, called the global balance. Ramírez and Mutharasan [51] were able to use an alternative oxygen mass balance that required only a

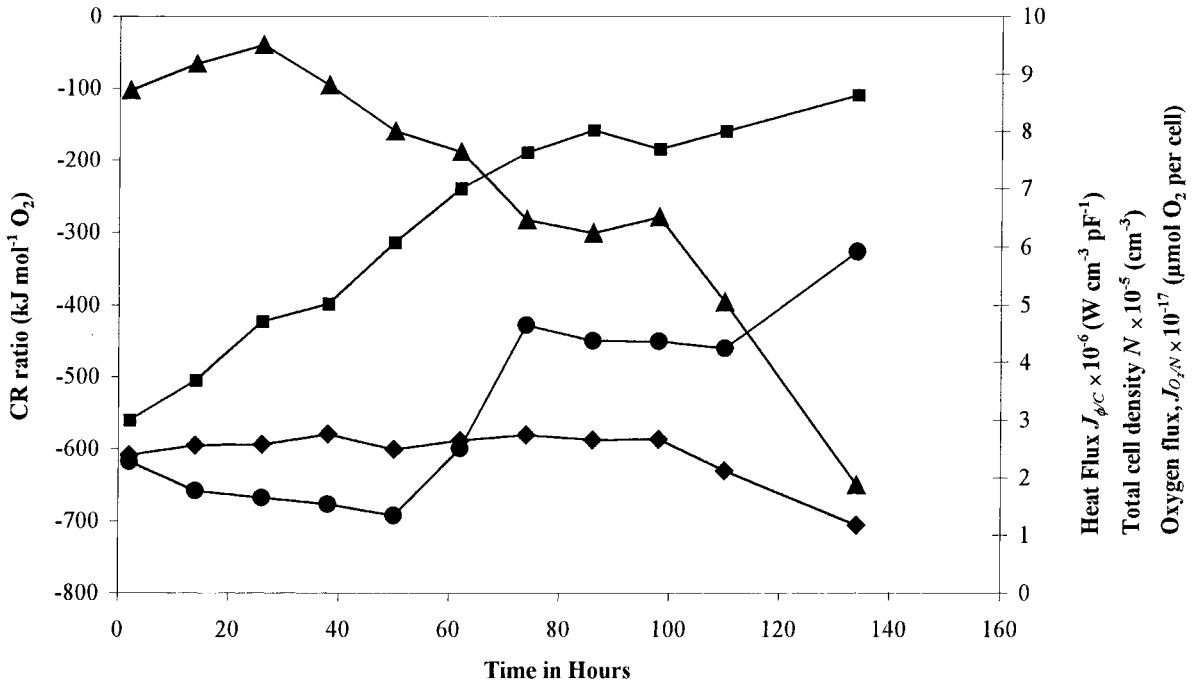

Fig. 6. The calorimetric:respirometric (CR) ratio, kJ mol^{-1} O_2 (●), of cultured CHO320 cells calculated from: (i) the continuous, on-line, traces at specified times for the heat flow rate (J_ϕ) divided by the change in capacitance (C) to give the heat flux ($J_{\phi/C}$) W cm^{-3} pF^{-1} (▲) and (ii) the oxygen flux, µmol O_2 per cell (◆) obtained off-line from the oxygen uptake rate divided by cell number concentration $J_{O_2/N}$. The pattern for the CR ratio should be compared against cell growth measured as the cell number concentration, (N) $\times 10^5$ cm^{-3}, (■).

conventional Clark-type electrode and a good software package. The balance was based on the liquid phase of the culture with constant dissolved oxygen concentration (DO). This method is known as the stationary liquid phase balance. In order to use it successfully, the molar fraction of oxygen has to be calculated from the set point of the flow rate of the three gases — oxygen, nitrogen and carbon dioxide that are fixed automatically to control the DO [52]. This is kept constant and OUR is then equal to the oxygen transfer rate (OTR) [53]. Then OUR is determined from OTR measurement,

