Biological/Life Sciences

AN ENTHALPY BALANCE APPROACH TO THE STUDY OF METABOLIC ACTIVITY IN MAMMALIAN CELLS*

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Abstract

After a formal explanation of Mayer's enthalpy balance method as applied to biological reaction rates, the history of its application is traced from Rubner's dog to accounting for the energy of muscle contraction. The introduction of microcalorimetry allowed the method generally to be used for cells in vitro and now particular emphasis can be paid to the growth of cells for the production of therapeutically-important heterologous proteins. In these systems, enthalpy balance studies contribute to defining catabolic processes, designing media, understanding the mechanisms of growth and controlling cultures using heat flux as an on-line sensor of metabolic activity.

Keywords: animal cell, enthalpy balance, heat flux, recombinant protein, stoichiometric coefficients

Introduction

In these words [1], "starting from Lavoisier's theory, according to which animal heat is the result of a process of combustion" [2] and using a working horse rather than a guinea pig, the physician Mayer deduced a figure for the mechanical equivalent of heat and in 1842 presented his theory of the conservation of energy. He went on to write "it follows that a general balance must be struck in the organism between receipts and expenditure or between work done and wear and tear", defining the former as "the evolution of heat" and the latter as "the amount of matter consumed", . . "so as to occasion growth and the renewal of worn-out solid parts" [2]. Already he recognized the "invariable relation between heat and work" but, in his definition of heat evolved by an organism, was he subconsciously acknowledging the irreversibility of life?

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The actual proof of applicability of the 1st Law to living systems was by Rubner [3] sixty years later who showed that a dog living for 45 days in a respiration calorimeter produced 72588 kJ (18.7 W) of heat energy at the same time that it utilized 72827 kJ of (net) nutrient (chemical) energy as calculated from the dog's respiratory metabolism ("slow combustion") and faecal and nitrogenous excretion [4]. The dog was adult and did not grow during the experiment. Otherwise there would have been a discrepancy such as Dubrunfaut [5] found in his massive experiment (21.4 ML!) on the heat produced by yeast in an alcoholic fermentation. The value which he obtained for the production of ethanol from 2559 kg of cane sugar was 38% lower than would have been expected from the Gay-Lussac equation [4]. The main reason for the difference probably is that the yeast organisms grew in the medium over the 4 days of the experiment. In coming to this conclusion, implicitly the principle of Mayer's general balance between receipts and expenditure has been employed – it is now known as an enthalpy balance – which needs a knowledge of energy and all material (mass) fluxes.

Formalization of the enthalpy balance equation

From the 1st Law of Thermodynamics and with certain conditions, the heat dissipation (flow) from a reaction, or set of reactions(r) per system size (heat flux), $J_{\Phi/V}$ (unit: W m^{-3}) must equal its enthalpy flux, $J_{\mathrm{H,r}}$ (unit: W m^{-3}) [6],

$$\frac{J_{\Phi/V}}{J_{\mathrm{H,r}}} = 1 \tag{1}$$

This ratio is called the enthalpy recovery and its value, in a complex system which does not exchange energy in the form of work (see Mayer above [1]), is unity (enthalpy is balanced) only if all the reactions and side reactions are held to account. In the strict sense of thermodynamics, this relationship holds only for a system at constant temperature and pressure when there is no (net) work. For living systems, heat dissipation is measured in an isothermal calorimeter in which usually the system is open and therefore at constant pressure (closed ampoules are sometimes used for short-term experiments with negligible pressure change). The growth process is irreversible which means that the (net) work term is zero. Within these assumptions, if the enthalpy recovery is more than unity, then the thermo-(bio)chemical analysis has failed to account for all the reactions. A recovery less than 1 indicates that there are undetected endothermic reactions.

Heat flux is the size-specific yield of the extensive quantity, heat flow, Φ (unit: W),

$$\Phi = \frac{\mathrm{d}Q}{\mathrm{d}t} \tag{2}$$

where Q is heat. In heat conduction calorimeters, Φ is the direct measurement, Q being obtained by integration of Φ in terms of time. The instrument registers the heat flowing from all of the measuring vessel and, in living systems, it is most common to divide it by the mass (m) or volume (V) of biological entity(ies) to obtain flux.

