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# Thermobiochemical evidence for the rapid metabolic rate in hybridoma cells genetically engineered to overexpress the anti-apoptotic protein bcl-2 in batch culture

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**Abstract**

A serious problem in the culture of animal cells to produce therapeutic proteins is apoptosis (programmed cell death) because it restricts the ability of the cell culture to yield the heterologous material. One means to delay apoptosis is genetically to engineer the anti-apoptotic gene *bcl-2* into the genome. This had been adopted elsewhere to give TB/C3 hybridoma cells that overexpressed bcl-2 (pEF bcl-2-MC1neopA plasmid) and a control, pEF, which contained a nonsense sequence (pEF-MC1neopA). In the spinner cultures used in the present investigation, bcl-2 cells grew for 60 h to give greater cell density at a faster specific rate and with prolonged better viability than the controls in which, after 36 h, the membrane integrity and the apoptotic index of the cells declined rapidly leading to death. Prior to 36 h, production of the monoclonal antibody was only slightly higher in the pEF control giving little evidence for the metabolic burden of its production on antibody synthesis. Bcl-2 synthesis, however, was associated with 125% increase in heat flow rate (HFR) measured in the chemically (triacetin) calibrated batch microcalorimeter. HFR is a function of the metabolic rate and it is suggested that its greater level in the bcl-2 cells than the control was caused by the increased protein production due to the *bcl-2* gene expression. The bcl-2 cells had an increased lactate flux compared with the control. The calorimetric–respirometric (CR) ratio confirmed the likelihood that glycolysis rather than glutaminolysis was the primary reason for this increase. It may be due to the limitation in mitochondrial capacity and/or the paucity of biosynthetic precursors leading to production of them from glucose.

The stationary liquid phase balance (SLPB) was shown to be the appropriate on-line method to measure the oxygen uptake rate (OUR) in the dilute cell suspensions grown in the tank bioreactor. The metabolic rate expressed as HFR was measured in a customised flow microcalorimeter, which had been calibrated using the enthalpy of hydrolysis produced by the new test reaction using methyl paraben. Both HFR and OUR declined before the decrease in cell density in batch culture, indicating that they were sensitive indicators of deteriorating environmental conditions. However, neither proved to be an early detector of the onset of apoptosis.


*Keywords:* Heat flow rate; Oxygen uptake rate; Apoptosis; Metabolic burden; Hybridoma cells

## 1. Introduction

One of the greatest problems in using animal cells in vitro to produce biopharmaceuticals is the phenomenon of programmed cell death, named apoptosis, that accounts for most of the loss of viability in cell cultures [1]. A likely strategy to alleviate the problem is genetically to engineer the human proto-oncogene, bcl-2 [2], which protects against apoptosis, into the genome of the cultured cells. To this end, Singh et al. [3] used an SV40-controlled plasmid carrying the *bcl-2* gene co-transfected with the *neo* gene that constitutively expressed neomycin phosphotransferase to destroy the antibiotic, geneticin G418 (pEF bcl-2-MC1neopA). A control was also available in which the *bcl-2* gene was replaced by a nonsense sequence (pEF-MC1neopA) so that there was only *neo* gene expression. As the experimental system, Singh et al. [3] used the TB/C3 cell line, an NS1-derived murine hybridoma producing monoclonal antibodies against IgG. One of the contentious issues in recombinant biology, however, is the degree to which the constitutive overexpression of proteins

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in genetically engineered cells retards their growth, creating the so-called "metabolic burden" (see [4]). It is conceivable that a point could be reached where the cost of protection against apoptosis in terms of providing substrates outweighs the product yield, reducing the efficiency of the process to below that of cells without the "anti-apoptotic" gene. The difficulty has been to discover an index of metabolism that unequivocally measures the burden [5] while being applicable to large-scale batch and fed-batch cultures.

Over recent years, it has been acknowledged that the overall metabolism of living matter can be quantitatively measured by direct calorimetry (see the series of reviews in [6]). This generalisation has a strong theoretical basis (see Section 2 and [7]), which was verified in off-line measurements of the extensive quantity, heat flow rate (HFR) on animal cells in vitro [8] before it was applied to mammalian cells in a bioreactor, as an on-line, ex situ analytical technique using flow microcalorimetry [9]. It was when comparing the typical, bell-shaped curve for the changes in cell number concentration with time against HFR that Guan et al. [9] noticed the latter started to decrease as an early event in the deterioration of the culture some hours before there was any effect on the cell growth measured as the increase in cell numbers. This correlated well with the effective exhaustion of the major substrates, glucose and glutamine. From this evidence, it was proposed that the intensive, cell-specific heat flux could be an excellent variable to act as a metabolic "sensor" but this required the on-line measurement of biomass. The best available on-line technique for assessing the quantity of viable biomass [10] was considered to be the impedance method using a dielectric spectrometer [11], the potential importance of which had been demonstrated for animal cells some years previously [12]. An investigation by flow cytometry showed that, providing there was little change in conductivity to give crosstalk [11], the magnitude of the capacitance signal was proportional to the volume fraction of viable cells [9]. In order to establish a baseline for the complex bioreactor studies and to discover if there is any evidence of the supposed metabolic burden, initial experiments with the TB/C3 cell line were carried out using spinner flasks and are described as the first aim of this investigation.