$$\mathrm{OUR} = k_\mathrm{L}a[C_\mathrm{L}^* - C_\mathrm{L}] \tag{11}$$

where $k_\mathrm{L}a$ is the volumetric mass transfer coefficient, C_L is measured with an oxygen electrode and kept constant by a PID controller and C_L^* is the dissolved oxygen concentration in equilibrium with the gaseous phase at the interface. The gas flow in the tubing needs to be maintained at a very high rate so that only a very small percentage of the oxygen (<0.5%) is consumed and, thus, it can be assumed that there is an oxygen equilibrium between the gas and liquid phases [51]. So, C_L^* can be calculated as

$$C_\mathrm{L}^* = B\frac{Pn_\mathrm{M}F_{\mathrm{O}_2}}{HF_\mathrm{T}} \tag{12}$$

where F_{O_2} and F_T are the oxygen and total gas flow rates, respectively, H the apparent Henry's constant for oxygen in medium, P the total pressure, n_M the molar concentration of the medium (assuming water), and B a conversion factor equal to 1000 when C_L^* is expressed in millimolar. Henry's constant can be readily obtained assuming that the oxygen concentration in medium saturated with air is 0.194 mM. This is a reasonable approximation taken from [54] for their culture medium, but strictly it should be determined for each recipe because the value for it depends on the degree of "salting out" by the constituents in each different medium.

For the stationary liquid phase balance, a disadvantage might be said to be that the $k_\mathrm{L}a$ has to be known

throughout the whole time of the culture [53]. It has been shown that the number ascribed to it may vary to an unacceptable degree in a sparged system because of the variability of the bubbles. An alternative of aeration through the surface of the bioreactor headspace has been found to be insufficient to provide enough oxygen for a fully aerated culture [55]. Recently, the Lausanne group proposed that the solution to this problem might be to use gas-permeable membranes that give bubble-free aeration [56]. Ducommun et al. [53] stated that such membranes provide a known, constant $k_L a$ to supply the requested amount of oxygen to the culture and, thus, control the partial pressure of DO. The material of the membrane was polytetrafluoroethylene (PTFE) (W.L. Gore and Associates GmbH, Putzbrunn, Germany) and it was used in the form of tubing that was fixed to a template in the bioreactor [52,53]. With a porosity of 80%, the material has a high permeability for gases with the air:liquid interface actually within the pores to minimise any disturbance and, thus, secure a stable value for $k_L a$. PTFE is very hydrophobic and this prevents the cells from adhering to it. In principle, the system developed in Lausanne [53] provides the cell culture with the exact amount of oxygen to keep the DO tension constant for the determination of OTR by calculation. If DO is kept constant all the time by adjusting the ratio of the gas mixture with a PID controller, then OTR is equal to OUR, the required variable.

The on-line determination of OUR by the stationary liquid phase balance of course provides those working in the field with a valuable variable for metabolic studies in its own right. It may even give an indirect measurement of biomass [53]. However, this may also be the answer to the quest for the continuous measurement of the CR ratio. If this succeeds, then it may well be possible to record to the enthalpy efficiency of the growth process from the CR ratio. This prospect is currently under investigation.

5. New developments for studying small samples

It has already been mentioned that the pharmaceutical industry is always seeking to evaluate new endpoints for measuring the metabolism of relatively small numbers of cells in multiple samples such as microtitre plates and tests in medical diagnostics. Pizziconi and Page [12] sought to measure the reaction between cells adherent to a thermopile and specific antibodies, in this case immunoglobulin E (IgE), to create a biosensor that acted as an immunoassay system. The essential prerequisite must be that the heat of reaction is greater than the metabolic heat of the cells. Using Mast cells as the cellular system, it was discovered by conventional calorimetry that the cell activation and exocytotic degranulation gave a noticeable heat effect, well above that of basal metabolism [12]. The specific IgE antigen activated the cells in a calcium-dependent process that results in degranulation to release chemical mediators such as histamine. The immunoassay system (see Fig. 7)