In the type of enthalpy balance approach described above [but see later – Eq. (12)], the reaction enthalpy flux is obtained from the reaction flux, $J_{B,r}$ (unit: mol s^{-1} m^{-3}) [6] and the theoretical standard molar reaction enthalpy, $\Delta_r H^o_B$ (unit: J mol^{-1}) [7],

$$J_{H,r} = J_{B,r}\Delta_r H^o_B \tag{3}$$

where the subscript B indicates "per mole of B" which also means that the reaction stoichiometry is defined in a form where the stoichiometric number (ν_B) of species B is either +1 or –1.

The reaction flux is the size specific advancement, ξ (flow or extent), of the reaction (unit: mol s^{-1}),

$$J_{B,r} = \frac{d_r\xi}{dt}V^{-1} \tag{4}$$

The advancement is,

$$d_r\xi = \frac{d_r n_i}{\nu_i} \tag{5}$$

where n (unit: mol) is the amount and ν is the stoichiometric number of species i.

According to von Stockar *et al.* [7], strictly the molar reaction enthalpy (unit: J mol^{-1}) of the j-th process is,

$$\Delta_r H_B = \left(\frac{\partial H}{\partial \xi_j}\right)_{T,p,n_j} \tag{6}$$

The partial molar enthalpy (unit: J mol^{-1}) of i is,

$$H_i = \left(\frac{\partial H}{\partial n_j}\right)_{T,p,n_j} \tag{7}$$

But H_i is concentration-dependent and thus it is not possible to assign it a value in a dynamic system. In practice, it is replaced by the concentration-independent standard molar enthalpy, H^o_i. In a living system, there are thousands of reactions but many of them involve substrates being converted to products by different pathways. By taking advantage of Hess's Law of Constant Heat Summation, it is possible to reduce the number considerably but, even so, there are a finite number of reactions which contribute to the total heat dissipation. Thus, the aggregate standard molar reaction enthalpy is,

$$\Delta_r H_B = \sum_i \nu_{i,j} H^o_{i,s} \tag{8}$$

where subscript s is the system. This is the chemical equivalent to heat dissipation.

Once these points are understood, the main difficulty in enthalpy balance studies lies in determining the correct value for the molar reaction enthalpies which are calculated from enthalpies of formation. It is vital to select accurate standard and reference states as well as to correct for side-effects and side reactions, many of which can give errors of at least 50% [7]. After having regard to these problems, von Stockar et al. [7] derived a theoretical equation which represented the enthalpy balance for a non-steady-state, open, pseudohomogeneous, multi-phase, living system in which several biochemical, chemical and physical transformation processes occur simultaneously; the details can be obtained from that review.

The value of the enthalpy balance

Following the lead given by Rubner [3], physiologists and nutritionists have used this method in association with whole-body calorimetry to study dietary requirements, thermogenesis and the effects of drugs, homoiothermy, exercise physiology in domestic animals and man, maximum efficiency of "meat production" in animals and the energy needs for optimal health in man [8]. For this purpose, foodstuffs and excreta have been assigned calorific values and oxycaloric equivalents; see, for instance, refs [9–11]. The importance of energy budgets extends to ecology in which energy flows are one of the most important factors [12]. It is in this field that it was first recognized that many animals, especially those in the aquatic environment [13], live in hypoxic, microxic and anoxic environments [14]. In these conditions, some groups of whole animals do not entirely obey Lavoisier's theory because at least a part of the net heat dissipation is from anaerobic processes. At very low environmental oxygen tension, these processes are exclusive. This is in contrast to the majority of animals which live in normoxic conditions where the products of individual internal anaerobic processes are recovered for aerobic processes. For instance, lactate produced in striated muscle as the result of exercise exceeding oxygen supply for the aerobic production of ATP [15] becomes a major substrate for gluconeogenesis in the liver – the Cori cycle [16]. In terms of an enthalpy balance, the whole animal is found simply to burn the carbohydrates and fats (and proteins) in its food. In contrast, animals that have evolved to live in environmental conditions of reduced oxygen tension (e.g. Arenicola – the lugworm) actually excrete lactate (acute physiological hypoxia) or, under long term environmental anoxia, utilize the succinate, propionate and acetate pathways [13]. In all these circumstances, the enthalpy balance is an extremely valuable device for checking the "receipts and expenditure". The relative complexities of constructing balances when the catabolic products are more reduced than carbon dioxide and water (combustion) have been fully rehearsed in microbiology in which a large number of the organisms are obligate or facultative anaerobes. In this respect, many of the difficulties posed by higher organisms with mixed aerobic and anaerobic metabolism, as well as those producing secondary products [16, 17], have been solved for microbial systems. In industrial terms, the efficiency of growth and production of target substances in bacteria and yeast is of paramount interest and it is with this in mind that a rigorous protocol has been drawn up to construct enthalpy balances for microbial growth [18].

