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Thermochimica Acta 309 (1998) 63–78

thermochimica acta

Probing the metabolism of genetically-engineered mammalian cells by heat flux[1]

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Received 15 July 1997; accepted 30 July 1997

Abstract

With the onset of the commercial production of target proteins by hybridoma and genetically engineered cells, there is a pressing requirement for biosensors to monitor on-line and in real-time their growth in culture. In terms of irreversible thermodynamics, most of the Gibbs energy provided in substrates for this process is dissipated as heat, with only a relatively small quantity being conserved in biomass and the target proteins. Calorimetry would appear, therefore, to be a strong contender for a metabolic probe. A flow microcalorimeter was modified for use with animal cells and tested using CHO 320 cells which produce the heterologous glycoprotein dimer, Interferon-γ. Preliminary results showed that heat flow did not follow the increase in cell-number concentration and it was realised that this technique must be combined with one to assess biomass on-line accurately to reflect metabolic activity. Dielectric spectroscopy was chosen because capacitance measurements of the culture measures the volume fraction of viable cells and not the dead ones. A commercial version, the viable cell monitor, was validated by parallel measurements of the volume of viable cells by flow cytometry. The combined probe showed that heat flux was a function of the specific growth rate which, in turn, was related to the fluxes for glucose and glutamine utilisation and possibly to the accumulation of toxic end products, lactate and ammonia. The calorimetric–respirometric (CR) ratio was highly negative during growth in the fully aerobic conditions, indicating simultaneous anaerobic metabolism during growth. The CR ratio gave a value consistent with solely oxidative processes once there was no growth. This might indicate that ATP demand was greater than could be satisfied by oxidative processes, but this was not the case since the cells were respiring at only ca. 60% of capacity, as judged by uncoupling with FCCP. It appears likely that lactate production was due to the need to produce biosynthetic precursors which were not supplied in the medium. A better medium design would probably decrease this requirement and thereby reduce the accumulation of toxic end products.

The heat flux probe was shown to be invaluable in exploring the metabolism of CHO 320 cells grown in batch culture.

Keywords: Capacitance measurement; Cell growth; Genetically engineered cells; Heat flux; Interferon-γ production

1. Introduction

In the last decade, the pharmaceutical industry has become increasingly interested in the large-scale use of animal cells in the production of monoclonal antibodies by hybridomas and of heterologous proteins with genetically engineered cells. [1–4]. Thus, it must

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[1]Presented at the Tenth Conference of the International Society for Biological Calorimetry, Ascona, Switzerland, 27–30 April, 1997.

0040-6031/98/$19.00
PII S0040-6031(97)00426-7

confront the problems of measuring the processes taking place in bioreactors that have confounded microbiologists over the years [5] and have made it difficult to optimise the productivity of these cultures. The lack of a range of robust and reliable on-line biosensors to measure cell growth and other metabolic variables [6] has also hampered the task of matching the complex media used for culture with the requirements of the cells. There is abundant evidence that the sub-optimal physiological conditions caused by the essentially empirical design of these media can result in the production of only relatively small quantities of, frequently, low-quality protein (see, e.g. Refs. [7–9]).

It is not often realised by biochemical engineers that a potentially excellent solution to this problem lies in thermodynamics and, specifically, in heat measurements. With rare exceptions [10], cells growing in a bioreactor constitute a thermodynamically open system [11]. Within the thermodynamic boundary (control volume [12]) of the vessel, the irreversible process of growth requires energy, a relatively small amount of which is stored in the accumulation and structuring of matter. The rest of it is expended on maintaining the physiological state of the cell [13,14] and in performing the internal work concerned with the functions of the cytoskeleton, such as intracellular vesicular transport [15]. For these processes, cells consume substrates which, in terms of energy analysis, possess high quality, useful energy known as Gibbs energy, dG. In the energy transformations required for maintenance and cytoskeletal activity, the quality is decreased and the remaining energy is dissipated as heat. Although the amount of internal work (dW) during the growth process may be small and the energy conservation in biomass accumulation modest, in the strict terms of irreversible thermodynamics [16], the growth of animal cells can be described as a dissipative rather than as a completely irreversible process, dD [17],

$$\mathrm{d}G = \mathrm{d}W + \mathrm{d}D \tag{1}$$

In these terms, the dissipated energy is related to the change in heat by,

$$\mathrm{d}Q = \mathrm{d}D + \mathrm{d}B \tag{2}$$

where dB is the bound energy which is the change in entropy (dS) times the absolute temperature (dB=T dS). Eq. (2) is recognised in the following form in relation to the change in enthalpy, dH [17,18],

$$\mathrm{d}H = \mathrm{d}G + \mathrm{d}B \tag{3}$$

From the foregoing, it can be seen that the growth rate of animal cells in a bioreactor can be measured by the heat flux using calorimetry when the absolute magnitude of the molar bound energy is negligible.

The relationship between metabolic activity of cell growth and heat dissipation has long been recognised and employed by an enlightened few in microbiology (see reviews in, e.g. Refs. [18,19]). In terms of instrumentation, there have been two distinct approaches using heat conduction calorimeters. For the one, the bioreactor constitutes a bench-scale, batch calorimeter [20] while in the other type [21] the cells are pumped from the culture flask to the flow vessel of a heat conduction microcalorimeter [22,23]. Until recently [24], the former did not have a low-enough detection limit for use with the typically dilute suspensions of animal cells ($<10^6$ cells per cm^3) and, in any case, is a dedicated culture tank of fixed size. While the latter has the complication of transmission lines between the two parts, in principle the calorimeter can be used in conjunction with bioreactors of any volume. In this laboratory, there has been much experience of flow calorimetry with animal cells [25,26] so this type of instrument was adopted for the present study of heat flux as a biosensor. It should be emphasised that the heat conduction calorimeter has one considerable advantage over other techniques as a biosensor because it directly measures the time-derivative of the heat, i.e. heat flow (Φ=dQ/dt), rather than the total heat, equivalent to concentration, alone.

In validating the design of the flow microcalorimetric assemblage, it is crucial to define the thermodynamic boundary of the system operating as a control volume and to ensure that within it the physical conditions for all the cells are identical at any one time. Guan et al. [27] decided on a system of which the boundary enclosed both the bioreactor and the calorimetric flow vessel, but not the instrument itself (Fig. 1). A series of tests have now been performed to show that the physiological conditions for the cells are similar in both parts of the combination and these are reported in this paper. In addition, since it is known that the physical volume of the calorimetric flow vessel is different to the thermal

