On-line heat flux measurements improve the culture medium for the growth and productivity of genetically engineered CHO cells

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Abstract

With the increasingly competitive commercial production of target proteins by hybridoma and genetically engineered cells, there is an urgent requirement for biosensors to monitor and control on-line and in real time the growth of cultured cells. Since growth is accompanied by an enthalpy change, heat dissipation measured by calorimetry could act as an index for metabolic flow rate. Recombinant CHO cell suspensions producing interferon- γ were pumped to an on-line flow calorimeter. The results showed that an early reflection of metabolic change is size-specific heat flux obtained from dividing heat flow rate by the capacitance change of the cell suspension, using the on-line probe of a dielectric spectroscope. Comparison of heat flux with glucose and glutamine fluxes indicated that the former most accurately reflected decreased metabolic activity. Possibly this was due to accumulation of lactate and ammonia resulting from catabolic substrates being used as biosynthetic precursors. Thus, the heat flux probe is an ideal on-line biosensor for fed-batch culture. A stoichiometric growth reaction was formulated and data for material and heat fluxes incorporated into it. This showed that cell demand for glucose and glutamine was in the stoichiometric ratio of $\sim 3:1$ rather than the $\sim 5:1$ in the medium. It was demonstrated that the set of stoichiometric coefficients in the reaction were related through the extent of reaction (advancement) to overall metabolic activity (flux). The fact that this approach can be used for medium optimisation is the basis for an amino-acid-enriched medium which improved cell growth while decreasing catabolic fluxes.

Abbreviations: dhfr – dihydrofolate reductase; FAF-BSA – fatty acid-free bovine serum albumin; FDA – fluorescein diacetate; HEPES – *N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); IFN- γ – interferon- γ ; ν_{S_1} , ν_{S_2} , ν_O , ν_L , ν_C , ν_N , ν_H , ν_X – stoichiometric coefficients for glucose, glutamine, oxygen, lactate, carbon dioxide, ammonia, water and biomass.

Introduction

In recent years, there has been increasing use of insect and mammalian cell lines to produce heterologous proteins, such as recombinant proteins, antibodies and vaccines (Spier et al., 1994; Funatsu et al., 1997). In most cases, the yield of these macromolecules has been low compared with the synthesis of biomass (Castro et al., 1995) and, in the case of recombinant glycoproteins at least, the quality has been poor (Hooker et al., 1995) supposedly owing to adverse physiological conditions (Jenkins, 1995). One of the most obvious reasons for these problems is that the

design of media has been essentially empirical in nature and not based on cellular requirements. In order to correct this deficiency by applying an analytical approach, it is necessary to achieve a greater quantitative understanding of metabolism. Recently, there has been considerable progress in this direction (Bonarius et al., 1996) so that it is possible to identify the quantitative requirements for both catabolic and anabolic substrates in the growth of particular cell lines and redesign the medium to optimise product yield (Xie and Wang, 1994, 1996b). These studies have also revealed that much of the lactate and ammonia accumulation that can be harmful in batch cultures (Borys et al.,

1994; Omasa et al., 1992), is due to the cells having to produce biosynthetic precursors from glucose and glutamine to counter medium deficiencies. At least in terms of glycolysis, this is certainly a more sensible reason for the build-up of lactate than the Crabtree effect being in operation despite allosteric regulation at phosphofructokinase.

The build-up of toxic products was one reason for the common adoption of fed-batch strategies in large-scale animal cell cultures. The other, of course, was the need to replenish the substrates, most clearly identified as glucose and glutamine. In most protocols for this approach (Bailey and Ollis, 1986), the concentrations for and timing of feeding for a specific growth rate was predetermined by experimentation and then applied without monitoring the resulting growth. In a few studies (see, for instance, Xie and Wang, 1996a), the increase in the number of cells has been recorded off-line and then more medium added when there was reduced growth. This is obviously unsatisfactory for routine industrial production but there are only two on-line techniques available to measure biomass (Zhou and Mulchandani, 1995), optical density probes usually with laser light (Wu et al., 1995) and radio-frequency dielectric spectroscopy (Harris et al., 1987). The former does not discriminate between living and dead cells whereas the latter measures capacitance of the cell culture which, for good theoretical reasons supported by experimentation, has been shown to be directly related to the volume fraction of viable cells (Davey et al., 1997; Guan and Kemp, 1998a). It is well known, however, that the reduction in bulk cell growth is a relatively late event in retarded metabolism (Guan et al., 1998). The resulting delayed response in terms of feeding could result in sub-optimal protein production. There is an urgent requirement, therefore, for on-line biosensors which will give an early indication of reduced metabolic flux.