Returning to complex animals, the role of physiologists is to study the interaction of structure, metabolism and function of tissues and organs. Starting from his design of a calorimeter to measure heat dissipation in milk fermentation (lactate) [19], Hill in 1912 [20] investigated the heat production by striated muscle isolated from the frog, noting the accumulation of lactate with repeated contractions of the preparation. Over the next 50 years [21], he and others showed that the heat dissipation in the contraction of healthy muscle adequately perfused with physiological fluid in vitro was mainly due to the splitting of phosphocreatine eventually to give ATP as the direct energetic source of contraction. The shortfall, revealed by enthalpy balances studies, between heat production explained by biochemical events and the observed value seems to be due to the movement at the beginning of contraction of calcium across the sarcolemma from the binding sites in the sarcoplasmic reticulum to sites on troponin C proximal to the actin-myosin-tropomyosin and troponin I-C-T complexes in the sarcoplasm [22]. It is likely that the change in entropy as a result of the movement of ions accounts for the "unexplained" heat production ($\Delta H = \Delta G + T\Delta S$) [23]. This is because the movement of the calcium ions causes a discharge of the condenser of the capacity C and temperature T present in excitable cells (muscle and nerve). The resulting change in enthalpy differs considerably from the Gibbs energy change by an amount $T\Delta S$, which can be shown to equal $\Delta G(T/C)(\Delta C/\Delta T)$. It has been demonstrated that $\Delta C/\Delta T$ is positive in living systems [23] and therefore heat will be released on discharge.

Calorimetric studies of organs and tissues isolated from animals [24] have now been paralleled by investigations of blood [25] and other tissue cells [26] in vitro which have been made possible by developments in flow and perfusion microcalorimetry [27]. In particular, biotechnological developments have provided an impetus to studies of the growth and metabolism of cells cultured in large-scale bioreactors after being engineered to produce medically important proteins [28].

Enthalpy balance studies of cultured cells

With the exception of blood cells and gametes, cells of an animal are, of course, adhesive and thus not naturally compatible with the batch or flow vessels of a calorimeter [29]. Only in a few cases have insertion vessels been designed to accept cells growing in monolayer (see, for instance, [30]) and then a large capacity calorimeter is required in order to accept them (e.g. SETARAM calorimeter [31]). In the majority of studies [28], the adopted practice was that first used for blood cells [25] of pumping a cell suspension through the flow vessel of a microcalorimeter [26–28] but it was soon realized that care has to be taken with naturally sticky cells to avoid blockage due to aggregates and damage to the cells by the action of the pump [32]. Fortunately many of the cell types used for large-scale culture have been adapted to grow in suspension (see later). In early studies using batch vessels, cells including those from blood tissue were allowed to sediment and exhibited the so-called "crowding" effect [33] (or "Uriah" effect [34]) in which catabolism becomes glycolytic with the production of lactate, seemingly because the local oxygen tension rapidly decreased in the non-stirred layer. The incorporation of stirrers into batch perfusion/titration vessels [35] provided a solution to this problem but it is a salu-

tary lesson not to forget the importance of oxygen – even in monolayer [36]. This is not to write that the production of lactate is necessarily an indication of poor oxygen tension; as will emerge, it is also a natural consequence of growth in cell numbers.