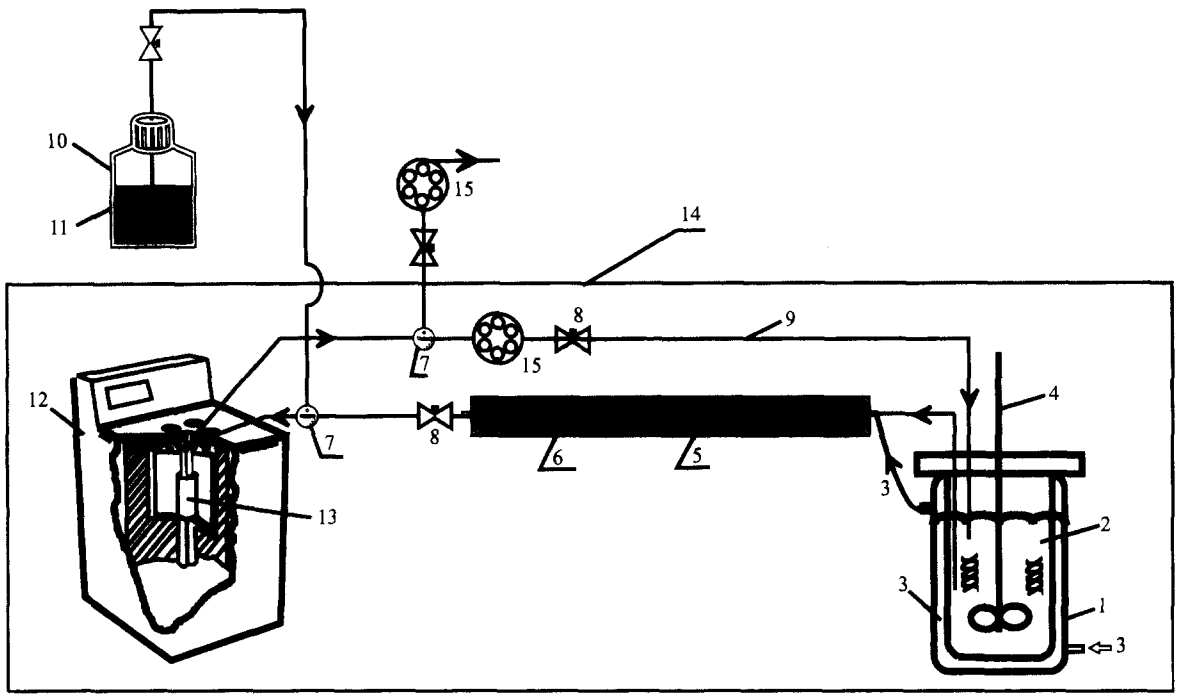

Fig. 1. A schematic diagram for the measurement of heat flow of bioreactor-cultured animal cells using a flow microcalorimeter. (1) bioreactor; (2) cultured cells; (3) jacket water for temperature control in the bioreactor; (4) agitator; (5) the outlet tubing of the jacket water is used for warming the cell suspension in the tubing leading to the microcalorimeter; (6) a non-conductive sponge-plastic pipe is used to reduce heat dissipation along the outlet jacket water attached to the PEEK tubing transmitting the cell suspension to the calorimeter; (7) PEEK T-piece; (8) PEEK two-way valve; (9) PEEK tubing (1 mm I.D.); (10) glass bottle holding sterilised medium for washing the flow vessel of the microcalorimeter through short-time interruption of the heat-flow measurement; (11) sterile medium for cleaning the flow vessel free of possible accumulated animal cells, which would result in overestimation of the heat flow for the cells in the bioreactor; (12) 4-channel thermometric microcalorimeter (TAM); (13) gold flow vessel assembly; (14) the boundary, enclosing essentially the bioreactor and the flow vessel of the calorimeter, is an open thermodynamic system for enthalpy balance studies; and (15) peristaltic pump. (Reproduced from Ref. [27] with permission of the publisher.)

volume [27], the confidence limits are established for the calibration assay by use of the hydrolysis of triacetin [28].

The cellular system used for this study is Chinese hamster ovary cells (CHO 320) genetically engineered to produce interferon-γ (IFN-γ) [29–31]. This immunomodulatory glycoprotein has important medical uses but cannot be isolated in sufficient quantities from natural sources to satisfy demand. At the same time, it is recognised that heterologous glycoproteins cannot be produced in the much more convenient microbial systems because these lack the endoplasmic reticulum and Golgi apparatus necessary for the appropriate post-translational modifications to the polypeptide backbone [32]. Hence, resort must be made to animal cells with their need for carefully controlled physiological conditions [30] and complex media [33,34]. Early results showed that heat flow per viable cell declined with time in culture during the growth phase [35,36], implying that, for the biosensor to be a valid monitor of metabolic activity, it should combine an on-line assay for the viable biomass with that of heat flow. This was achieved using a dielectric spectroscope that measures the capacitance of a cell culture [37] which closely approximates to the volume fraction of viable cells [38]. The results of detecting the heat flux variable throughout the course of batch cultures of CHO 320 cells will be described in this paper and its potential will be discussed in the light of the results from other metabolic assays.

2. Experimental

2.1. Cell Culture

The experimental material was suspension-adapted CHO 320 cells genetically engineered to produce IFN-γ [29]. Originally, co-transfection of the dihydrofolate reductase (*dhfr*) and *IFN-γ* genes into a dhfr-deficient mutant of the CHO-K1 cell line was by a plasmid derived from a pSV2-dhfr vector controlled by an SV-40 early promoter. Amplification was by selection with 0.1 μM methotrexate. The cells were grown in RPMI 1640 medium (Life Technologies, Paisley, UK) based on Dulbecco PBS and supplemented from Sigma (Poole, UK) with 2 mM glutamine, 20 mM HEPES, 4 mM sodium bicarbonate, 5 g dm^{-3} BSA free of fatty acids (PENTAX bovine albumen, Miles, Kankakee, IL), 50 mg dm^{-3} gentamycin and all the other compounds specified by Hayter et al. [29].

2.2. Bioreactor and on-line sensors

The 3-L bioreactor system (Applicon, Tewksbury, UK) contained 1.5 dm^3 cell suspension which was stirred at 60 rpm with a marine-type impeller. The four sensor inputs of the control unit were used for:

(a) measuring the culture temperature with a platinum resistance thermometer and maintaining it at 37°C;
(b) monitoring the medium pH with a glass combination electrode and controlling it at 7.25±0.02 using 0.1 N NaOH and 0.1 N HCl;
(c) recording the concentration of dissolved oxygen with an Ingold polarographic sensor and maintaining it at 55% of the air-saturated medium by sparging with filtered oxygen; and
(d) the signal from the probe of a dielectric spectroscope (Viable Cell Monitor, Aber Instruments, Aberystwyth, UK) operating at a radio frequency of 0.5 MHz [37,38].

The data were acquired using Applicon BioWatch software and analysed with the aid of Excel v.7.0a.

2.3. Flow microcalorimeter

The details of this system are available elsewhere [27] but, in brief, the heat flow of the cells was measured at 37°C in the gold flow-through vessel of a thermal activity monitor (Thermometric AB, Järfälla, Sweden). As shown in Fig. 1, the bioreactor was connected to the calorimeter by thermostated PEEK tubing (1 mm i.d) and the return was via a Jubile peristaltic pump (H.J. Guldener, Zurich, Switzerland) with Viton tubing (1.3 mm I.D.) in which the cell suspension was pulled back to the culture flask. The flow rate was 35 $cm^3 h^{-1}$ and, in order to avoid trapping the cells in the coils of the heat exchanger and flow vessel, the calorimeter was modified to permit downward flow. The flow vessel was calibrated daily with the in situ electrical Joule heater and periodically using the exothermic hydrolysis of triacetin [27,28]. Before an experiment, the transmission lines and flow vessel were sterilised using Decon 90 detergent and 70% ethanol, followed by copious amounts of sterile Dulbecco PBS and finally the complete culture medium. Data were logged by Oroboros DatLab AQ and analysed by the complementary DatGraf 2.1 (Oroboros, Innsbruck, Austria).