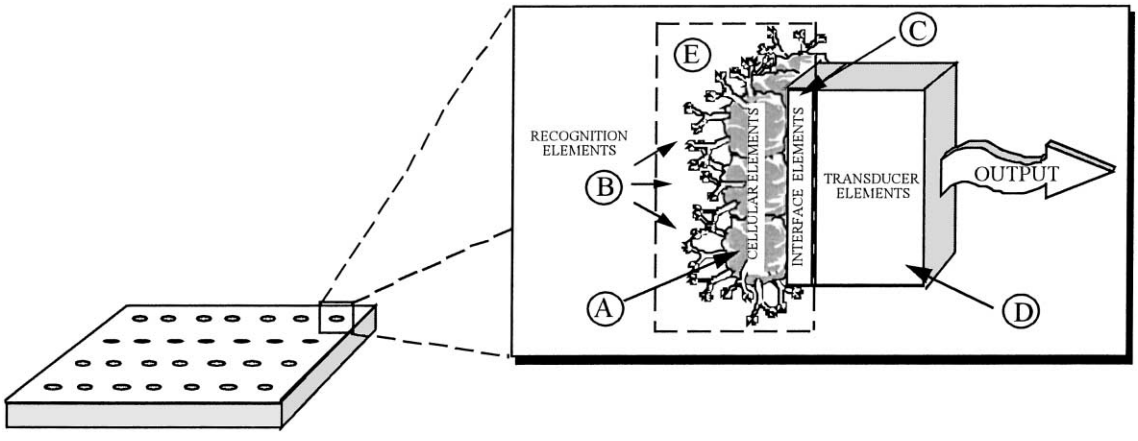

Fig. 7. Cell-based hybrid biosensor immunoassay system. The inset depicts the arrangement of the key hybrid sensor components. For details of the system A–E, see the text (reproduced from [19] with permission of the publishers).

consisted of (A) living mast cells, with (B) the IgE antigen recognition system, bound by natural extracellular matrix (ECM) proteins at the cell/transducer interface (C) to a highly sensitive, thin-film thermopile transducer (D). It is possible to amplify the heat produced by reacting the mediators with known ligands (E). It is also desirable to multiplex the system, using a sensing array, as depicted conceptually in Fig. 7 [12].

The prototype thermopiles were thin films of antimony and bismuth metals in regular patterns to form pairs of thermopile junctions on a 38 μm Mylar© support. They were altered so that the living cells in culture medium were in a small microwells, sited directly over both the active and the reference junctions. The cells were immobilised with fibronectin over the active junctions only (Fig. 8), so that the thermopile electromotive force was proportional only to the temperature difference between the active and the reference junctions. Local temperature gradients were minimised using a small stirrer. In the experiments, the cells were activated with the calcium ionophore A23187, and there was a relatively rapid increase in heat production within 20 min. The quantity of heat obtained was comparable to that found for the same reaction conducted at the same time in a heat flow microcalorimeter.

The authors realised that, for some applications, it would be an advantage to have a faster response time and a greater thermal output [12]. Page and Pizziconi [57] considered by that this aim could be achieved by incorporating into the assay a complex of extracellular enzyme systems that exothermically degrade mediators released during degranulation (see Fig. 7E). Histamine and chondroitin sulphate were the targets. They were catalysed by duets of diamine oxidase plus catalase and chondroitin ABC lyase plus sulphatase, respectively. It was found that the enzyme amplification