An early use of the enthalpy balance method in studying the metabolism of tissue cells was by Eftimiadi and Rialdi [37] who thermobiochemically characterized fresh human neutrophils. The role of these cells in vitro is to kill foreign microbes after engulfing them, using active oxygen metabolites (e.g. superoxide) and hydrogen peroxide rapidly produced ("burst") by reduction of oxygen using NADPH generated by the pentose phosphate pathway (PPP). When suspended in a simple glucose-containing, phosphate-buffered saline and pumped to the flow vessel of an LKB heat conduction microcalorimeter [29], it was shown using Eqs (1–3) and (8) that resting neutrophils which have few mitochondria produced energy entirely by PPP (shown by the carbon-1/carbon-6 ratio) and glycolysis (Table 1). When the cells were stimulated to produce the respiratory burst by phorbol-12-myristate-13-acetate (PMA), there was a rapid increase in heat production and oxygen consumption (Table 1). The calculated heat flux was almost entirely explained by the activity of the PPP with only 17% being due to glycolysis compared to 64% in resting cells. Any lactate produced by neutrophils in vivo in the blood and surrounding tissues would of course be conveyed to the liver and act as a substrate for gluconeogenesis, with the recycled glucose probably taking part in aerobic processes.

While the neutrophils were freshly obtained from blood and maintained in a simple defined medium, the second example of the use of the enthalpy balance method for studying cellular metabolism is an established cell line (CCRF-CEM) originating as a neoplastic (cancer) lymphoma and adapted to grow in suspension in a complex medium containing 2 mM glutamine and 10% (*v/v*) calf serum which was buffered to pH 7.17±0.03 with 20 mM HEPES [28, 38]. The cells were maintained in suspension by stirring in the perfusion vessel of a Thermometric TAM microcalorimeter (Thermometric AB, Järfälla, Sweden) [35]. The heat flux was 12 pW per cell and three pathways were identified – respiration, glycolysis and glu-

Table 1 Thermobiochemical characterization of fresh human neutrophils: interpreted and summarized from Eftimiadi and Rialdi [37] by Kemp [28]; and now abbreviated to show the enthalpy recovery

Metabolic fluxes	Metabolic states	
	Resting	PMA-activated (10 $\mu g\ cm^{-3}$)
Heat flux[a] (pW per cell)	2.5	9.8
Oxygen consumption (fmol h^{-1} per cell)	7.95	60
Lactate production (fmol h^{-1} per cell)	111	103
Carbon dioxide evolution (fmol h^{-1} per cell)	–	48

[a]The calculated "resting" heat flux (pW per cell) is 1.8 (glycolysis) +1.0 (PPP) = 2.8; the actual value was 2.5. The calculated total "burst" heat (nW per cell) is 4.03 (glycolysis) +19.52 (PPP) = 23.55; the actual value was 23.5. The reaction to produce the oxygen metabolites via PPP is: $NADPH + O_2 \xrightarrow{NADPH_{oxidase}} O_2^- + NADP^+ + H^+$. The molar reaction enthalpy ($\Delta_r H_B$) for the above under the conditions of the incubation mixture was calculated as –2884 kJ mol^{-1} [37].

taminolysis; and then quantified after HPLC separation. Because lactate and CO_2 production are common to two pathways, radioisotopic labelling was essential to assign quantities to each flux. In this way it was possible to show that practically all the glucose was converted to lactate with only 1% appearing as CO_2. Thermobiochemical calculations [28] revealed that glycolysis accounted for 37% of the heat flux and respiration for 10%. The data for the conversion of glutamine were incomplete but it would appear that, of the remaining 53% of the recorded heat production, glutamine oxidation to lactate and CO_2 (glutaminolysis – see later) was responsible for 17%. Thus, the explained heat production was 64% (7.8 pW per cell) of the measured value. Of the remaining 36%, all but 2% could be accounted for by the surplus oxygen consumption, using the average value for the oxycaloric equivalent (Thornton's Rule) of – 450 kJ mol^{-1} [39]. It will be recalled that the cells were grown in a medium containing calf serum. It appears likely that the abundant fatty acids in this undefined body fluid were oxidized by the cells.

Enthalpy balances for hybridoma cells

In the body, antibodies are produced by mature B lymphocytes which no longer proliferate and thus cannot form the basis of cell cultures for mass production of the required proteins. Millstein solved this problem by separating the B lymphocytes from other blood components and fusing them with cells of a cancerous lymphocyte cell line [40]. The resulting hybrid retained the antibody-producing properties of the mature cell and the growth characteristics ("immortalized") of the cancer cell.