In bioenergetic terms, the metabolic process in higher organisms can be regarded as the continuous flux of electrons from the chemical bonds of substrates to oxygen in mitochondria. Within these organelles, this flux is used to maintain the proton flux, the energy of which is harnessed to the synthesis of ATP. In growth, some of the electrons are conserved in the accumulation and structuring of biomass (entropy dissipation of Gibbs energy) but the vast majority is lost as H_2O . In the overall process, the high quality, useful energy (Gibbs energy) in the chemical bonds of substrates is used mainly to produce ATP endergonically for the coupled energetic demands of ATP hydroly-

sis in (i) anabolism; (ii) maintaining the physiological state of the cell (Roels, 1983); and (iii) performing the internal work concerned with the functions of the cytoskeleton, such as intracellular vesicular transport. In the energy transformation to synthesise ATP, the quality of the energy is reduced and so this part of the Gibbs energy from substrates is dissipated as heat. On the reasonable assumption that ATP demands for maintenance [(ii) above] and internal work [(iii) above] during growth are constant, it can be seen that the rate of growth is directly related to the rate of heat dissipation. Since growth cannot be reversed, strictly it must be considered within the concepts of Irreversible Thermodynamics (Prigogine, 1967). The minority of the Gibbs energy is dissipated entropically in growth and the majority is dissipated as heat. Put simply, the above considerations mean that the growth of animal cells can be measured directly by calorimetry (Kemp, 1993).

In order to translate the above thermodynamic theory into the practice of an on-line biosensor, the calorimeter should be of the heat conduction type in which the heat from the cells flows to a heat sink through a thermopile (Wadsö, 1994). The reason is this gives an instant rate ($d\Phi = dQ/dt$, where Φ is heat flow rate and Q is heat). Unless the bioreactor was actually to be made a calorimeter, the cell suspension has to be pumped to the *ex situ* calorimetric measuring vessel and then returned to it. So, the flow calorimeter has the complication of transmission lines between the two parts, but in principle it can be used in conjunction with bioreactors of any volume. Guan et al. (1997) have described a suitably modified instrument but, in early experiments, it was noted that the heat flow rate declined considerably before the end of cell growth (Kemp and Guan, 1997). This suggested that a more sensitive indication of metabolic activity might be size-specific heat flow rate, termed heat flux. It was already known that dielectric spectroscopy is an on-line, *in situ* probe of the volume fraction of viable animal cells (Davey et al., 1997). Subsequent experiments have shown the combination of flow microcalorimetry with dielectric spectroscopy to give heat flux on-line provides a reliable indication of metabolic activity (Guan et al., 1998).

The mammalian cell system in this study was Chinese hamster ovary cells (CHO 320) genetically engineered to overexpress interferon- γ (IFN- γ) (Hayter et al., 1991) constitutively and secrete it into a bicarbonate-buffered medium. These cells require carefully controlled physiological conditions (Hayter

et al., 1992) and well-designed, complex media (Castro et al., 1992), without which there is a reduction in the quality of the product (Hooker et al., 1995). One of the aims of this investigation is to show that the onset of unfavourable conditions which results in a decline in metabolic activity is indicated by the heat flux measurements.

Biochemical analysis of the fate of the major metabolites allows the construction of an equation for the growth reaction with stoichiometric coefficients for the individual components (Guan and Kemp, 1996; Kemp and Guan, 1997). In common with ordinary chemical reactions, this reaction can be formulated to include heat dissipation which expresses both the thermodynamics and kinetics of growth and, moreover, allows the validity of the reaction to be tested by the enthalpy balance approach (Kemp, 1993; Kemp et al., 1997; von Stockar et al., 1993). The stoichiometric coefficients in the growth reaction reveal cellular demand for substrates and can be a basis for medium improvement. The second aim of the current study is to identify that demand.