2.4. Off-line measurements

Samples were drawn from the bioreactor at regular time intervals and subjected to a range of assays. Cell density and viability, as well as oxygen consumption, were determined immediately but the rest of the sample was rapidly frozen and only thawed at the time of a series of assays conducted on all the samples of one batch culture.

Cell numbers were estimated using a Coulter Counter (Model D, Coulter, Luton, UK) and viability was determined by mixing fluorescein diacetate and ethidium bromide with cells [39] and viewing under a Zeiss fluorescence microscope with epifluorescence attachment (Zeiss, Luton, UK).

Oxygen consumption was assessed using an Oroboros Oxygraph two-channel respirometer (Anton Paar, Graz, Austria) [40,41]. The cell suspension, 2.3 cm^3, was added to each chamber and stirred with a magnet at 400 rpm. Stepwise and continuous titrations were performed by inserting the long (85 mm), thin (0.35 mm I.D.) needle of a 500 mm^3 Hamilton syringe into the central channel of the plunger for the chamber and injecting solutions with the aid of an Oroboros Alge motorised driver (Titration–Injection Micropump – TIP – Alge Elekronik GmBH, A-6893

Lustenau, Austria) [42]. Carbonyl cyanide *p*-trifluoroethoxyphenylhydrazone (FCCP), rotenone and antimycin A were purchased from Sigma (Poole, Dorset) and made up as ethanolic solutions. The final concentration of ethanol in the test and control cell suspensions did not exceed 100 mM which is considered to be harmless to the cells. The data were logged on Paar Oxydat software and analysed by Excel v.7.0.

The ammonium concentration and pH of the cell suspension were measured with a pH/ion meter (Model PHM95; Radiometer Analytical A/S, Copenhagen, Denmark) using an ammonium electrode (Model F2322NH4) and a combined pH electrode Model pHC2406) with a data acquisition program (Radiometer DMP/MeterLab) as described previously [43]. IFN-γ concentration was assayed by a specific antibody ELISA technique [29]. The amounts of glucose, glutamine and lactate were determined, after deproteination of the sample, using respectively Sigma kits 635, GLN-2 and 826 [43].

3. Results and discussion

3.1. Proof of the system

A substantial part of the validity for using heat flux as a variable to measure metabolic activity during cell growth is to ensure that there are uniform conditions within the delimited thermodynamic boundary. For the present system, it was decided that the infinitely thin boundary should be in the wall of the transmission tubing and calorimetric flow vessel as well as around the bioreactor [27]. Therefore, the physiological conditions must be the same in the transmission tubing and calorimetric flow vessel as in the bioreactor. When considering this matter, it should be remembered that mammalian cells are in dilute suspension and grow at a relatively slow rate (doubling time, t_d>~20 h) compared with microbes (t_d<~1 h). In order to consider the possible conditions in the flow vessel, it was calculated that the residence time of a cell flowing in the tubing to the end of that vessel (3.75 cm^3) was 400 s, which causes minimal changes to substrates, for instance, glucose (initially $\sim 1\times10^{-2}$ M) flux is 8.7×10^{-11} s^{-1} per 10^6 cells between 24 and 36 h of a batch culture (see Table 1). The only substrate that could be limiting is oxygen which has the comparatively low solubility of 190×10^{-9} mol dm^{-3} in the medium used for these experiments. From the data in Table 1, the amount of oxygen consumed by cells at a density of 1×10^6 cm^{-3} is <6% saturation (11×10^{-9} mol dm^3). In any case, although it is frequently not appreciated, mitochondrial oxygen flux is maintained at comparatively low levels of oxygen [41]. The oxygen flux of CHO 320 cells diluted in fresh medium was constant (ca. 3.0×10^{-17} mol s^{-1} per cell) at concentrations of oxygen dissolved in the medium from 100% saturation to as low as 30% (55×10^{-9} mol dm^{-3}, see Fig. 2). Gaseous diffusion into the medium through the walls of the PEEK and Viton tubing is minimal, the diffusion coefficients being determined as 1.0×10^5 and 9.1×10^4 cm^2 s^{-1}, respectively. In these tests, Viton was found to be the preferred pump tubing, with considerably less diffusion than, for instance, Aliprene (diffusion coefficient 1.4×10^5 cm^2 s^{-1}).

Table 1
Metabolic fluxes per CHO 320 cell calculated from the data for a typical batch culture

Time (h)	26–38	50–62	74–86
Heat ($W\times10^{-11}$)	1.87	1.61	1.19
Oxygen (mol$\times10^{-17}$ s^{-1})	2.75	2.71	2.65
CR ratio (kJ mol^{-1})	−680	−594	−450
Glucose (mol$\times10^{-17}$ s^{-1})	8.70	5.39	0.92
Glutamine (mol$\times10^{-17}$ s^{-1})	1.83	1.00	0.02
Lactate (mol$\times10^{-17}$ s^{-1})	14.98	6.73	0.14
Ammonia (mol$\times10^{-17}$ s^{-1})	1.17	0.70	0.11
IFN-γ ($\times10^{-7}$ units)	2.12	1.11	0.32
IFN-γ[a] ($\times10^{-19}$ mol)	1.47	0.83	0.20
IFN-γ ($\times10^{-15}$ g)	7.07	3.99	0.91
Protein flux ($\times10^{-15}$ g)	2.60	1.58	0.69
μ (h^{-1})	0.019	0.01	0.004
Protein content ($\times10^{-10}$ g)	4.65	5.91	5.66
Cell diameter (μm)	12.4	12.4	12.6

[a] The specific activity for 48 kD IFN-γ dimer is 3×10^7 units mg^{-1}

Concern is often expressed about the possible damage to cells by the peristaltic pump which, of course, works by "squeezing" the tubing and contents. As a safeguard, the pump was placed after the flow vessel (see Fig. 1). However, if the low flow rate (35 cm^3 h^{-1}) is considered in comparison with the large bulk volume, it is obvious that any damaged cells would be diluted over 40-fold in the bioreactor. Earlier tests had also shown that the damage was negligible

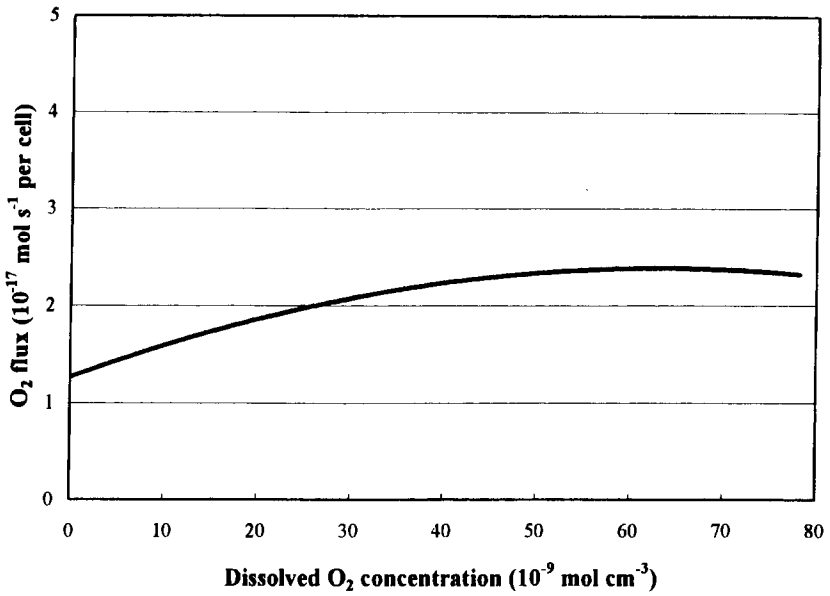

Fig. 2. CHO 320 cells growing exponentially in a normoxic (190×10^{-9} mol O_2 cm^{-3}) bicarbonate buffered medium and at a density of 4.0×10^5 cm^{-3} were allowed to respire in the Oxygraph until there were hypoxic conditions. Oxygen flux remained constant until 30% saturation (60×10^{-9} mol O_2 cm^{-3}) after which the concentration of oxygen appeared to limit respiratory flux.