For many years, it was held that, providing mammalian cells were cultured under controlled, fully aerobic conditions, oxidative processes fulfilled all the energy demands in metabolism [13]. With the discovery of appropriate analytical tools in biochemistry, it was realised that, for stationary cultures, anaerobic processes leading to the production of lactate by glycolysis could become dominant, probably because not enough of the sparingly water-soluble oxygen could be delivered to the cells in the absence of the carrier, haemoglobin [14]. When highly controlled bioreactors for fully aerobic cultures were developed [15], however, it was noticed that, even under these essentially favourable conditions, lactate was excreted from cells as a toxic product [16–18]. It is generally considered that the presence of lactate in the medium has a deleterious effect on cellular processes, and particularly on post-translational changes [19]. Although minimising the production of lactate is a complex issue in the catabolic processes of cell growth (see earlier), it is probable that to measure its glycolytic production from glucose geared to biosynthetic needs is a good index of cell growth [9,20].

Lactate can be produced by the partial or complete oxidation of exogenous glutamine in the culture medium as well as being formed anaerobically under fully aerobic conditions as a by-product of the synthesis of anabolic precursors [21,22]. Therefore, chemical assays tend to over-estimate the glycolytic production of lactate. As a consequence, the most accurate, non-isotopic estimation of lactate and the one in exact relation to the stoichiometric growth reaction of cells is combinatorial calorimetry in which a ratio is obtained between the heat flow rate (or its size-specific flux) measured directly by a calorimeter and the oxygen uptake rate (or flux) obtained by indirect calorimetry. It was Lavoisier who first pioneered the twin combination for living organisms and in physiology the theoretical value that should be obtained become known as the oxycaloric equivalent. It was found to have similar value for the combustion of all carbon compounds because of the regularity for the heat evolved per equivalent of oxygen, as first reported by Thornton [23]. The most widely accepted average value, known as Thornton's rule, is $-115\,\mathrm{kJ\,deg^{-1}}$ of reductance (per equivalent of the available electrons) [24]. Kemp and Gnaiger [25] first systematically applied combinatorial calorimetry to animal cells in vitro and termed the ratio of the data from the two methods, the calorimetric–respirometric (CR) ratio (see Section 2). They calculated that, for fully aerobic systems, the ratio varied from $-430$ to $-480\,\mathrm{kJ\,mol^{-1}}$ $O_2$; an average of $450\,\mathrm{kJ\,mol^{-1}} \pm 15\%$ [26]. When there are anaerobic processes under fully aerobic conditions, the heat produced by these reactions is additive to the oxycaloric equivalent to give a more negative CR ratio.

Although rarely used in practice, all calorimeters must be calibrated with an authenticated chemical reaction as well as electrically with a resister of known value [27]. Because metabolism is a slow reaction, it is important that the reaction rate of the chemical calibration is correspondingly slow. It is generally accepted that the most suitable reaction with this property is the imidazole-catalysed hydrolysis of triacetin [28,29]. The stirred insertion vessel used in the calorimetric measurements of the cells grown in spinner flasks was calibrated by s means [30]. However, doubts have been raised about the suitability of the triacetin reaction for use in flow calorimeters [31]. It was reasoned that, although the enthalpy is relatively large, the rate constant is small and, therefore, the rate of change of signal in the flow vessel is insufficient to be reliably followed by the instrument. O'Neill et al. [31] maintained it is necessary to employ a secondary test reaction in flow calorimeters, i.e. one for which the derived parameters can be traced back to, and validated by, the test reaction in the triacetin calibrated batch calorimeter. The base catalysed hydrolysis of methyl paraben has the

appropriate physico-chemical properties. The reaction is described as pseudo first order in nature, that it is first order in the presence of excess base. The heat flow of the reaction was used to calibrate the flow vessel.

If there were any indications that off-line heat flow rate measurements on spinner cultures indicated changes to metabolism in the batch culture of hybridoma cells, it was our intention to employ the technology to on-line bioreactor-controlled batch cultures. It is generally considered that the chances of detecting subtle metabolic changes are much better in on-line systems because of the continuous record. It should apply to the problem of apoptosis in particular because one of the triggers to initiate it [30] is the mitochondrial permeability transition (MPT), which can occur at any stage of the batch culture when there are adverse conditions. MPT has been detected by a change in oxygen uptake rate (OUR) [30] and certainly should be accompanied by an increase in HFR because the MPT uncouples respiration [7]. If these changes do indeed occur then, unless they are totally compensatory, they should be reflected in the values for the CR ratio at the critical time. The problem with the idea of using OUR, however, is that the normal polarographic method to determine it with a Clark-type electrode [32] is not sensitive enough for the low cell number concentrations found in the early stages of batch cultures of animal cells, typically starting at $2 \times 10^5\ \mathrm{cm}^{-3}$ [9]. Ruffieux et al. [33] reviewed alternatives, including that requiring expensive mass spectrometry [34,35], and came to the conclusion the method that involves constructing the stationary liquid phase balance (SLPB) was the most acceptable in terms of sensitivity, ease of use and cost. Others, particularly Ramírez and Mutharasan [36], had previously utilised this method after it had been pioneered by Miller et al. [37]. Ducommun et al. [38] described the modern refinement of this method in terms of the precision flow meters and the highly porous, hydrophobic polytetrafluoroethylene (PTFE) tubing used in addition to the bulk phase/headspace interface to aerate the culture and thus give a stable oxygen mass transfer coefficient ($k_\mathrm{L}a$) (see Section 2). The second aim of this investigation is to construct the required instrumentation to apply the SLPB method to cells in bioreactor-type batch cultures and to use it to measure the OUR of the hybridoma cells simultaneously with the HFR, prior to employing the CR ratio on-line as an indicator for changes in the metabolic balance in these cultures.