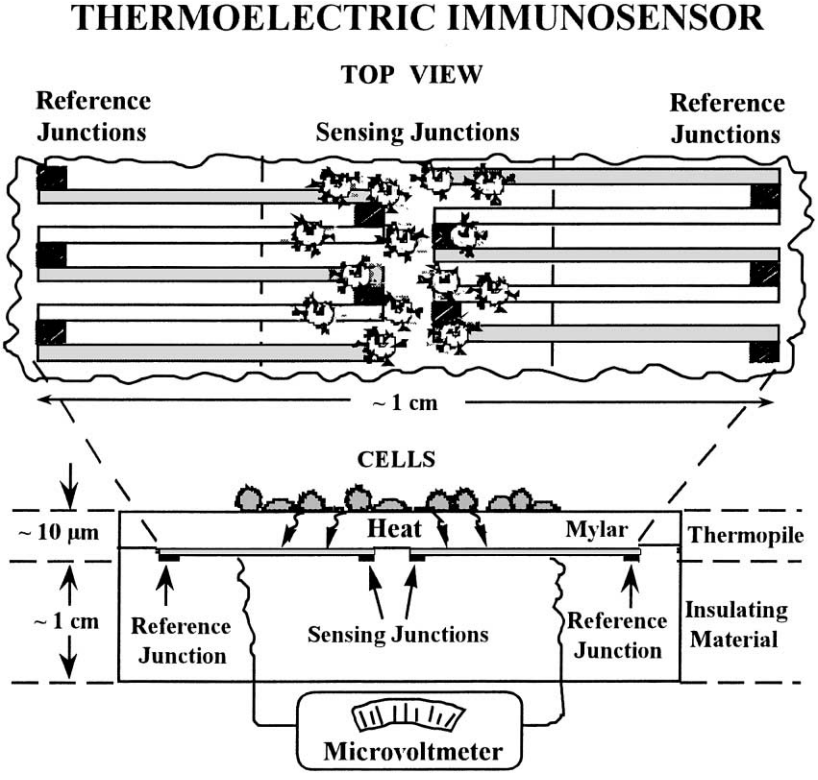

Fig. 8. Schematic representation of a thin-film whole cell biosensor prototype. The top view shows the placement of living cells over active sensing junctions (reproduced from [19] with permission of the publishers).

reduced the time for peak increase in heat production to about 5 min and increased the magnitude of the response by up to nine-fold. The work was then extended to a real antigenic system, rather than the ionophore model and the group chose dinitrophenylated albumin [58]. With enzyme amplification, the thermal output was more than sufficient to pick out the reaction and the thermal response time was as little as 3 min. Such hybrid immunobiosensors hold considerable promise and not only in medical immunology. The technology could be extended to other types of cellular interaction that produces exothermic responses, such as cellular interactions with growth factors, hormones, cytokines, neurotransmitters, etc. and even certain groups of pharmaceutical drugs.

An alternative technique to the above-mentioned thermoelectric device for achieving high throughput of data is the recently described infrared imaging of cells cultured in multi-well microtitre plates [13]. It can be seen in Fig. 9 that the apparatus is a thermo-electrically cooled Adema Thermovision 900 Infrared System AB camera (Marietta, GA, USA) in a precisely controlled ($\pm 0.02^{\circ}$C) incubator. Minimum thermal noise is secured by placing the plate in a ''black box'' within the incubator and positioning it on a ''black body'' plate holder with uniform heat conductivity made from a secret polymer developed in GlaxoWellcome, Inc. Using 384-well microtitre plates, Paulik and coworkers [12,59] validated this real-time thermal monitor using C3H10T1/2 human adipocytes and yeast exposed for 10 min to respiratory effectors and the β3-adrenoceptor agonists, CL316243 (anti-obesity agent) and troglitazone (antidiabetic drug). The resolution of this system was found to be 2×10^{-3}°C compared with $\sim 10^{-5}$°C for the thermoelectric device [12] and 10^{-6}°C for heat conduction microcalorimetry.

It seems certain that, in comparison to conventional heat conduction microcalorimetry, developments in IC calorimetry will allow [14], (i) small sample mass;