[27]. In order to further emphasise the benign nature of pumping, a small volume of stirred cell suspension (5 cm^3) was repeatedly circulated around a loop of Viton pump tubing (0.8 cm^3 internal volume) at 35 cm^3 h^{-1}. Viability was checked by the dual fluorescence test [39] at approximately every fifth cycle and found only to deteriorate from the bulk value of 95% to below 90% at between 10 and 15 cycles.

It would seem reasonable from the sum of all these tests to conclude that the physiological conditions of the cells in the calorimetric flow vessel were sufficiently similar to those of the bulk in the bioreactor to permit the two containers to be considered as one environment and to justify the construction of enthalpy balances within a single control volume.

Comment should be made in this section on the finding that the thermal volume of the flow vessel (0.7 cm^3, see Ref. [27]) was considerably larger than the nominal physical volume (0.6 cm^3). Wadsö [44,45] has long stressed the need for chemical calibration of all calorimetric vessels, even those of simple geometry, for which it is now possible to purchase insertion heaters. The requirement for a chemical test is greater with respect to flow vessels for which it is almost impossible to determine a position for an electrical heater so that it faithfully mimics heat conduction from the flowing sample to the heat sink. The fact that there was a difference, therefore, came as no surprise but the scale was unexpected and led to several repeat tests which varied by ±7% to show that triacetin hydrolysis gives a reproducible estimate of vessel volume.

3.2. Heat-flow measurements

Initial experiments measuring the heat flow of animal cells growing in a batch culture [35,36,43] indicated that thermal measurement was not a straightforward assay for biomass (see Fig. 3). After 50 h the heat flow declined, whereas the number of viable cells continued to increase for a further 36 h. This is unlike many microorganisms growing in a defined medium where it has been found that the quantity of biomass can be calculated from the integral of heat flow [46,47]. On the face of it, there seems no reason for the difference but it might be suggested that it is due either to medium deficiency or to the accumulation of toxic end products. In terms of the former, it is known that the medium in use has not been optimised for these cells [29–31], although improvements based on

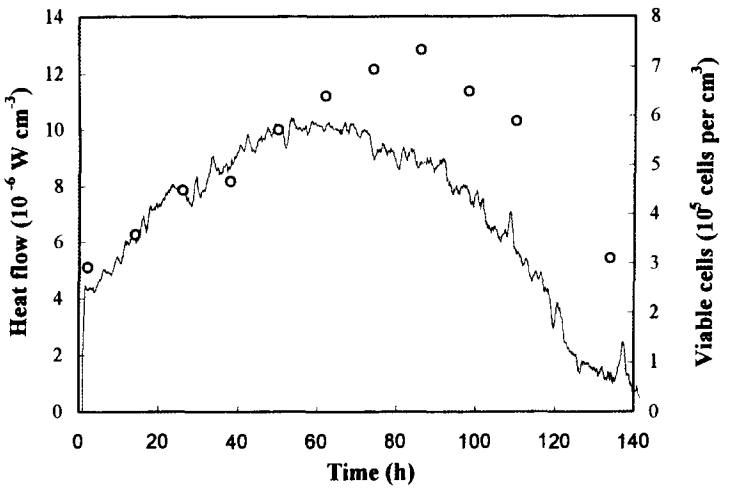

Fig. 3. Heat flow of growing CHO 320 cells measured on-line by the microcalorimeter and scaled to the unit bulk volume of culture medium (———). Estimates of cell density (○) were made at discrete time intervals (reproduced from Ref. [36] with permission of the publisher).

a statistical approach have led to a series of changes [33,34]. An alternative, more rational method of medium design has been suggested in which the stoichiometry of the growth reaction was determined in order to define demand for substrates from enthalpy [48] and mass [49] balances. As regards toxic products, there are very many reports that the accumulations of ammonia [50,51] and lactate [52,53] have deleterious effects on cell metabolism, and thereby growth.

3.3. Heat flux as an on-line metabolic probe

Once it was decided that the intensive quantity, scalar heat flux (W m^3), rather than total heat flow must be used at present for animal cell cultures, there seemed to be only two on-line options to estimate biomass, laser-light nephelometry and dielectric spectroscopy [6]. On the basis that the latter measures only viable cells [37], this technique was validated by comparison with off-line assessments of the volume fraction of viable cells obtained by flow cytometry in combination with the fluorescein diacetate stain [35,36,43]. Results showed that the two records of biomass accretion were similar and, thus, the viable cell monitor was chosen to pair with calorimetry. Dielectric spectroscopy measures the capacitance (C, in farads, F) of the culture and the value for the cells is obtained by subtraction of the reading for the medium ($C-C_0$). This expression is abbreviated as C in the symbol for heat flux, $J_{\Phi(C)}$. The results of a typical batch-culture experiment, complete with off-line assays for crucial metabolites are reproduced in Fig. 4. Setting up the parameters and variables as well as measuring the conductivity of the culture takes 2 h after the cells have been introduced; hence, the start of the measuring period is (zero +2 h), during which time the cells consume substrates. The value for heat flux was calculated as a continuous record off-line from the on-line heat flow and capacitance readings. In Fig. 4, heat flux was at or above the initial value for approximately the first 40 h of the culture during which cell numbers increased by 60% and IFN-γ accumulated at a rapid rate. Many of the substances in the medium can act as substrates for catabolism and/or as biosynthetic precursors, with amino acids particularly relevant to the latter but, remembering that BSA free of fatty acids was used in these studies, the principal catabolites in the growth reaction were glucose and glutamine, the consumption of which is shown in Fig. 4. The fluxes were calculated and are shown as Table 1. On the basis of these results, the following growth reaction has been constructed [35,36,43],

$$\nu_{S_1}[\text{Glucose}] + \nu_{S_2}[\text{Glutamine}] + \nu_O O_2 = [\text{Biomass}] + \nu_p[\text{product}] + \nu_L[\text{Lactate}] + \nu_C CO_2 + \nu_N NH_3 + \nu_H H_2O + \Delta_{met}H_X \quad (4)$$