## 2. Theoretical

It is necessary to use a heat conduction calorimeter [39] for this type of study because it measures the heat flow *rate* ($\Phi = \mathrm{d}Q/\mathrm{d}t$, watts, W). Thus, the measurement gives the kinetics of the process as well as providing thermodynamic information on such properties as enthalpy change, $\Delta H$. Gnaiger [40] advanced the idea that heat flow is effectively the rate of thermal (th) advancement, $\mathrm{d}_\mathrm{th}\xi/\mathrm{d}t$, in the energy transformations. The advancement, or the extent of reaction as it is sometimes known, is an important concept in energy transformation because it is expressed explicitly in terms of the stoichiometric coefficients, $v_i$, of the $i$th species in the specified reaction. In other words it is exactly equivalent to the set of stoichiometric coefficients that describe a reaction, even one as seemingly complex as the growth reaction [6,41].

What is not always appreciated is that the phrase metabolic rate simply denotes the rate of advancement of the aggregated biochemical reactions in the growth reaction [41] of living cells, $\mathrm{d}\xi_\mathrm{B}/\mathrm{d}t$, or their flux when the rate is expressed as specific to mass, $(1/X)(\mathrm{d}\xi_\mathrm{B}/\mathrm{d}t)$ where $X$ represents the amount of biomass. The thermal advancement of energy transformation, $\mathrm{d}_\mathrm{th}\xi$, is related to $\mathrm{d}\xi_\mathrm{B}$ by the expression [41],

$$\mathrm{d}_\mathrm{th}\xi = v_i\,\Delta H_{\mathrm{B},i}\mathrm{d}\xi_\mathrm{B} \tag{1}$$

where $\Delta H_{\mathrm{B},i}$ is the molar enthalpy of the reaction in terms of species $i$ [6]. The change in thermal advancement, $\mathrm{d}_\mathrm{th}\xi$ is exactly equivalent to the change in heat $\mathrm{d}Q$. Thus, heat flow is a function of the metabolic rate.

With respect to the oxygen measurements (indirect calorimetry), the principle for using the SLPB is that, because the dissolved oxygen concentration, $C_\mathrm{L}$, is maintained constant, the oxygen transfer rate (OTR) must be equal to the OUR. Therefore, an oxygen balance in the liquid phase yields:

$$\mathrm{OTR} = k_\mathrm{L}a[C_\mathrm{L}^* - C_\mathrm{L}] \tag{2}$$

where $C_\mathrm{L}^*$ is the dissolved oxygen concentration in equilibrium with the gaseous phase in the PTFE tubing. The gas flow in the tubing is 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 oxygen is in equilibrium between the gas and liquid phases [28,36,42]. So, $C_\mathrm{L}^*$ can be calculated as

$$C_\mathrm{L}^* = B\frac{P n_\mathrm{M} F_{\mathrm{O}_2}}{H F_\mathrm{T}} \tag{3}$$

where $F_{\mathrm{O}_2}$ and $F_\mathrm{T}$ are the oxygen and total gas flow rates respectively, $H$ is the apparent Henry's constant for oxygen in the medium, $P$ is the total pressure, $n_\mathrm{M}$ is the molar concentration of the medium (assuming water), and $B$ is a conversion factor equal to 1000 when $C_\mathrm{L}^*$ is expressed in millimolar quantities. Henry's constant can be readily obtained assuming that the oxygen concentration in the medium saturated with air is 0.194 mM [37]. At constant DO, OUR is related to the specific $\mathrm{O}_2$ uptake rate ($q_{\mathrm{O}_2}$, $\mathrm{mol\,h}^{-1}$ per cell) at a given cell number concentration ($N$, cells $\mathrm{m}^{-3}$), as follows:

$$\mathrm{OUR} = q_{\mathrm{O}_2} N \tag{4}$$

When it is necessary for thermodynamic and energetic reasons to express the results of the OUR measurements in

terms of the heat fluxand/or molar reaction enthalpy, the appropriate oxycaloric equivalent, $\Delta_k H_{O_2}$, is applied to the data for oxygen flux ($J_{O_2}$),

$$J_Q = \Delta_k H_{O_2} J_{O_2} \quad (5)$$

With reference to experimentation by combinatorial calorimetry, the theoretical oxycaloric equivalent is the same as the expected ratio of the calorimetric heat flux and the respirometric oxygen flux, the CR ratio,

$$\text{CR ratio} = \frac{J_Q}{J_{O_2}} \quad (6)$$

Gnaiger and Kemp [26] established that the highly exothermic CR ratios in animal cells are due to the integration of anaerobic pathways with aerobic metabolism. They showed that the net production of the most common anaerobic end product, lactate, is accompanied by a dissipative catabolic enthalpy change, $\Delta_k H_{Lac}$, of $-80\,\text{kJ mol}^{-1}$ when the acid is buffered in the cytosol. However, the plasma membrane is very permeable to lactate (and pyruvate) and so the enthalpy change depends on the nature of the buffer in the medium [14]. It is $-80\,\text{kJ mol}^{-1}$ when excreted into a bicarbonate buffer, $-59\,\text{kJ mol}^{-1}$ into a phosphate buffer and $-77\,\text{kJ mol}^{-1}$ into 20 mM HEPES buffer. The molar amount of lactate (Lac) produced per unit amount of oxygen consumed ($Lac/O_2$) is a good indication of the relative extent of the aerobic glycolysis [26]. The catabolic ($k$) heat change per mol $O_2$, $\Delta_k H_{(ox+anox)}$ (CR ratio), is then calculated as

$$\Delta_k H_{(ox+anox)} = \Delta_k H_{O_2} + \frac{Lac}{O_2} \times \Delta_k H_{Lac} \quad (7)$$

Similar equations can be constructed for other anaerobic products (p). In a generalised equation, P can be substituted for Lac and, if there is more than one such product, the heat effect is additive, $\sum_p Lac/O_2 \times \Delta_k H_p$.