Table 2 Flux, J_B, of major metabolites of 2C11-12 mouse macrophage hybridoma cells suspended in a simple incubation medium (Dulbecco PBS with per dm^3, 5.5 mmol glucose; 2 mmol glutamine; 2 g bovine serum albumin (BSA) and 20 mmol HEPES, pH 7.2±0.05) and triggered into a respiratory burst by PMA (average of 5 experiments from [41])

Major metabolites	Reaction flux[a] (pmol s^{-1} per 10^6 cells ±SEM)
Glucose	–51±5
Glutamine	–19±2
Glutamate	+5±0.05
Aspartate	+0.5±0.07
Serine	+1±0.01
Alanine	+3±0.3
Pyruvate	+6±0.4
Lactate	+34±3
Succinate	+0.5±0.04
Oxygen	–109±7
Carbon dioxide	+105±19
Ammonia	+18±2

[a]Positive fluxes indicate net production and negative ones mean net consumption.

This procedure has since been extended to other types of mature white cells and a hybridoma (2C11–12) formed by fusion of mouse macrophages with a lymphosarcoma cell line has been used to discover the major pathways used by the cells in culture [41]. Similar to the neutrophils mentioned earlier, these hybridoma cells undergo a respiratory burst when triggered by PMA. The main difference is that these cells continue to grow and thus require glutamine which is a vital precursor for purines and pyrimidines. As can be seen in Table 2, both glucose and glutamine were heavily utilized by the cells which consumed oxygen and produced considerable amounts of lactate, carbon dioxide and ammonia. In addition, there was net accumulation of some amino acids. Assessing the relative importance of respiration and glutaminolysis is difficult because both evolve CO_2; similarly, lactate is produced in both glycolysis and glutamine oxidation. All that is known for certain is that oxidative processes accounted for much of the observed heat flux. Given Thornton's Rule that the average oxycaloric equivalent of (cellular) carbon substrates is –450 kJ mol^{-1} [39], then the consumption of oxygen (109 pmol s^{-1} per 10^6 cells) represents 49 pW per cell, an enthalpy recovery of 58/49 =1.18. Isotopic trace studies were necessary to assign the correct quantities to the reaction fluxes shown in Table 2. The results given in Table 3 indicate that, while most of the glucose retrieved by column chromatography had been respired, a significant amount had been converted to lactate, with a little to pyruvate, in glycolysis. Some of the glutamine was converted to glutamate and part was oxidized to lactate and CO_2. As with glucose, however, a considerable amount was not retrieved as catabolic products (35% of glucose carbon and 45% of glutamine carbon). It is reasonable to assume that the missing carbon was incorporated into anabolic products because both glucose and glutamine and their metabolites are known to be biosynthetic precursors [42]. Some evidence for this mechanism in 2C11–12 cells can be seen in Table 2 in the accumulation of alanine, aspartate and serine.

Table 3 Accumulation rates (pmol s^{-1} per 10^6 cell) calculated from specific activity of relevant substrate for metabolites produced from the substrates [U-^{14}C]glucose and [U-^{14}C]glutamine (%) by triggered 2C11-12 mouse macrophage hybridoma cells in standard incubation medium – see Table 2. Values indicate the percentage of substrate carbon retrieved in the compound. (average 4 experiments from [41])

	Glucose metabolites	(%)	Glutamine metabolites	(%)
Lactate	30±2	(30)	4±0.5	(21)
Pyruvate	6±0.5	(6)	0.2±0.1	(0.9)
Carbon dioxide	90±7	(29)	7±1	(7)
Glutamate	–		5±0.5	(26)
Total		(65)		(54.9)

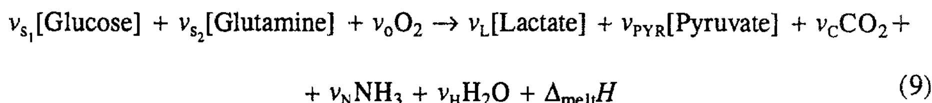
Using the data from Table 4 for the reaction fluxes and calculating the molar reaction enthalpies to include anaerobic processes, the enthalpy recovery for triggered 2C11–12 cells was 58/50.45=1.15 (Table 4), sufficiently close to unity to allow