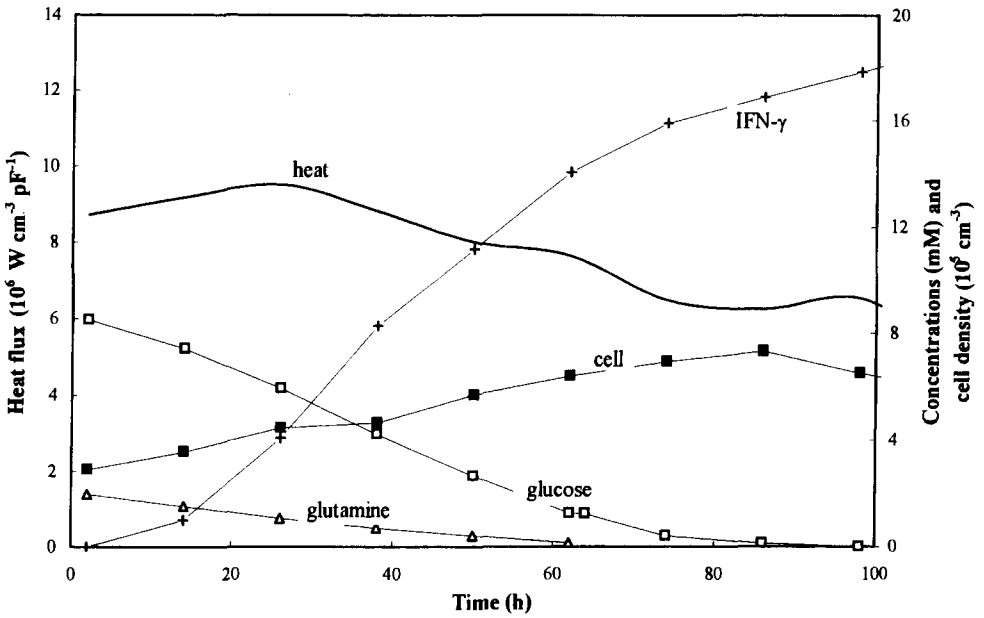

Fig. 4. On-line measurement of heat flow and capacitance calculated off-line as heat flux (———) with off-line measurements of cell density (■) and changes in the concentrations of glucose (□), glutamine (△) and IFN-γ (+) for a batch culture of CHO 320 cells.

where ν_I are the stoichiometric coefficients and $\Delta_{met}H_X$ the enthalpy change of the reaction for the formation of biomass, X. The validity of Eq. (4) has been demonstrated by the enthalpy balance method [54,55] which gave a recovery close to 1 [36,47].

In the middle stages of the culture (40–70 h), heat flux declined and glutamine, in particular, was completely consumed by the cells (see Fig. 4). Cell growth and the secretion of IFN-γ continued at an appreciable pace. Important changes in rate were suspected, however, and these are highlighted in Table 1. The specific growth rate and the flux of IFN-γ had declined coincident with lesser fluxes of the major catabolites. The exhaustion of glutamine is significant because the most essential need for this amino acid is in purine and pyrimidine synthesis [56] and, if it is absent, of course DNA synthesis comes to a halt [57,58]. It would appear that glutamine frequently shows a Monod-type effect on specific growth rate [59] which can be due to a stoichiometric limitation rather than a kinetic one, at least at concentrations close to 0.5 mM [57–60]. The continuation of cell growth beyond the point of total absence of the amino acid may well be due to the fact that cells which have completed *S* phase of the cycle do not require the substance for division, providing there is a suitable energy source. There is a body of evidence that synthesis of constitutively secreted, recombinant proteins is related to growth and specifically to the nature of the early promoter [61]. In the present studies, this is derived from SV40, for which there is some evidence of gene expression being dependent on S phase [62]. The production of IFN-γ would appear to be related to cell growth (see Table 1 and Ref. [63]).

In the third phase of the culture (>70 h), overall cell growth gradually decreased but the cells remained viable and continued to produce IFN-γ for some considerable time (see Fig. 4). Even though glucose was totally depleted at 86 h, the heat flux profile ceased to decline and, in fact, assumed a new plateau. It will be seen in Table 1, however, that the specific growth rate and IFN-γ flux were considerably less than earlier in the culture period and reflected the lack of the primary substrates. It is of interest that the cells remained alive, presumably by utilising amino acids as a gluconeogenic source and, possibly, even consuming lactate derived from glycolysis and glutaminolysis [64,65]. Since IFN-γ was still being produced albeit at a lower flux and as the expression of the IFN-γ gene is thought to be growth-related, it must be that a

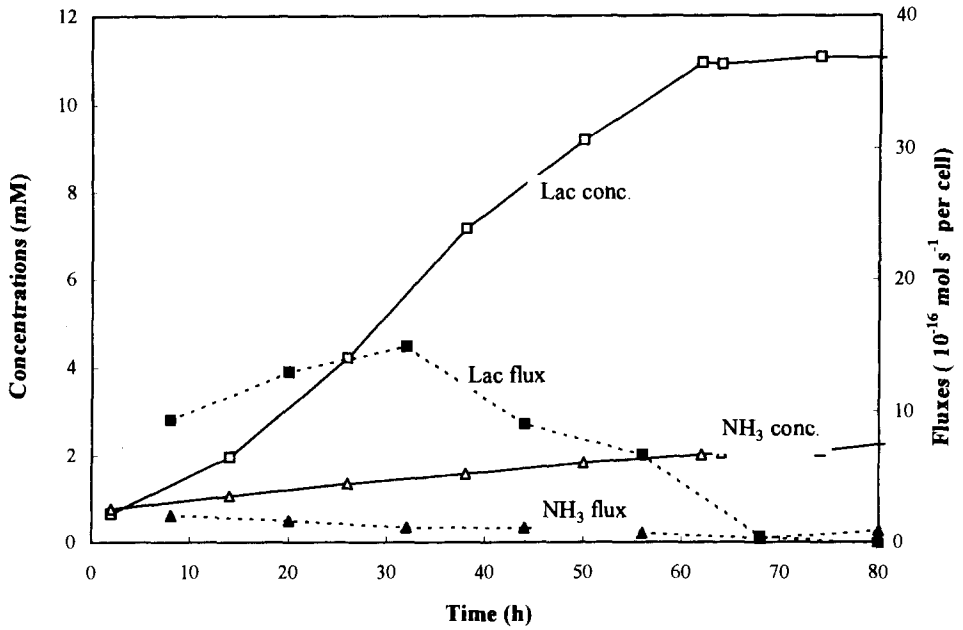

Fig. 5. Accumulation in the same culture depicted in Fig. 4 of the potentially toxic end products, lactate (□) and ammonia (△). When the fluxes were calculated, it was obvious that lactate flux (■) was greatest during the early stages of batch-cell growth, declining rapidly thereafter. The highest ammonia flux (▲) was also in the initial phase of growth.

reasonable proportion of the cells are actually growing and dividing during this period. The lower specific growth rate, then, reflects the shifting balance between these cells and ones that have died and possibly undergone lysis.

After 86 h, viable cell numbers begin to decline and this trend accelerates to the end of the experimental period. The most probable reason is the exhaustion of all possible substrates, though the accumulation of toxic end products may also contribute to the problem. As can be seen in Fig. 5, considerable quantities of lactate (11 mM) and ammonium (>2 mM) ions are in the medium by 80 h. However, the flux of lactate was highest during the period of strongest specific growth rate (see Table 1) and declined to such an extent that it was very low at 74–86 h. There are many reports of these two end products being harmful to cells [50–53], though Hayter et al. [66] did experiments with cells of this type which indicated that concentrations of these orders were not toxic.