On the subject of the chemical calibration of the flow calorimeter using the base catalysed hydrolysis of methyl paraben, it is well established that the heat flow ($\Phi$, see above) for a first-order reaction can be expressed by the following [31]:

$$\Phi = -FCH(1 - e^{-k/\tau})e^{-k/t} \quad (8)$$

where $F$ is the flow rate ($\text{dm}^3\,\text{s}^{-1}$), $C$ the concentration ($\text{mol dm}^{-3}$), $H$ the molar enthalpy change ($\text{kJ mol}^{-1}$), $k_1$ the first-order rate constant ($\text{s}^{-1}$), $t$ the time (s), and $\tau$ is the residence time (s). An enthalpy change of $-51\,\text{kJ mol}^{-1}$ was used, which previously had been shown valid for the temperature range of 20–52 °C [31]. The rate constant was determined from a plot of the natural log of the heat output versus time and the residence time was derived using Eq. (8). The residence time, $\tau$, is related to the thermal volume, $V_c$, as follows:

$$\tau = \frac{V_c}{F} \quad (9)$$

## 3. Experimental

### 3.1. Cells, culture conditions, membrane integrity and apoptotic index

The NS1 derived murine hybridoma, TB/C3, used in this investigation produces antibodies against human Immunoglobulin G (IgG), [43]. The two variants were cells that were: (i) stably transfected with the bcl-2 expression plasmid (PEF bcl-2-MClneopA), known as TB/C3 bcl-2; and (ii) transfected with a control plasmid (pEF-MClneopA)—TB/C3 pEF. Both lines express neomycin phosphotransferase constitutively; the TB/C3 bcl-2 cells in addition constitutively overexpresses the bcl-2 protein. The mammalian-type neomycin, geneticin G418, was only used to maintain the transfection in the cells by passage in it every 10 weeks [44]. This is necessary because the number of plasmids in the mammalian genome is diluted with successive cell generations.

The cells were routinely cultured in 350 $\text{cm}^3$ spinner flasks using RPMI 1640 medium (Gibco, UK) supplemented with 5% (v/v) fetal calf serum (Gibco, UK) and 2 mM L-alanyl-L-glutamine (Glutamax©, Gibco, UK), and contained the antibiotics, streptomycin (100 $\mu\text{g cm}^{-3}$) and penicillin (100 units $\text{cm}^{-3}$). The medium was buffered to pH 7.2 with 24 mM sodium bicarbonate. The cells were counted with a modified Fuchs–Rosenthal haemocytometer and seeded into the batch culture at an initial density of $2 \times 10^5$ cells $\text{cm}^{-3}$. The flasks were incubated at 37 °C and stirred at the constant 200 rpm. Headspace gassing of the culture medium using 5% (v/v) $CO_2$ in air was performed after the initial inoculation and at every subsequent sample removal.

Cell viability was assessed by the trypan blue dye exclusion method [44]. The result was expressed as the percentage membrane intact cells with respect to the total cell population to give the index of membrane integrity (MI). The apoptotic index (AI) was measured using fluorescence microscopy (excitation 488 nm) to identify nuclear condensation after staining with acridine orange and propidium iodide [44]. The concentrations of IgG monoclonal antibody produced by both cell lines were assayed using an ELISA method [45].

### 3.2. Off-line measurements of catabolic variables and monoclonal antibody in spinner culture

The OUR of the cell suspensions was measured off-line using an Oroboros Oxygraph twin-channel respirometer (Innsbruck, Austria) [46] with high-resolution Orbisphere electrodes (Model 2120). The cell suspension, 2 $\text{cm}^3$, was added to each chamber maintained at 37 °C and stirred at 450 rpm with a customised Oroboros magnet. Prior to measurement, the instrument was calibrated at 0 and 100% $O_2$ [46].

The HFR of the cells was measured off-line using a stirred 4 $\text{cm}^3$ stainless steel perfusion/titration vessel in a

Thermal Activity Monitor (TAM) batch microcalorimeter (Model 2277, Thermometric AB, Järfälla, Sweden) [47]. The calorimeter was routinely calibrated electrically with the 50 Ω resister and chemically by the hydrolysis of triacetin at the required stirring rate [26–30]. A 3 $cm^3$ aliquot of the cell suspension was transferred to the perfusion/titration vessel and stirred with a KelF turbine [48] at 60 rpm for the duration of the experiment. The vessel was slowly lowered into the measuring cup according to the manufacturer's instructions and the data was collected using the Applikon BioXpert data acquisition program (Applikon Ltd., Tewksbury, Glous.). Lactate and glucose estimations were carried out by HPLC using a Rezex reversed phase organic acid column (Phenomenex UK Ltd.) calibrated with the appropriate standards.