For the energy transformations in growth, it is crucial to realise the central importance of the extent of reaction, the advancement ($\xi = n_B/\nu_B$ where ν is the stoichiometric coefficient, n is the change of molar number, and B is the amount concentration of the entities (reactants and products)), the rate of conversion, $d\xi/dt$, is the metabolic (flow) rate, a kinetic term which can be made size specific as the metabolic flux (J_B),

$$J_B = d\xi/Xdt \quad (1)$$

Since heat dissipation measured calorimetrically as heat flow rate is integral to the kinetics of the reaction, it can be regarded as the rate of thermal (th) advancement ($\Phi = d_{th}\xi/dt$, and thus can indicate the metabolic rate. Guan and Kemp (1996, 1998c) showed theoretically that this rate is related to the exact proportions of the rate of any of the components in the growth reaction, that is to the set of stoichiometric coefficients. From the foregoing, it is established that the thermal component, obtained by integrating the heat flow rate, is directly related to the stoichiometric coefficients. The third aim of this paper is to demonstrate this direct relationship. It means that the suitability of a given medium can be shown by heat flux measurements which, consequently, can be employed in optimising the medium in terms of metabolic activity. Progress in that direction is the final aim of this investigation.

Materials and methods

Cell culture

The cells were a suspension-adapted line which had been co-transfected with dihydrofolate reductase (dhfr) and IFN- γ genes by a plasmid derived from a pSV2-dhfr vector controlled by an SV-40 early promoter (Hayter et al., 1991). Amplification was by selection with 0.1 μ M methotrexate. The medium originally used to grow these cells was RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 1 mM pyruvate, 0.1 mM alanine, freshly-added 2mM glutamine and 24 mM sodium bicarbonate from Sigma (Poole, U.K.) plus 5 g dm⁻³ fatty acid-free (FAF) BSA (Pentex bovine albumen 82-047, Bayer diagnostics division, Kankakee, IL 60901, USA), 50 mg dm⁻³ gentamycin and all the other trace compounds specified by Hayter et al. (1991). Changes were made to this *original* medium designed to improve growth and productivity. The alterations made in an empirical approach, to give an *improved* medium were 7.5 g dm⁻³ FAF-BSA, 2.5 mM pyruvate and 0.2 mM alanine, (recommended by Castro et al., 1992); 0.5% (v/v) Pentex ExCytex VLE which is a 'very low endotoxin lipoprotein' mixture containing 9.7 g dm⁻³ cholesterol and 15.4 g dm⁻³ protein (Jenkins et al., 1994); and increases in the concentrations of all the amino acids (apart from glutamine) in RPMI 1640 medium to give finally 0.5 mM (Xie and Wang, 1994). Cells were routinely cultured in 0.5 dm³ and 2 dm³ spinner vessels [Techne Ltd., Cambridge, U.K.] in the original medium and gassed with 5% CO₂ in air. Concentrated suspensions for bioreactor batch cultures were prepared by centrifugation at 80 $\times$ g for 3 min.

The 3 dm³ tank bioreactor (F.T. Applikon Ltd., Tewksbury, U.K.) contained 1.5 dm³ cell suspension with the cells injected as a concentrate using a 50-cm³ syringe. The suspension was stirred at 60 rpm with a marine-type impeller (Guan et al., 1998). The control unit has four analogue inputs which were used for: (a) maintaining the culture temperature at 37 °C using a platinum resistance thermometer; (b) controlling medium pH at 7.25 $\pm$ 0.02 using 0.1 N NaOH and sparged CO₂ with a glass combination electrode; (c) measuring the oxygen concentration using an Ingold polarographic sensor and keeping it at 55% of the air-saturated medium with filtered air or oxygen; and (d) the heat flow rate signal from the calorimeter (see details later). The Applikon BioXpert software for acquiring, smoothing by the moving average technique

and analysing the data has the capacity for many more than four digital inputs and an AD/DA converter, PCL-812PG PC-Labcard supplied by Applikon, was used to digitise the analogue capacitance and conductivity signals from the on-line probe of a Viable Cell Monitor (Aber Instruments, Ltd., Aberystwyth, U.K.) operating as a dielectric spectrometer at a radio frequency of 0.5 MHz (Davey et al., 1997; Guan and Kemp, 1998a). The baseline value for medium alone was subtracted from that of the cell suspension using BioXpert so that changes in the on-line signal were due to the volume fraction of viable cells (Guan et al., 1998). Monitoring conductivity was necessary because any marked changes in it affects the capacitance readings.