It is well established that *N*-glycosylation of IFN-γ by CHO 320 cells in culture is variable, seeming to change according to the physiological conditions [8] including those caused by cell aggregation [67]. This dimeric glycoprotein [68] should have two long oligosaccharide chains (4 kD each) and low fidelity can be due either to the absence of one or each of the chains (macroheterogeneity, see Refs. [7,9,66,69]) or to smaller changes within the chains leading to triantennary and truncated glycan structures (microheterogeneity, see Ref. [8]). Since the present experiments were performed under similar conditions to those found in these reports, there is every reason to suppose low-fidelity IFN-γ was produced, at least later in the culture. One of the lesser realised roles of glutamine is in the formation of amino sugars from fructose-6-phosphate. The use in the present study of BSA free of fatty acids might also contribute to a low-quality product.

3.4. Oxygen flux and the calorimetric–respirometric (CR) ratio

One of the most surprising findings in this study was that, in contrast to the changes in substrate and heat fluxes, oxygen consumption remained essentially the same throughout the period of culture (see Table 1). During net cell growth, this was associated with a

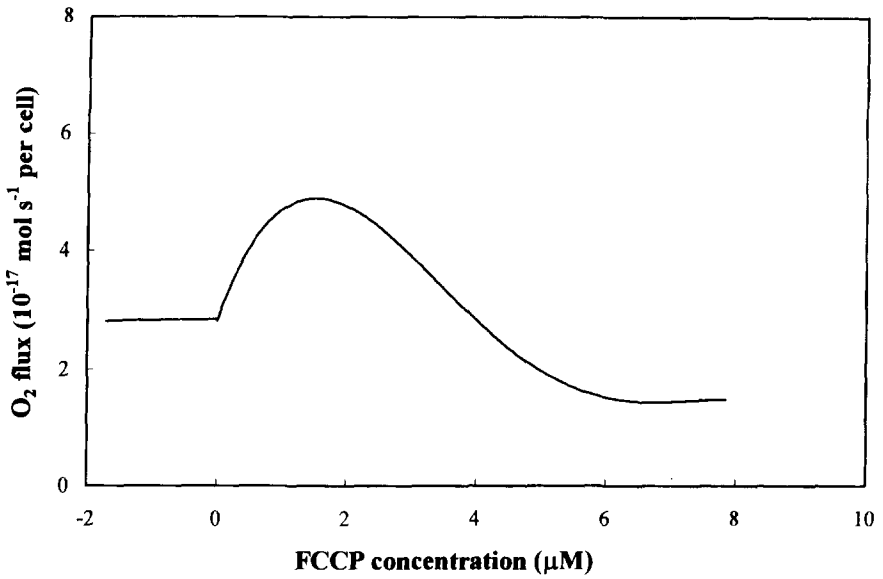

Fig. 6. Mitochondrial capacity was determined in the Oxygraph respirometer by continuous titration with the Oroboros Alge TIP at 0.02 μl s^{-1} of the uncoupler, 500 μM FCCP into a suspension of cells (8.8×10^5 cm^{-3}). Note that the maximal increase in oxygen flux occurred at 1.8 μM FCCP and that inhibition was evident at higher concentrations.

considerable lactate flux. It has already been established that oxygen flux was constant even under hypoxic conditions in culture (see Fig. 2), so there is no reason to suspect that the Pasteur effect is responsible for the amount of lactate. It is possible, however, that these cells exhibit the Crabtree effect, with excess glucose being converted to lactate with conservation of NADH. It is more likely, though, that the glycolytic pathway in tightly-coupled mammalian cells responds to allosteric inhibition of phosphofructokinase by effectors such as citrate and AMP. If this were the case then, conceivably, it is a limitation in mitochondrial capacity that causes a shortfall in ATP supply with the response of increased glycolytic flux to meet the demands of a high biosynthetic flux in these genetically-engineered cells.

For the above reason, the respiratory chain was uncoupled from ATP synthesis by continuous titration with FCCP [42,70]. The amount of it required maximally to stimulate respiration in CHO 320 cells was found to be 1.8 μM (Fig. 6). At this concentration, the respiratory rate had increased by 66% showing that the mitochondria were not operating at full capacity under normal conditions. At higher concentrations, the characteristic feature of uncouplers in causing inhibition of oxygen flux was observed in this system (Fig. 6). Despite this reassurance that the increased oxygen flux was probably due to uncoupling, an experiment was performed to inhibit the respiratory chain after the addition of FCCP. As can be seen in Fig. 7, oxygen flux for cells newly resuspended in fresh medium was established to be 3.0×10^{-17} mol s^{-1} per cell. Then 5 mM succinate was added and this only slightly increased respiration, meaning that the large majority of the cells had not been damaged by the centrifugation [70]. FCCP was then added at a sub-optimal concentration to avoid the possibility of inhibition due to biological variability. Once it was established by the increased oxygen flux that the chain was uncoupled, it was inhibited at complex I by 0.5 μM rotenone [71]. Oxygen flux was reduced to nearly 0.5×10^{-17} mol s^{-1} per cell corresponding to 85% inhibition and this was only slightly further inhibited by the addition of 2.5 μM antimycin A which blocks the electron flow from succinate at complex III and indicated the level of damaged cells. These experiments showed conclusively that the mitochondria were operating at 40% below capacity and had the potential for increased ATP production. It is clear that the reduction of pyruvate to lactate was not due to a limitation of the ability of oxidative phosphorylation to supply ATP.

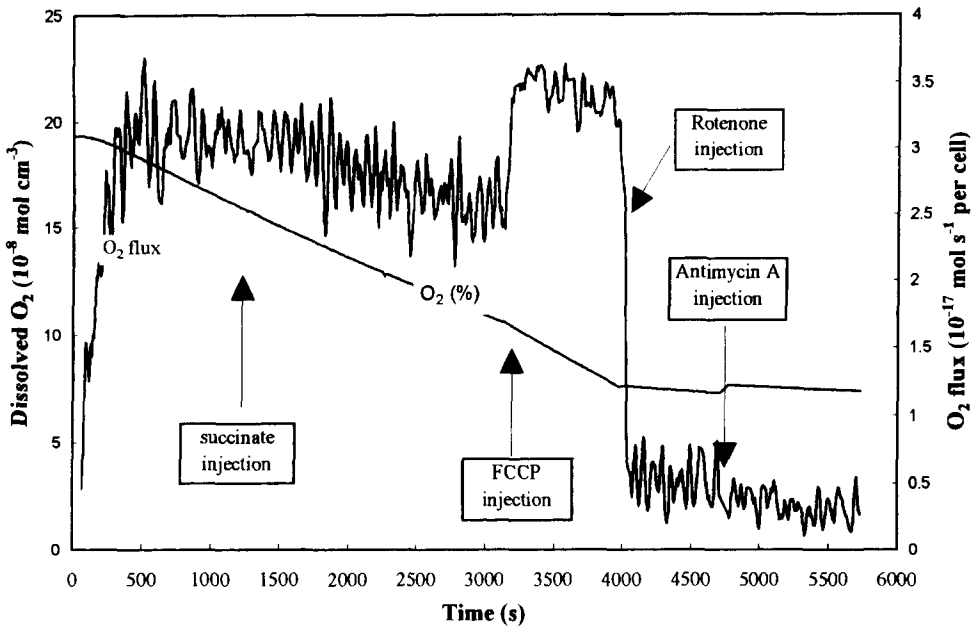

Fig. 7. Stimulation of the respiratory rate of CHO cells using 1.6 μM FCCP after the addition of 5 mM succinate to check on damage by the centrifugation used to concentrate the cells to 1×10^6 cm^{-3}. An increased oxygen flux was followed by a considerable inhibition on addition of 0.5 μM rotenone. When 2.5 μM antimycin A was injected into the suspension, a further slight inhibition occurred, indicating that at least some of the cells were "leaky" to succinate.