### 3.3. On-line cell culture in the bioreactor and measurement of its catabolic variables

A 3 l jacketed tank bioreactor (Applikon) containing the 2000 $cm^3$ suspension of TB/C3 bcl-2 cells in the RPMI 1640-based medium (see Section 3.1) was used in all the experiments. Cells were inoculated at an initial cell density of $2.5 \times 10^5$ cells $cm^{-3}$. The pH of the culture medium was maintained at $7.2 \pm 0.05$ using 0.5 M NaOH and 0.5 M HCl. A marine-type impeller was used for stirring at 60 rpm with a tip speed of 0.33 cm $s^{-1}$. The temperature of the culture was $37 \pm 0.2$ °C, using a water jacket. As described previously [9], all these basic parameters were acquired by the Applikon BioXpert software and maintained constant by the Biocontroller. Aeration of the medium was achieved by circulating $CO_2$, $N_2$ and $O_2$ at the constant gas flow rate of 450 $cm^3$ $min^{-1}$ into 1 m highly porous PTFE tubing (W.L. Gore and Associates GmbH Putzbrunn, Germany) fixed in the bioreactor [38]. The flow rate of the individual gases was controlled by a calibrated mass flow meter (Brooks, Veenendaal, The Netherlands), connected to the BioXpert-containing PC by a digital–analog converter card. The gas mixture passed to the headspace before leaving the bioreactor through a cooled, jacketed condenser. This had the effect of increasing the surface area available for gaseous exchange. The concentration of dissolved $O_2$ (DO) in the medium was measured with a polargraphic oxygen electrode (Ingold, Urdorf, Switzerland). A PID control program was developed using the BioXpert software to maintain the DO at the constant level of 80% by altering the composition of gas supplied to the tubing with the mass control meters, whilst keeping the gas flow constant at 450 $cm^3$ $min^{-1}$. Several experiments were performed to optimise the PID control algorithm. The oxygen diffusion coefficient, $k_L a$, was calculated from initial 'gassing in' experiments prior to inoculating the medium in the bioreactor with the cells [38].

The HFR of the suspended cells was measured continuously ex situ at $37 \pm 0.001$ °C using a thermometric TAM flow microcalorimeter exactly as described in [9] but replacing the standard gold measuring vessel with the customised, stainless steel one of 1 $cm^3$ nominal volume, as detailed in [49]. This was calibrated electrically as described in Section 3.2 and chemically using methyl paraben (see Section 3.4). The cell suspension was drawn through the flow vessel at 100 $cm^3$ $h^{-1}$ by a Jubile peristaltic pump (H.J. Guldener, Zürich, Switzerland) placed distal to the flow vessel.

Samples were taken at regular intervals for counting the cells by haemocytometry (see Section 3.1).

### 3.4. Chemical calibration of the calorimetric flow vessel

For the first order, base catalysed hydrolysis of methyl paraben, 0.3808 g methyl paraben (Sigma Poole, UK) was added to 50 $cm^3$ 0.5 M NaOH (Sigma Poole, UK) with vigorous stirring. in a water bath at 37 °C. The reaction mixture was then pumped at different flow rates in the range of 30–180 $cm^3$ $h^{-1}$ in a continuous loop through the flow vessel in the microcalorimeter set to the temperature of $310 \pm 0.001$ K. The data were collected using Applikon BioXpert data acquisition software, and analysed using Microsoft Excel. As expected from Eq. (9) and shown experimentally by O'Neill et al. [31], a good linear fit was obtained from the plot of $\tau$ versus $1/F$. The experimentally derived thermal volume was found to be $1.28 \pm 0.2$ $cm^3$, which differs significantly from the nominal (engineered) volume of 1 $cm^3$ [49]. This means that some of the tubing downstream from the engineered measuring vessel was included in the "effective" volume of the vessel [31].

## 4. Results and Discussion

The results from the cells grown in spinner flasks showed that both cell types produced very similar numbers of cells until 36 h (see Fig. 1A). At the later stages of the batch cultures, the data confirmed the findings by Simpson et al. [43] from cell counts and microscopic observation of living, necrotic and apoptotic cells that the constitutive overexpression of the *bcl-2* gene allowed the TB/C3 bcl-2 hybridoma cells to continue growth during the time (48–60 h) at which the TB/C3 pEF control cells that do not express the bcl-2 protein showed considerable decline in viable numbers (see Fig. 1A). By 48 h, the pEF viable cell number was beginning to decrease and afterwards there was both a marked loss of MI measured by trypan blue exclusion (Fig. 1B) and an increase in the nuclear condensation and therefore the AI, as indicated by the fluorescent stains, acridine orange and propidium iodide (Fig. 1C). These two effects resulted in the rapid loss of viable pEF cells after 48 h (Fig. 1A). In this case, the amount of native bcl-2 protein in the pEF cells presumably was insufficient to regulate the MPT [50] that is said to cause the downstream events leading to necrosis and an increase in the apoptotic index (see [51]).

The average fluxes of some crucial metabolic variables for the two cell lines in the period from 24 to 36 h are depicted in Table 1. Close scrutiny of the differences in metabolism