Flow microcalorimeter

Cellular heat flow was measured at 37 °C using the 24-carot gold flow-through vessel of a Thermal Activity Monitor (Thermometric AB, Järfälla, Sweden) modified for downward flow (Guan et al., 1997) and connected to the bioreactor by thermostated PEEK tubing (1 mm i.d.; Jour Research, Onsala, Sweden), which has an extremely low diffusivity to gases. The cell suspension was pumped at 35 cm³ hr⁻¹ by a Jubile peristaltic pump (H.J. Guldener, Zurich, Switzerland) with Viton tubing (1.3 mm i.d.; diffusion coefficient 9.1×10^4 cm² s⁻¹). The amplification was 100 µW. The flow vessel was electrically calibrated at the beginning of the experiment and by the well-characterised, chemical hydrolysis of triacetin every month (Wadsö 1994). This showed that the thermal volume was within 2% of its nominal volume (0.6 cm³). There was a careful protocol for cleaning the flow system with Decon 90 and sterilising it with 70% ethanol (Guan et al., 1997). When not processed using BioXpert, data were collected using Oroboros DatLab AQ software and analysed by the complementary DatGraf 2.1 (Oroboros, Innsbruck, Austria).

Off-line measurements

Samples were removed from the bioreactor at regular intervals and tested for a range of variables. Most of these were conducted as a series at the end of the culture period so the sample was frozen, but not until estimates were made of cell volume using a Skatron Argus flow cytometer (Skatron Ltd., P.O. Box 34, Newmarket, Suffolk, U.K.) after staining with fluorescein diacetate (FDA) (Guan et al., 1998), cell density by the conductimetric Coulter counter (Coulter

Ltd, Luton, U.K.) and viability using FDA and ethidium bromide on a Zeiss epifluorescence microscope (Kemp, 1995). Oxygen consumption was measured using an Oroboros Oxygraph two-channel respirometer (Anton Paar, Graz, Austria) (Gnaiger et al., 1995). The cell suspension, 2.3 cm³, was added to each chamber and stirred with a magnet at 400 rpm (Kemp and Guan, 1998). The rate of revolution was chosen by experimentation to give the least damage to cells for the comparatively short (<1-h) periods of measurement. The ammonium ion concentration and pH of thawed samples were assessed using a pH/ion meter (Model PHN95; Radiometer Analytical A/S, Copenhagen, Denmark) (Guan et al., 1998). IFN-γ concentration was assayed using a monoclonal antibody ELISA technique (Hayter et al., 1991) with anti-IFN antibody 2B10 supplied by Professor N. Jenkins (then at Kent University). The amounts of glucose, glutamine and lactate were determined, after deproteination with 0.6 M perchloric acid, using respectively Sigma kits 635, GLN-2 and 826 (Guan et al., 1998).

Results and discussion

Cell growth and productivity

As can be seen in Figure 1, the growth of CHO 320 cells had an initial slow phase up to 36 hr followed by a period during which there was a greater increase in viable cell numbers. After 84 hr, the rate slowed and growth had stopped altogether by 120 hr. Then there was a decline in the number of viable cells. This was similar in both general form and extent of growth to previous findings for this type of culture maintained in bicarbonate- (Hayter et al., 1991) and HEPES-buffered (Guan et al., 1998), serum-free media. One of the most probable reasons for the poor proliferation was the complete absence of lipid, even to the extent of using FAF-BSA in order to simplify the enthalpy balance. Jenkins et al. (1994) showed for the present cell line that addition of lipid supplements to the medium considerably improved growth. Overexpression of the IFN-γ gene resulted in the accumulation of the recombinant protein (Figure 1) for 130 hr, but the rate appeared to slow with the decrease in cell growth. The relationship between growth and foreign protein production is better illustrated in Figure 2 in which the time integral of viable cells is plotted against the total cytokine production. The curve demonstrates that this is a growth-associated product. The slope of the line is