The alternative explanation is that the lactate from glycolysis was formed as a by-product of the need for biosynthetic precursors, such as serine in the formation of phospholipids [72]. These are derived from three carbons in glucose, with the remainder being converted to pyruvate. From the foregoing evidence, it is seen that the demand for ATP is being satisfied by oxidative processes, and that pyruvate reduction is primarily to conserve the NADH cycle with an "incidental" net 2 ATP from the pathway. Another source of lactate is glutaminolysis which is the incomplete oxidation of glutamine in the second half of the Krebs cycle essentially to produce amino acids [65,66,72]. This produces 6-9 ATP and, thus, provides a part of the cellular energy requirements. The magnitude appears to vary depending on cell type [60,73–76] and ranges from 35% to as high as 70% of ATP production [59]. There have been imprecise suggestions that cells actually *require* glutamine for their energy needs. It is much more likely that glutaminolysis is due to the inadequate design of media, particularly with respect to provision of appropriate amounts of both essential and so-called non-essential amino acids, the latter frequently being absent. Recent evidence in support for this contention is that comprehensive analyses of cellular metabolic requirements by a stoichiometric approach [77,78] and one using mass balances [79] has resulted in the design of more complex and refined media with consequentially lower lactate and ammonia production. Cells take advantage of energy provision from glycolysis and glutaminolysis and, in a coupled system, decrease respiration. It is significant that the pyruvate formed in glutamine breakdown is not further oxidised but, as in glycolysis, is reduced to lactate. No comment can be made upon the role of the pentose phosphate pathway because the required experimentation has not been completed but, undoubtedly, the carbon flux through it will be large because of the need for reducing power (NADPH) in biosynthesis. Bonarius et al. [79] have shown that this pathway is predominant in glucose utilisation for a particular hybridoma cell line. In terms of the validity of the heat flux probe, the quantity of heat produced in the combustion of glucose is the same irrespective of the pathway (Hess' Law).

The simultaneous involvement of anaerobic processes with oxidative pathways in catabolism can be quantified by using the CR ratio [26,35,36,55,80]. By Thornton's Rule, oxidative processes cannot produce more heat than ≈ -450 kJ mol^{-1} O_2 – the oxycaloric equivalent ($\Delta_k H_{O_2}$) [80] with the enthalpies of anaerobic pathways being additive to this quantity. In Table 1, the CR ratio during early growth is highly negative probably due to lactate production in glycolysis. For the growth reaction, r, this hypothesis can be tested,

$$\Delta_r H_{O_2(ox+anox)} = \Delta_k H_{O_2} + (Lac/O_2)\Delta_r H_{Lac} \quad (5)$$

where $\Delta_r H_{Lac}$ is the enthalpy change for the formation of lactate from glucose, which has a molar value of -77 kJ mol^{-1} when excreted into 20 mM HEPES buffer [55]. Taking the data from Table 1 at 26–38 h and using the oxycaloric equivalent for glucose (-470 kJ mol^{-1}; see Ref. [80]), this means that glycolysis accounted for only half of the lactate (7.5×10^{-17} mol s^{-1}). Even in the unlikely event of all the ammonia in the medium being produced in glutaminolysis, this would still only account for 0.67×10^{-17} mol s^{-1} (NH_4^+/lactate stoichiometry of 2). This means that no explanation can be given for 6.5×10^{-17} s^{-1} lactate. Nevertheless, the highly exothermic CR ratio during growth was a measure of the demand for glycolytic intermediates as biosynthetic precursors. This explanation is supported by the pattern of changes to lactate flux during growth in which the highest flux was seen (Fig. 5) when there was a rapid specific growth rate (see Table 1). When growth stopped, metabolism became singularly aerobic. It could be argued that proper medium design would ensure that there was no resort to anaerobic catabolism under aerobic conditions. If measuring the CR ratio could be made an on-line process, then it would be possible to monitor growth continuously and detect deviations from the ideal physiological conditions.

3.5. Relationship of heat flux to cell growth

On the reasonable assumptions that the energy for cell maintenance *during growth* was constant and that ATP production was coupled to demand, it could be expected that the heat produced in growth would be directly related to the specific growth rate. As can be seen in Fig. 8, this is precisely the result of plotting one against the other – as the specific growth rate

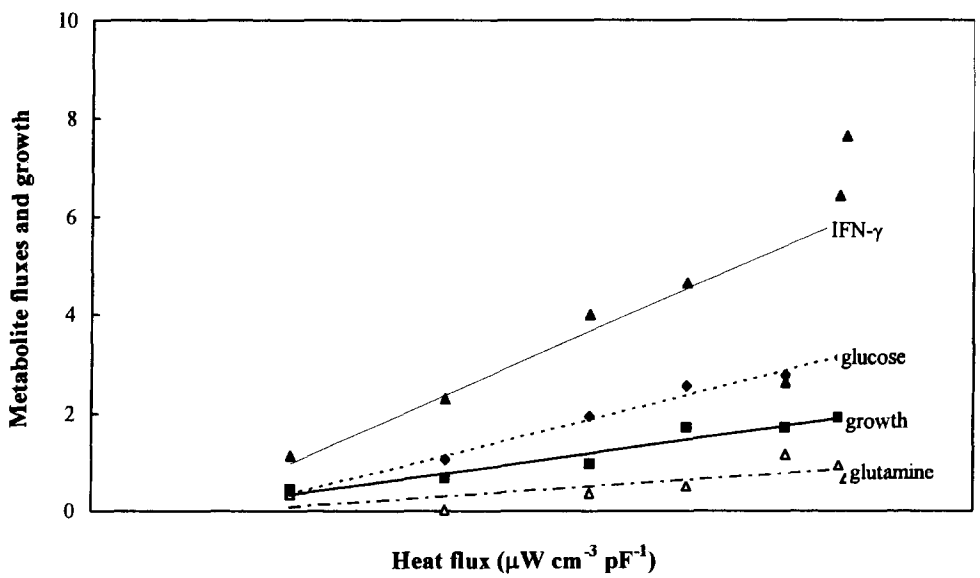

Fig. 8. Heat flux is plotted as a function of specific growth rate (■) showing the monotonic relationship. This dependence extends to the fluxes for $10^4\times$IFN-γ production flux, IU s^{-1} per cell (▲) and the major catabolites, $10^7\times$glucose consumption flux, mol s^{-1} per cell (◆) and $10^7\times$glutamine consumption flux, mol s^{-1} per cell (△).

declined during culture, so there was a monotonic decrease in heat flux. In the case of the CHO 320 cell culture, this was probably due to the substrate(s) becoming limiting to the rate of growth. In terms of irreversible thermodynamics, this would mean less Gibbs energy available for growth, so a smaller quantity was dissipated as heat. This is depicted in terms of substrate flux in Fig. 8. As mentioned previously, there is every reason to believe that IFN-γ production is growth-related [62], so its flux also declined monotonically with the other fluxes.