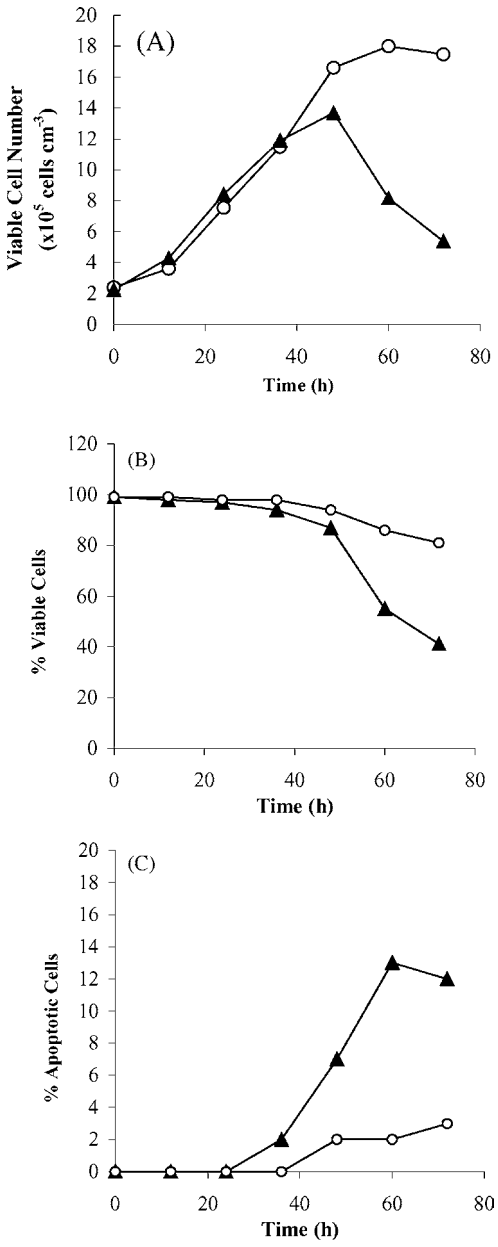


Fig. 1. Representative curves comparing growth with time in spinner culture of TB/C3 bcl-2 (○) and TB/C3 pEF (▲) cells suspended in RPMI medium with 5% FCS: (A) viable cell numbers, (B) percentage viability, (C) percentage apoptotic cells.

between the pEF and bcl-2 cells revealed that the specific growth rate ($\mu$) was significantly (Student's $t$-test: $P < 0.05$) ~20% faster in the latter and that the flux for the production of the monoclonal antibody was depressed by over 8%. On the other hand, the catabolic fluxes ($J$) of glucose, oxygen and lactate in the bcl-2 cells were markedly higher than in the control. This may well have been caused by the demands of the recombinant bcl-2 production for ATP synthesis [52] and/or to provide the biosynthetic precursors not, or no longer, available in the medium (see [53]). Guan and Kemp [54] showed that the requirement for these precursors was accompanied by lactate production and was due to the medium not being optimised for the supply of substrates and the necessary precursors. It can be seen from the theoretical treatment leading to Eq. (7) that lactate production under aerobic conditions makes the CR ratio more negative than the oxycaloric equivalent for glucose, $-470\,\mathrm{kJ\,mol^{-1}}$ $O_2$ [26]. The experimental CR ratio in both cell types typically gave a value that indicates the glycolytic pathway was active under aerobic conditions (Table 1) because the data were more exothermic than the appropriate oxycaloric equivalent. As in other reported cases [6,25,53], the highly exothermic CR ratio for the hybridoma cells was probably an indicator of cell growth. It is interesting that $CR_{exp}$ ratio for the bcl-2 cells was almost 10% more negative than for the pEF cells (Table 1) in line with the finding that the specific growth rate for the bcl-2 cells was faster than the control. There are two possible explanations: (i) the production of recombinant bcl-2 protein required biosynthetic precursors from glucose; and/or (ii) the mitochondria had reached their respiratory capacity and the demanded ATP had to come by substrate phosphorylation. The off-line measurements of lactate were incorporated into the calculation shown in Eq. (7) to give the calculated CR ratio using the $J_{Lac}/J_{O_2}$ ratio (see Table 1). Although the $CR_{cal}$ ratio for the pEF cells was similar to the $CR_{exp}$ ratio, it was inexplicably different, but probably not significantly so, for the bcl-2 cells.

An interesting aspect of the results from the spinner cultures is that the bcl cells produced slightly but not significantly less (Student's $t$-test: $P > 0.1$) monoclonal antibody than the pEF cells over the first 36 h in culture before the production by the latter was arrested, owing to increasing cell death (Table 1). This possibility was recognised in the experimental design for the present work and two of the possible reasons may have been: (a) competition with the bcl-2 protein for the available biosynthetic machinery in the anabolic pathway; and/or (b) the demand in the amount of chemical energy (exergy) in the form of ATP available for the anabolic process exceeding mitochondrial supply (similar to (ii) above). Circumstantial evidence that the overexpression of bcl-2 protein accelerated the metabolic rate of the cells comes from the fact depicted in Fig. 2 that the heat and oxygen fluxes of the bcl-2 cells were considerably greater by 125% than in the control during the first 36 h despite the fact that the specific growth rate was faster by only 20% (Table 1). The experiment was truncated at 60 h because the variability in the usually high degree of cell death after that time may well be the cause of the low reproducibility of the data.

The fact that the heat flux is a function of the metabolic flux is demonstrated theoretically in Eq. (1). The relationship of the heat flux to the ATP flux derives from the fact that, in fully aerobic metabolism, the cycle of ATP production and degradation is coupled to demand in anabolism and work [52,54]. It is reasoned that, since the overall enthalpy change of anabolism is normally close to zero [55], it is the catabolic pathway coupled to the ATP cycle that is reflected in the heat

Table 1
Comparison of the crucial metabolic fluxes ($J$), the specific growth rate ($\mu$) and the calorimetric–respirometric (CR) ratio between TB/C3 pEF and TB/C3 bcl-2 cells grown in spinner flasks on RPMI 1640 medium with 5% (v/v) FCS

| Cell lines | $\mu$ ($\times 10^{-2}\,h^{-1}$) ($\pm$S.E.) | $J_{Glc}$ ($\times 10^{-17}$ mol s$^{-1}$ per cell) | $J_{Lac}$ ($\times 10^{-17}$ mol s$^{-1}$ per cell) | $J_{O_2}$ ($\times 10^{-17}$ mol s$^{-1}$ per cell) | $J_{\Phi/N}$ (pW per cell) ($N$) | $J_{mAb}$ ($\times 10^{-7}$ ng s$^{-1}$ per cell) ($\pm$S.E.) | $CR_{exp}$ ratio (kJ mol$^{-1}$ $O_2$) | $CR_{cal}$ (kJ mol$^{-1}$ $O_2$) | $J_{Lac}/J_{Glc}$ | $J_{Lac}/J_{O_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| TB/C3 pEF | 3.54 $\pm$ 0.24 | 4.3 | 2.9 | 3.8 | 19.9 | 5.9 $\pm$ 0.4 | −524 | −511 | 0.67 | 0.76 |
| TB/C3 bcl-2 | 4.21 $\pm$ 0.29 | 6.2 | 6.6 | 7.8 | 44.8 | 5.4 $\pm$ 0.4 | −574 | −518 | 1.06 | 0.85 |