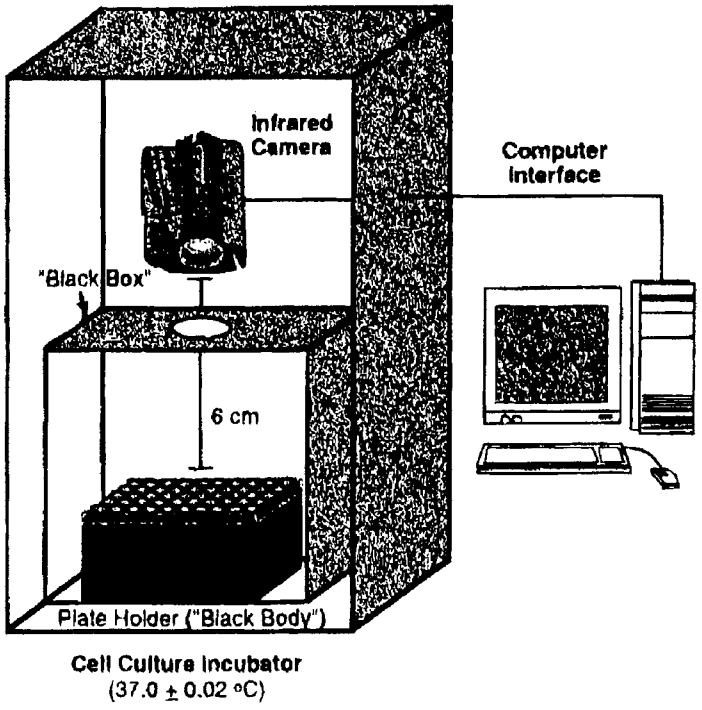

Fig. 9. Apparatus for measuring infrared thermography in cell culture. The ''black box'' and the ''black body'' minimise the thermal noise (i.e. reflection and air currents) from the culture plates and the surrounding environment. The use of an incubator also prevents fluctuations in the surrounding temperatures and improves cellular responses and viability. The camera monitors the real-time heat production from the cells in culture with images recorded by a central processing unit for further data analysis (reproduced from [19] with permission of the publishers).

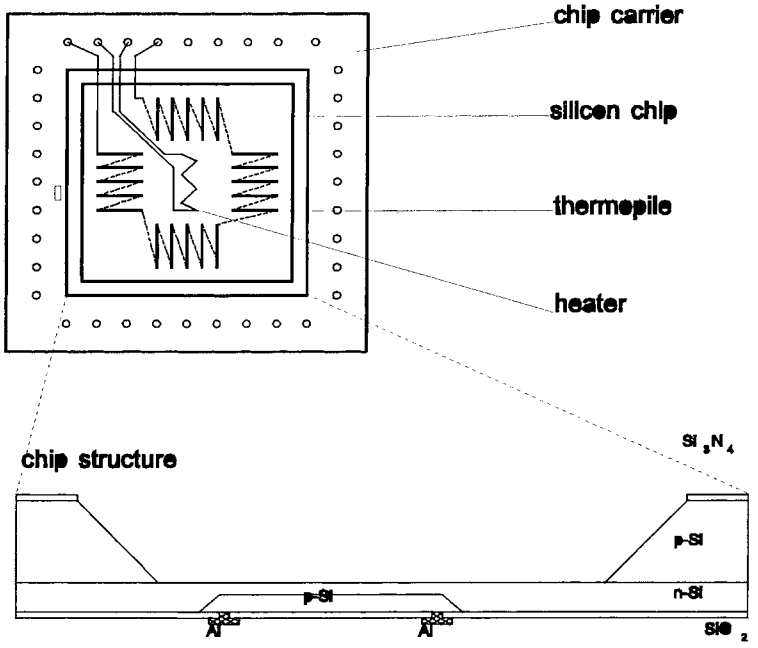

Fig. 10. Scheme of a silicon chip with an integrated thermopile and calibration heater (reproduced from [14] with permission of the publishers).

(ii) lower detection limit; (iii) high resolution for a succession of thermal events; (iv) faster equilibration time and (v) the probability of multiple-channel equipment to match microtitre plates. It will be seen in Fig. 10 that the IC consists of a thin Si-chip with a rim for stabilisation, assembled in a massive chip carrier. The chip itself is a planar structure with a self-supporting membrane. The sensitive sphere for detecting the heat is in the middle of the membrane where there is the Joule calibration resistor. The thermopile is integrated between the sensitive sphere and the rim. This measures the temperature difference created as a consequence of the heat flow through the membrane containing the thermocouples. A number of different types of IC calorimeter have been designed from a simple liquid-batch model to more complex flow calorimeters and even a temperature-scanning version [60]. Although these have only been used to measure heat changes in chemical reactions until now, there seems no reason why adaptations cannot be made for the IC calorimeter to be employed for cellular physiological and pharmacological investigations.

6. Conclusions

It seems that the use of cellular calorimetry in the pharmaceutical industry has not been as great as might have been expected from the powerful nature of it. Cell culture for producing drugs and other macromolecules in medicine is clearly an inefficient process that could well benefit from the rigours of a thermodynamic approach (see [19]). One of the most powerful tools in this field is the CR ratio [46] and the chances of fully employing it to explore the metabolic rationale for the physiological behaviour of cultured cells has been enhanced by improvements to the on-line measurement of oxygen flux that involves the stationary liquid phase balance [52,53]. With the introduction of computerised machinery to make compounds unsullied by humans, there is considerable pressure on the pharmaceutical industry to increase the throughput of testing the nascent compounds for biological activity. If it is realised that heat flux is an expression of overall metabolic flux, then the types of novel devices described in this mini-review will find considerable form in this field.

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