Figure 1. The growth of CHO 320 cells in a controlled bioreactor with the constitutive production of recombinant interferon- γ . The curves are for viable cells (x) and cumulative cytokine secretion (*).

equal to the average IFN- γ production flux up to that time and was found to be 254 IU hr^{-1} per 10^6 cells in the growth phase. The association of production with cell growth is in agreement with evidence that foreign gene overexpression has a dependence on the stage of the cell cycle (Gu et al., 1993). The promoter in the present case is derived from the SV40 tumour virus and it seems probable that this only allows expression during the S phase (Kubbies and Stockinger, 1990).

Calorimetric measurements and the metabolic fluxes

Estimations of the utilisation of the two most prominent catabolic substrates (Figure 3) revealed that glutamine was consumed at a rate which meant that less than 0.5 mM remained at 108 hr. It has frequently been reported that, at about this concentration, there is a Monod-type effect on cell growth (Jeong and Wang, 1995). Nevertheless, growth continued for at least a further 12 hr (see Figure 1) and it only ceased at about the same time (120 hr) as glutamine exhaustion. Continuation of cell growth under glutamine limita-

tion beyond 108 hr might have been due to the fact that cells which have completed S phase of the cycle do not require the amino acid for division, providing there is an energy source. This is supplied of course by glucose which was available throughout the culture period (Figure 3), though it is worth noting that its consumption slowed after 108 hr. Heat flow rate had already reached a peak by 96 hr which was earlier than the possible limitations to metabolism due to substrate exhaustion. It is possible that other environmental factors contributed to cause the attenuated heat flow rate, most notably the accumulation over time of lactate (Omasa et al., 1992) and ammonium ions (Borys et al., 1994) in the medium (see Figure 3). Lactate rose to $\sim 10 \text{ mM}$ and ammonium ions to 1.5 mM and, although these concentrations were well below the toxic levels for these cells reported by Hayter et al. (1991), it is still possible that they were the reason for the reduced metabolic rate which seemed to presage an end to cell growth. The fact that value for heat flow rate peaked before that of cell numbers indicated that the size-specific heat flux would be a

Figure 2. Presentation of the cumulative production of IFN- γ versus the integral of viable cells.

highly sensitive indicator of metabolic activity (Guan et al., 1998). Conventionally the intensive quantity is obtained from dividing by mass but there is now the availability of dielectric spectroscopy which measures the volume fraction of viable cells (Davey et al., 1997).

The data for the on-line *ex-situ* heat flow rate shown in Figure 3 were converted to volume-specific heat flux (J_{Φ}/C) by division with capacitance measured on-line and *in situ* by dielectric spectroscopy (Harris et al., 1987). Because heat conduction calorimeters give the flow *rate* of heat, the heat flux measurement is a continuous record of the kinetics of metabolism. In contrast, the fluxes for glucose and glutamine can only be obtained from the concentrations of the substances at two consecutive times and this causes a delay in detecting changes in metabolic activity. Thus, the first metabolic flux points were at 6 hr, the midpoint of the first two discrete measurements. In order to make comparisons, heat flux data were treated in the same manner (Figure 4). Initially, the metabolic activity represented by these data was

low, possibly owing to the trauma of centrifugation and re-suspension in fresh medium. It then quickly rose to the highest value. By 60 hr heat flux had decreased and this continued well before the change in glucose flux at 96 to 108 hr. The curve described a course, however, which was much closer to the pattern of glutamine flux. The highest point in this was in the period from 24 to 36 hr. This was coincident with the peak metabolic flux as seen from the heat flux data. The specific growth rate, however, was highest in the following measurement interval (36 to 48 hr; $\mu = 0.026 \text{ hr}^{-1}$). This is thought reasonable because purine and pyrimidine synthesis involving glutamine must occur prior to S phase and subsequent cleavage in the cell cycle. This hypothesis could be tested by observing if the addition of, for instance, hypoxanthine and thymidine prolongs the period of highest metabolic rate. These results clearly showed that the heat flux probe is an early indicator of changing metabolic activity, in this case possibly due to altered glutamine flux. It is also likely that other environmental factors

Figure 3. Concentration profiles for the major catabolic metabolites in a controlled batch culture of CHO 320 cells with on-line monitor of heat flow rate (—) to monitor metabolic activity. Glucose (□) and glutamine (◇) consumption is depicted with their metabolic products, ammonia (■) and lactate (▲).

had a detrimental effect though, apart from ammonia and lactate, none has been identified for this culture system.