It was indicated earlier that cell growth was thermodynamically dissipative rather than completely irreversible with some energy being accumulated in the macromolecular structure of the biomass. This can be represented by considering the numbers of available electrons per unit carbon atom of the substrates and the biomass, the so-called degree of reduction, γ [10,13,14,19,47,81]. The value for glucose is four and that for glutamine is 3.6. At present, the elemental analysis of CHO 320 biomass is not available and, in fact, it seems only one elemental analysis has been carried out on animal cells. Bushell et al. [82] obtained an "empirical formula" for the biomass of a murine hybridoma, PQXB1/2, of $CH_{1.7}N_{0.25}O_{0.25}$; so γ_B=4.45. On the reasonable assumption that CHO 320 biomass has a similar formula, this degree of reduction means that, while some amounts of the two substrates in the present cultures are oxidised to CO_2 (no electrons, of course) and many electrons are "excreted" in lactate, a considerable number are conserved in biomass. In the case of genetically-engineered cells, this would include the amplified gene number and the expressed dhfr enzyme. The target recombinant protein, in these experiments IFN-γ, would also conserve electrons but it is secreted and no longer part of the cell. It is still within the control volume, however, and must feature in an electron balance. Many of the valence electrons conserved in biomass are incorporated via the glycolytic process and, thus, much of the Gibbs energy available from glucose is dissipated as chemical entropy in the biomass rather than as heat [83].

3.6. Coupled ATP cycle and the "metabolic burden"

Under the steady state conditions of cell growth, endothermic ATP generation (supply) is tightly coupled to exothermic utilisation (demand). Furthermore, the catabolic process is coupled to ATP supply and ATP demand is coupled to anabolic processes, which have a net enthalpy change close to zero [45]. Since the change in entropy for the oxidation of glucose [16] is small, the molar reaction Gibbs energies for this pathway is very similar to the oxycaloric equivalent. It was possible, therefore, to calculate the ATP yield per Joule using the ATP stoichiometric coefficient of oxygen of 33/6=5.5 ATP/O_2 (this is from Ref. [84], but it is not universally agreed). For the aforementioned reasons (also see Ref. [83]), the glycolytic pathway leading to the formation lactate has a considerable entropy change, meaning that the molar Gibbs energy is different to the molar enthalpy change. For the calculation, this was taken to be 50% (see Ref. [83]) and the mechanistic stoichiometry was taken to be 1.0 ATP/lactate [85]. Since much of the glutamine is incorporated into biomass as a by-product of glutaminolysis, it was assumed that the entropy change for glutamine was also considerable, necessitating a similar correction. The ATP stoichiometric coefficient for this process was calculated from Beavis [84] to be 9/1.5=6 ATP/O_2. The data are given in Table 2. Knowing the relative activities of the three pathways (see Table 1), the ATP turnover at three selected time intervals during the growth of the cells was calculated from the yield (see Table 2). Then, it was also possible to assign the proportion derived from oxidative processes compared with Gibbs energy release from glycolysis. The turnover was matched against ATP requirements in biosynthesis, knowing the molecular weights of IFN-γ and dhfr and adopting the stoichiometric ATP requirements for proteins and biomass, in general, calculated by Xie and Wang [86]. The results showed (Table 2) that the amplified gene expression system demanded ca. 40% of the ATP required in biosynthesis in the period of most active growth (26–38 h). It is well-known that the expression of foreign genes in microorganisms slows growth. It has been claimed sometimes that the same is true for animal cell systems [87,88]. It has been thought that, unless there is spare ATP production capacity in the normal counterparts of transfected cells, the availability of ATP may well limit the rate of growth since the rate of production of the foreign proteins is forced by selection of the amplified system, in the case of CHO 320 cells by methotrexate. The present experiments

Table 2
ATP turnover calculated from heat flux data and the calculated utilisation of it in biosynthesis

ATP yield per J (small entropy change)			
		(mol ATP $J^{-1} \times 10^{-5}$)	
Glucose respiration (ATP/O_2 ratio=5.5)		1.39	
Glycolysis (ATP/Lactate ratio=1.0)		1.59	
Glutaminolysis (ATP/O_2 ratio=6.0)		1.32	
ATP turnover			
Time (h)	**26–38**	**50–62**	**74–86**
ATP turnover (mol$\times 10^{-16}$)	2.80	2.49	1.74
ATP per O_2	1.51	1.20	1.46
Gibbs energy release	1.39	1.29	0.28
ATP requirements in biosynthesis			
IFN-γ (mol$\times 10^{-16}$)	0.32	0.18	0.04
Dihydrofolate reductase (mol$\times 10^{-16}$)	0.33	0.19	0.05
Cell biosynthesis (mol$\times 10^{-16}$)	1.08	0.66	0.01
Sum (mol$\times 10^{-16}$)	*1.73*	*1.03*	*0.10*
"Spare" ATP (mol$\times 10^{-16}$)	1.17	1.46	1.64
Fate of substrates			
"Biosynthetic" glucose (%)	17	34	48
"Biosynthetic" glutamine (%)	68	65	61
From radioisotope experiments, complete oxidation "non-biosynthetic" glucose (%)	9.7	10.1	91.7

(see Figs. 6 and 7) indicate that the mitochondria of even the genetically-engineered CHO 320 cells were not operating to full capacity. It is not possible at this stage to rule out restrictions at the level of translation.

A proportion of the ATP flux could not be accounted for in the biosynthetic process – the so-called "spare" ATP (Table 2). Some may have been required in the internal work associated with the cytoskeleton which possesses several motor ATPases associated with microtubules and microfilaments. The rest would have been needed for maintenance, with prominent demands including those of membrane pump ATPases. It is conceivable that the changed physiological conditions, such as an osmotic pressure change, at the end of the growth phase could have placed a demand on active transport pumps.

4. Conclusions

These studies have shown that scalar heat flux is a credible on-line monitor of metabolic activity and thereby the growth of CHO 320 mammalian cells in real time. The monotonic decline in heat flux with decreasing specific growth rate was explained in terms of substrate limitation which meant that proportionally less of the Gibbs energy was dissipated as heat. It seems likely that the large quantities of glucose and glutamine used as biosynthetic precursors were needed because the medium was inadequately supplied with certain other nutrients, especially amino acids. Good medium design would minimise this inefficiency and reduce the accumulation of toxic lactate and ammonia. Nevertheless, it is envisaged that heat flux could become a control variable in fed-batch culture, as well as in continuous ones. Since the CR ratio can detect anaerobic processes that occur during aerobic metabolism usually as a result of the imbalance in the supply of amino acids from the medium, it seems certain that the best probe would combine on-line measurements in real time of heat and oxygen fluxes with the specific growth rate assessed as capacitance by dielectric spectroscopy.

Acknowledgements

This paper is dedicated to our former respected colleague and still trusted friend, Dr Peter Evans, who was "rationalised" away from us in the pursuit of the twin-headed, false god of "economic gain" and academic "excellence".

We are grateful for thoughtful discussions with Ted Battley, Erich Gnaiger, Lena Gustafsson and Christer Larsson and for the skilled technical assistance of Phil Lloyd and Alice Williams; as well as the respirometry team of Stuart Martin, Rachel Ryan and Agnes Toth.

The research was aided by generous grants from the BBSRC (nos. 2/3680 and 2/TO3789).

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