Table 4 Reaction enthalphy flux of triggered C11-12 mouse macrophage hybridoma cells calculated from the measured reaction flux (Tables 2 and 3) and theoretical molar reaction enthalpy for each reaction pathway and compared against the heat flux, $J_{\Phi/N}$

Reactions	Molar reaction enthalpy[a], $\Delta_r H_B$/ kJ mol^{-1}	Reaction enthalpy flux $J_{\Phi/N}$ for triggered cells/ μW per 10^6 cells
Actual heat production		58
Calculated enthalpy flux from data in Table 3 for		
(a) Oxidation of		
(i) Gln to Lac	–695	2.8
(ii) Gln to Pyr	–890	–0.05
(iii) Glc to CO_2	–3018	–45.3
(b) Glycolysis of		
(i) Glc to Lac	–153	–2.3
Total		50.45

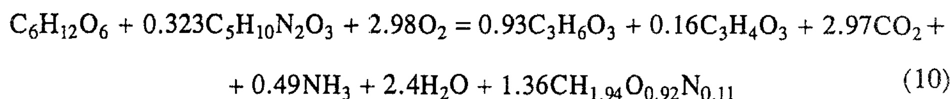
[a]Calculated from Wilhoit [43].

the following stoichiometric equation to be written by Guan and Kemp [44] for the catabolic process, known as a half-reaction,



$$\nu_{S_1}[\text{Glucose}] + \nu_{S_2}[\text{Glutamine}] + \nu_o O_2 \rightarrow \nu_L[\text{Lactate}] + \nu_{PYR}[\text{Pyruvate}] + \nu_C CO_2 + \\ + \nu_N NH_3 + \nu_H H_2O + \Delta_{melt}H \qquad (9)$$

where $\Delta_{met}H$ is the enthalpy change of this reaction. In Eq. (9), it is assumed that the enthalpy of anabolism ($\Delta_{ana}H$) is zero [4, 45] so that the pathways for glucose and glutamine metabolites which act as biosynthetic precursors (Table 4), make no contribution to cellular heat dissipation. The complete growth reaction requires the addition of "ν_X [biomass]" to the right hand side of Eq. (9). Taking the data from Table 2 and calculating the stoichiometry gives [44],



$$C_6H_{12}O_6 + 0.323C_5H_{10}N_2O_3 + 2.98O_2 = 0.93C_3H_6O_3 + 0.16C_3H_4O_3 + 2.97CO_2 + \\ + 0.49NH_3 + 2.4H_2O + 1.36CH_{1.94}O_{0.92}N_{0.11} \qquad (10)$$

One of the advantages of writing a growth equation is that it reveals the requirement by the cells for substrates, rather than the supply actually fed to them. For instance, the incubation medium for 2C11–12 cells contains glucose and glutamine in the molar ratio of 2.75. A glance at Eq. (10) reveals that the cells "demand" these substrates in the ratio 3.1: 1. This is obviously not important for the short-term incubation of hybridoma cells but most such cells are required to produce optimal quantities of protein antibodies over considerable periods (see, for instance [46]) and

correct medium design is an important aid to that end. For such cells producing antibodies, the term "v_P [product)" should be introduced as the second expression on the right hand side of Eq. (9).

Genetically-engineered cells

On an increasing scale, animal cells in vitro are being genetically-engineered and grown in large-scale culture to produce recombinant proteins. This is because many of the important therapeutic macromolecules are glycosylated proteins which require for synthesis, machinery only present in the endoplasmic reticulum and Golgi apparatus [47]. Animal cells are, of course, much more complex than microbes and their physiological requirements are correspondingly more difficult to fulfill in order to obtain optimal growth and production of heterologous proteins. Many of the problems, for instance control of physiological parameters/variables and medium design, are common to both hybridoma and genetically-engineered cells (see above section) but the potential contribution of thermobiochemical studies to this biotechnological field is illustrated by an example of the latter. Enthalpy balance studies not only enable researchers to define the catabolic processes of cultured cells but, as already shown, to make a contribution to medium design, to understand the mechanisms of growth ("towards a white box") and to control the growth process through heat flux as an on-line sensor of metabolic activity.