It can be seen from Figure 5 that capacitance measurements showed a decline after cell growth had ceased at approximately 120 hr. In theory (Harris et al., 1987), there are three possible reasons for this change; they are (i) reduction in cell numbers, (ii) decrease in viability, and (iii) reduction in cell size. In practice, all three occurred in these studies. The data in Figure 1 show the loss in cell numbers and this was probably due to necrosis. The routine viability studies (see Figure 5) revealed the decline in the numbers of living cells and the presence of more dead ones, with the latter being due to both necrotic and apoptotic processes. Finally, flow cytometry (Figure 5) indicated that, late in the cell culture when there was no net increase in cell numbers, cell volume decreased to a marked extent. This is a classic symptom of apoptosis (Mastrangelo and Betenbaugh, 1998). Many environmental factors have been reported to be initiators of this process of programmed cell

death. Among the most likely in the present situation is the exhaustion of growth factor (insulin) and nutrient deprivation. A third possibility for cells in culture, that of oxygen limitation, cannot apply in these studies because 50% medium saturation for this gas is a carefully controlled parameter. The strongest candidate is the deprivation of amino acids. Simpson et al. (1998) has shown that exhaustion of any single amino acid in the culture medium induces apoptosis. This is the main reason why care must be taken when considering the reduction of glutamine in the medium, particularly in fed-batch cultures. Besides consideration of kinetic limitation (Guan et al., 1998), it is easy to induce apoptosis by glutamine depletion. For hybridoma cells, the onset of the apoptotic cascade has been prevented by overexpression of the apoptosis-suppressor gene, *bcl-2* (Singh et al., 1996; Simpson et al., 1997) or the familial *bcl-x_L* (Kim et al., 1997) to give more of the protein on the outer mitochondrial membrane. A more appropriate approach for genetically engineered cells, however, might be early detection and restorative action by feeding fresh medium or inject-

Figure 4. Heat flux compared with other metabolic fluxes in the growth of CHO cells. The material fluxes can only be calculated by two measurements for each 12-h period. For valid comparison, the on-line signal for heat flux is treated as discrete measurements in the same way. The graph compared the heat flux (+) with the specific growth rate (Δ) and the material fluxes of glucose (□), glutamine (◇), lactate (▲) and ammonia (■).

ing apoptosis inhibitors such as antioxidants to the culture (Mastrangelo and Betenbaugh, 1998). A clue to finding an early, on-line indicator is the fact that initiators of apoptosis cause the release of cytochrome c from mitochondria (Liu et al., 1996) with the resultant disruption to membrane potential and cessation of oxygen consumption (Adachi et al., 1997). This change should be detected by the heat flux probe and, with more sensitivity, by calorimetry, combining calorimetry with oxygen uptake measurements (Gnaiger and Kemp, 1990).

Stoichiometric modelling

It has been shown (Guan and Kemp, 1996; Guan et al., 1998) that the chemical events depicted in Figure 4 can be described by the following generalised growth reaction (r), which relates material flows to enthalpy flow ($J_{H,r}$).

$$\nu_C \text{ CO}_2 + \nu_N \text{ NH}_3 + \nu_H \text{ H}_2\text{O} + \nu_X \left(\int_{t_1}^{t_2} J_{H,r} dt \right) \quad (2)$$

in which there is explicit use of stoichiometric coefficients ν_i for the i th species. These constitute a set of constant values only for a given metabolic state and thus must change to reflect different such states. In other words, a given stoichiometric reaction only applies as long as there is no alteration in the yields (Guan et al., 1998) including that of heat ($Y'_{Q/X}$). This formalises what is observed in Figure 4. Any given growth reaction can be validated by the enthalpy balance approach (Kemp, 1993; Kemp et al., 1997). It will be noted that amino acids other than glutamine are not included in Equation (2). This is because it is likely that anabolic processes make negligible contribution to the enthalpy change during growth (Guan and Kemp, 1996, 1998a, b) and thus do not participate in the enthalpy balance.