The cited values were averaged from measurements made at 24 and 36 h and were the mean of three experiments. $CR_{exp}$ was calculated from the off-line HFR and OUR data whereas $CR_{cal}$ was calculated according to Eq. (7) using the $J_{Lac}/J_{O_2}$ ratio.

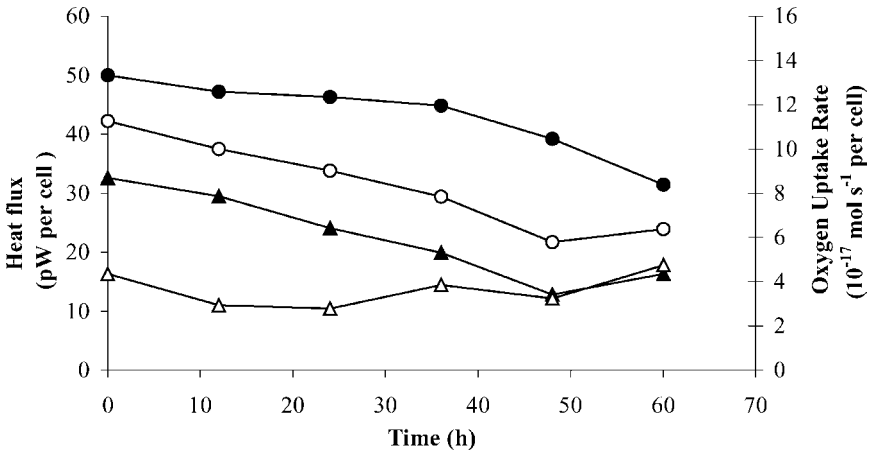


Fig. 2. Representative heat flux (solid symbols) and oxygen flux (open symbols) curves of TB/C3 pEF (▲, △) and TB/C3 bcl-2 (●, ○) cells grown for 60 h in spinner cultures on RPMI 1640 medium plus 5% FCS.

flux. As mentioned earlier, it is possible that the demand for ATP exceeded supply by oxidative and substrate phosphorylation and that this limited protein synthesis in terms of the production of monoclonal antibody (see Table 1). Further experimentation would be required, however, to demonstrate that the mitochondria were operating at respiratory capacity, using an uncoupler to give the 100% marker [56] and/or mitochondrial fluorescence intensity with the cationic dye, rhodamine 123, quantitatively detected by flow cytometry [5]. It is not clear why the heat and oxygen fluxes increased in the pEF cells after 48 h but it may have been owing to the adverse changes occurring to the cell culture during this period. As seen in Fig. 1, there were increases in the MI (Fig. 1B) and AI (Fig. 1C) indices, which means that the cells were dying not only because of necrosis but also they were undergoing degradative biochemical alterations owing to apoptosis [50]. It is conceivable that the processes involved in this phase produced heat. Increased glycolysis might have been another reason for the increase in heat flux, although there was no evidence of increased lactate production in the pEF cells after 48 h (Fig. 3). In fact, it would appear that the CR ratio became less negative towards the end of the culture period, signifying the decreased intensity of anaerobic processes with the cessation of cell growth. These results also showed the course of glucose consumption by the two cell types. As could be expected from the heat flux data, more glucose was utilised by the bcl-2 cells than the pEF cells (Fig. 3). Some scientists (e.g. [57,58]) favour substrate limitation as the reason for the metabolic burden but, at least in the first 36 h, glucose was not limiting to the metabolic flux of the bcl-2 cells. It is likely that in both cases glucose became rate limiting later in the batch culture and this was mirrored in the apparent decrease in lactate excretion with the increasing age of the batch culture towards senility. In fact, in the case of the pEF cells, at least, there was some evidence of lactate consumption under conditions of

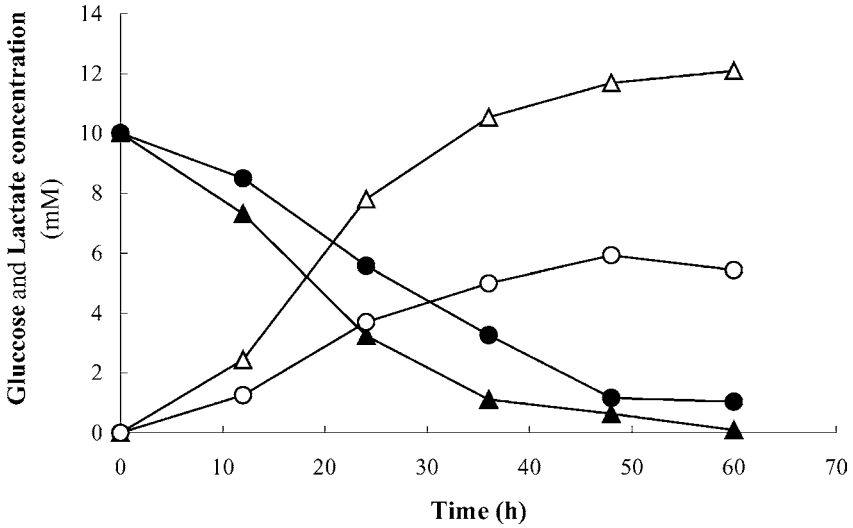


Fig. 3. Representative curves showing the changes of glucose (solid symbols) and lactate (open symbols) concentrations during the batch culture of TB/C3 pEF (▲, △) and TB/C3 bcl-2 (●, ○) cells grown in RPMI 1640 medium with 5% FCS for 60 h using spinner cultures.