Chinese hamster ovary cells (CHO 320) producing interferon-γ (IFN-γ) in batch culture [48] have been used as a demonstration of the potential for measurements of heat dissipation. They were grown in an RPMI-based defined medium containing, inter alia, 11 mM glucose, 2 mM glutamine and 5 mg cm^{-3} fatty-acid free BSA. The pH was 7.25± 0.03 and the buffer was 24 mM sodium bicarbonate until otherwise specified. Viability was measured by the fluorescein diacetate/ethidium bromide method [49]. Heat dissipation was measured *ex situ* by circulating the cells continuously from the bioreactor to the flow vessel of a TAM microcalorimeter [32]. Biomass was monitored in situ with the probe of a dielectric spectrometer op-

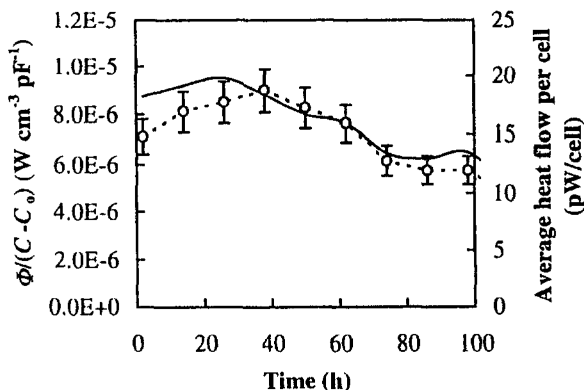


Fig. 1 On-line heat flux measurements (—) and heat flow per viable CHO 320 cell (o) over 100 h of a batch culture. The culture system was buffered by 4 mM bicarbonate and 20 mM HEPES

timized to record the capacitance of animal cells in suspension (Viable Cell Monitor (VCM), Aber Instruments Ltd, Aberystwyth, UK). An evaluation indicated that when the analogue signal is smoothed by the moving-average method, this instrument accurately measures the volume fraction of viable cells at densities as low as 3×10^5 per cm^3 [50]. At present, the two signals are matched off-line to give the heat dissipation per unit capacitance (C) as the size-specific quantity heat flux, $J_{\Phi/C}$, but, in the future, it is envisaged that this will be performed in real time to realize the potential of it as a control variable. Figure 1 compares the heat fluxes associated with on-line biomass measurement (capacitance of the cell suspension by VCM) and with off-line detection (cell number counting and viability estimation by a Coulter counter and fluorescence staining respectively) [51]. It can be seen that there is a good agreement between these two techniques. The significance of this

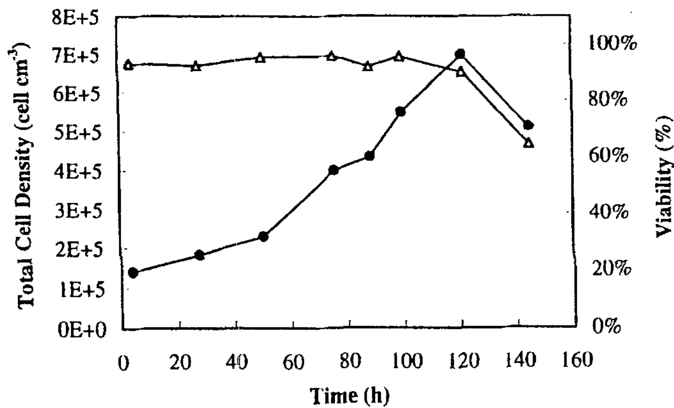


Fig. 2 The growth curve of CHO 320 cell line in a batch culture using a bioreactor system. • total cell, Δ viability (%)