It will be seen from Equation (2) that growth can be represented by the set of stoichiometric coefficients with that for IFN- γ being subsumed with biomass be-

Figure 5. The capacitance signal of the dielectric spectroscopy is compared with the average viable cell volume and cell viability. Capacitance (—) was plotted with changes in cell viability ($\square$) and volume (Δ).

cause, as products of translation, the two cannot be distinguished,

$$\vec{v} = (\nu_{S_1} \ \nu_{S_2} \ \nu_O \ 1(\text{cell}) \ \nu_L \ \nu_C \ \nu_N \ \nu_H \ Q) \quad (3)$$

where Q is the integral of the heat flow rate. Since Equation (3) shows that there is a one-to-one corresponding relationship between the metabolic flux and the stoichiometry of the growth reaction, it follows that,

$$\frac{1}{X} \frac{d\xi}{dt} \leftrightarrow \vec{v} \quad (4)$$

Since heat flux, J_{th} is a form of metabolic flux, Equation (4) can be converted as,

$$\nu_i = f(J_{th}) \quad (5)$$

The strength of this relationship is illustrated from data for the utilisation of glucose, glutamine and oxygen in

relation to heat flux for the following stoichiometric ratios in the period 0–120 hr shown in Figure 4,

$$\nu_{Glc}/\nu_O = 0.0581 J_{th}^2 - 2.0949 J_{th} + 19.663 \quad (6)$$

$$\nu_{Gln}/\nu_O = 0.0279 J_{th}^2 - 1.045 J_{th} + 9.8561 \quad (7)$$

From Equations (6) and (7), it can be seen (Figure 6) that, for the specified range, heat flux is a monotonically increasing function of these ratios and therefore a valid probe of metabolic activity. It can be used in fed-batch cultures to indicate the point at which cells should be fed nutrients. At present, off-line samples are taken for chemical analyses and/or cell counts and changes in the appropriate flux and/or cell number are used as a rather late indicator of the need for nutrient (Xie and Wang, 1994, 1996a, b; Xie et al., 1997). It can be suggested from the present results that, once a

Figure 6. The heat flux over a specified set of values is compared against the stoichiometric ratios for the consumption of glucose (s_1) and glutamine (s_2) to oxygen. The data for the two specific cases [see Equations (6) and (7)] show that the relationship of heat flux to glucose ($\square$) and glutamine ($\diamond$) fluxes.

culture system has been defined in metabolic terms, the heat flux probe is an ideal way for early intervention to control feeding by an automated on-line technique. The probe is universally applicable to all animal cell cultures because every metabolic state can be represented by a set of stoichiometric coefficients and the heat equivalent of them, in same form as that in Equation (3). In both hybridoma cells and tightly regulated recombinant cells which have consecutive phases of growth and protein production, there are correspondingly different sets of stoichiometric coefficients and consequently dissimilar plateaux for heat flux at steady and quasi-steady states. Since each metabolic state demands a different medium composition, the heat flux probe can be used to optimise each

medium and monitor changes in cellular requirements for components in the medium.

The growth equation can be used for any given period in culture to indicate the demand by the cells for catabolic nutrients, as opposed to the amounts supplied in the medium (Guan and Kemp, 1996, 1998b). As an example, when data illustrated in Figure 4 were incorporated into Equation (2), it was shown that the strongly growing cells needed a stoichiometric ratio of $\sim 3:1$ for glucose/glutamine as opposed to the $\sim 5:1$ supplied to them. This explains why glutamine is exhausted prior to glucose in this culture system. Clearly growth would be prolonged if the two catabolic nutrients were fed to the cells in the correct ratio. Even in fed-batch culture, however, it is unlikely that the metabolic state would remain the same (no steady

Figure 7. Changes in the concentrations of the major catabolic metabolites during the batch culture of CHO 320 cells grown in the improved medium. Cell growth (x) is shown together with the concentrations of glucose (□), glutamine (◇) and lactate (▲) at each time. Also shown is the constitutive secretion of IFN- γ (*).

state) and, therefore, according to Equation (2) the stoichiometry of the reaction would change with time in culture. Any active fed-batch regime must take account of such alterations. It is also crucial, perhaps paramount, to ensure that neither substrate becomes rate-limiting in terms of their Monod constants. These questions are being addressed at present by using continuous cultures prior to experiments in the fed-batch regime.