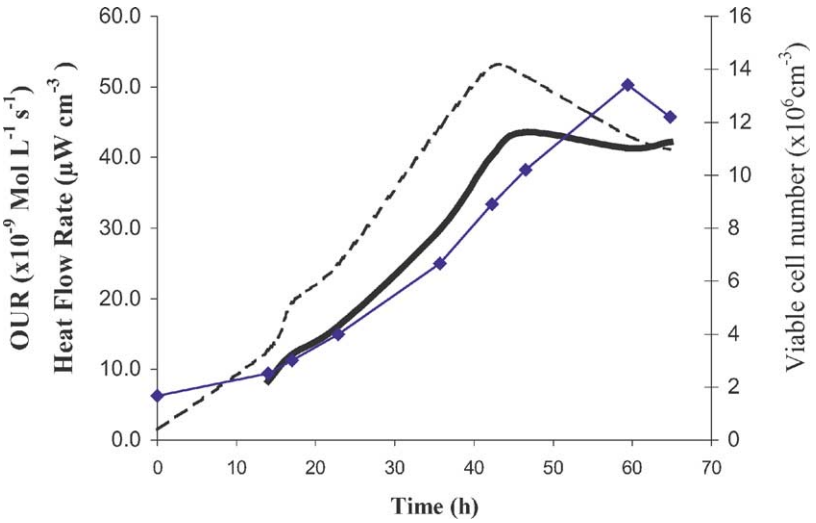


Fig. 4. Typical on-line traces for heat flow rate (- - -) and oxygen uptake rate (—) compared with off-line counts of the viable cell density (■), during the batch culture of TB/C3 bcl-2 cells in the Applikon tank bioreactor.

little glycolysis as indicated by the CR ratio. It has not been shown that these cells are able to oxidise lactate but that is a possible explanation for the decreasing amount of lactate in the medium later in the culture period. If there is indeed a metabolic burden, some favour substrate limitation as the reason for it but, at least in the first 36 h, glucose was not limiting to the bcl-2 cells. In late exponential growth, glucose became quickly exhausted and cells would have had to resort to gluconeogenesis to supply glucose for essential metabolic requirements.

Turning to the tank batch cultures, the on-line curves for HFR and OUR of the growing TB/C3 bcl-2 cells are shown in Fig. 4. The decreases in the values for the two metabolic variables from approximately 44 h onwards, occurred whilst the cells were still increasing in number. This prediction of the forthcoming termination of cell growth was first reported for batch cultures of CHO 320 cells [8,9] in which, similarly to the data expressed in Fig. 4, the HFR reduced in value before the peak of cell growth. This finding was interpreted as meaning that the cells in G1 phase of the cell cycle were still able to divide once more despite the fact that the metabolic rate was in decline, probably because of substrate exhaustion and/or the accumulation of toxic metabolic by-products [9]. On this basis, Guan et al. [9] suggested that on-line, instantaneous measurements of scalar heat flux obtained by using the capacitance measurement of viable cell mass to give the intensive value would be an excellent control variable to indicate the metabolic status of the cells (see [59]). However, even though for the spinner cultures at least the percentage apoptotic bcl-2 cells was 10% at 48 h (see Fig. 1C), OUR and HFR measurements in the tank bioreactor unfortunately gave no direct indication of the putative MPT (see [60]). Although it is reasonable to assume from the spinner cultures that apoptosis was prevalent in the bioreactor at 48 h, it is possible that the averaging effect of many cells in a large volume would mask the detection of it by MPT.

## 5. Conclusions

In line with the first aim, our studies of spinner cultures showed that the overexpression of bcl-2 protein in TB/C3 hybridoma cells caused a significant increase in the metabolic flux as measured by the heat flux. Additional evidence for the accelerated metabolism was provided by the greater glucose and lactate fluxes. The specific growth rate was faster than in the controls but there was a small decrease in the monoclonal antibody flux. This indicated that overexpression of the bcl protein caused a small metabolic burden to the cells. The increased heat flux probably was due to the greater demand for mitochondrial ATP because the formation of each peptide bond requires four to five ATP molecules [52]. Because the capacity of the mitochondria to produce ATP must be limited, then unfulfilled demand for ATP may cause an increased glycolytic flux and also the reductions in specific growth rate and the production of particular proteins. It is also possible that substrate limitation may cause the metabolic burden [61] but this was not the case at least for glucose. Alternatively, a given cell type has a finite number of ribosomes [62] and thus the bottleneck could be at the level. The further experimentation detailed above is required to answer these issues.

Extending our findings to the second aim, the results for the bioreactor-controlled cultures saw the successful adoption of SLPB as the means to measure OUR but neither it nor HFR, despite their early detection of the onset of adverse conditions for cell growth, revealed the MPT [60] as a possible trigger for the onset of apoptosis. Nevertheless, both OUR and HFR, together with the CR ratio, have potential in discovering how changes in such medium ingredients as amino acids [43], including glutamine [63], dissolved oxygen [63], $Mg^{2+}$, $Ca^{2+}$ and $K^{+}$ ions [64] and vitamins [65] induce the apoptotic cascade.

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