Improved medium

The correct ratio of the major catabolic substrates is only one of many factors important in medium design. As already mentioned, the accumulation of ammonia and lactate is a strong possibility for causing the deterioration of cell cultures with the resulting reduction in the quantity and quality of their foreign products. Much of this is because glucose and glutamine are being utilised inappropriately as biosynthetic precursors (Kemp et al., 1997; Kemp and Guan, 1997, 1998).

In their stoichiometric approach to medium design, Xie and Wang (1994, 1996a, b) have identified the need for considerably more amino acids in the medium and found that supplying the demand led to ~60 fold less lactate flux and ~4 fold less ammonia flux. This consideration led to the design of a modified medium which contained increased amino acids according to the Xie-Wang (1994) formulation, as well as a lipid supplement (Jenkins et al., 1994) and more of some other ingredients (Castro et al., 1992). In a stepwise approach, this trial medium retained a glc/gln ratio of ~5:1 and the results (Figure 7) showed that, while both glucose and glutamine were depleted from the improved medium at approximately the same time as they were exhausted in the original medium (Figure 3), the total number of viable cells grown in the culture was virtually three times greater than in previous cultures. It is also important to note that there was a more rapid accumulation of IFN- γ in improved medium (Figure 7) than in the original one (Figure 1).

Table 1. Representative average fluxes of the major metabolites for CHO 320 cells growing in the original and the improved media in the period of 36 to 60 hr during batch culture in bioreactors

Media	Fluxes				
	μ (h ⁻¹)	glucose (mol s ⁻¹ per cell)	glutamine (mol s ⁻¹ per cell)	lactate (mol s ⁻¹ per cell)	IFN- γ (IU hr ⁻¹ per cell)
The improved	0.049	4.11×10^{-17}	1.67×10^{-17}	5.28×10^{-17}	6.06×10^{-4}
The original	0.028	5.81×10^{-17}	1.78×10^{-17}	1.08×10^{-16}	4.82×10^{-4}

The medium was, however, not bespoke as it was not possible to make a complete stoichiometric analysis along the lines pioneered by Xie and Wang (1994, 1996a, b). This is probably the main reason why there was still considerable production of lactate. The degree of improvement on the original medium is shown more clearly in Table 1 which shows that the glucose flux was considerably reduced in the improved medium compared with the increase in specific growth rate. More significantly, the flux of IFN- γ was more than 25% greater than in the original medium, proving that increased growth was not at the expense of productivity.

Although there was considerably more of the amino acids in the improved medium, there was not much difference between the two glutamine fluxes. This indicated that little of the particular amino acid was transaminated to other ones in the original medium. Support for the contention that less glucose was being used as biosynthetic precursors comes from the fact that there was reduced lactate flux. It is likely that this will be further decreased when the two major catabolic substrates are supplied in the correct stoichiometric ratio. The increased demand for ATP in biosynthesis was fulfilled by increased respiration as evidenced by the oxygen flux at 6.83×10^{-17} mol s⁻¹ per cell compared with 2.75×10^{-17} mol s⁻¹ per cell in the original medium. This was further demonstrated by preliminary results using 1.6 μ M *p*-trifluorophenylhydrazine as an uncoupler to indicate that the mitochondria were operating at 80% of capacity (unpublished data), rather than at 64% as in the original medium (Kemp and Guan, 1998).

Conclusions

This work has demonstrated that the on-line heat flux probe is a robust biosensor for detecting early changes in metabolic activity. Heat flux could be employed as

the control variable in fed-batch cultures because the heat from cells is related to the set of stoichiometric coefficients for the growth reaction at any defined metabolic state. In identifying the level of metabolic activity, heat flux can be employed to optimise culture media for the maximum production of high quality heterologous proteins.

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