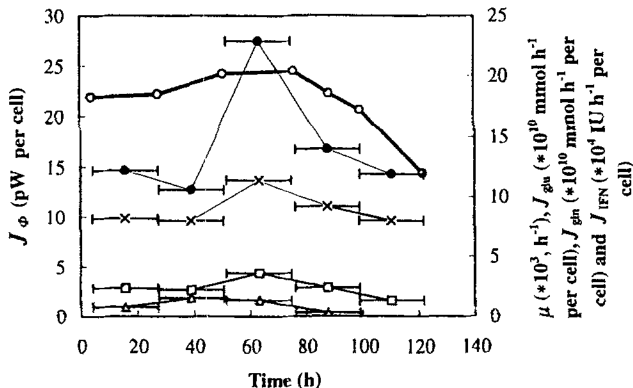
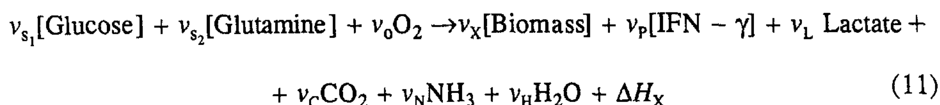


Fig. 3 Comparison of heat flux (J_Φ) with fluxes of glucose (J_{glc}), glutamine (J_{gln}) and IFN-γ (J_{IFN}), and specific growth rate (μ) during the batch cultivation of CHO 320 cells, heat flux O, glucose flux □, glutamine flux Δ, IFN-γ flux ×, specific growth rate •. The bars indicate the period over which the discrete off-line measurements are made to give the individual average values for fluxes

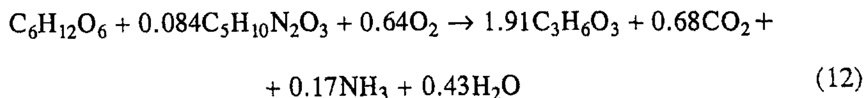
work is that heat flux can now be measured on-line in real-time with good reliability [52].

To illustrate the potential of on-line measurement, Kemp *et al.* [51] grew cells in a 2-L bioreactor at a controlled oxygen saturation from an initial inoculum of 1.3×10^{-5} per cm^3 to 7×10^5 per cm^3 in 120 h (see Fig. 2). After that time, viability rapidly decreased, resulting in an overall decline in cell numbers. The calculated record of heat flux (Fig. 3) was reasonably constant until 80 h. Since it has already been established that this flux reflects the intensity of the catabolic process, the reaction fluxes of the major participants were estimated by measuring the amounts at discrete time intervals and differentiating the resulting data. It will be seen in Fig. 3 that the fluxes of glucose, glutamine and IFN-γ all decreased in the time interval after the reduced heat flux signal became evident. It will also be noticed that the specific growth rate and the flux of IFN-γ production were reduced for the time period 60–80 h and at subsequent intervals. Following the form of the growth reaction for 2C11–12 cells Eq. (9), Guan and Kemp [44] thought the following would be appropriate for CHO 320 cells from the data in Fig. 3,



$$\nu_{s_1}[\text{Glucose}] + \nu_{s_2}[\text{Glutamine}] + \nu_O O_2 \rightarrow \nu_X[\text{Biomass}] + \nu_P[\text{IFN} - \gamma] + \nu_L \text{ Lactate} + \\ + \nu_C CO_2 + \nu_N NH_3 + \nu_H H_2O + \Delta H_X \quad (11)$$

The heat flux strictly is a measure of the catabolic half-reaction, Eq. (9). Taking a representative time period (51–76 h) for Figs 2 and 3, this can be written as,



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

The validity of constructing this description of the catabolic half-reaction can be evaluated by taking a different approach to that summarized by Eq. (3). For the present purpose, the change in enthalpy was calculated from the stoichiometric coefficients in Eq. (12) and the appropriate enthalpies of formation ($\Delta_f H^o$) for the sums of all the reactants and all the products, corrected for side reactions (from the data of Wilholt [43, 44]). After adjusting for the appropriate reaction flux [7], it was possible to estimate the enthalpy recovery at nearly 100%, which fully qualified Eq. (12). From this equation which was originally in Ref. [44], it is possible to make some interesting observations.

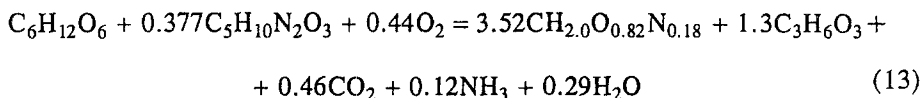
The first is that the minority of the glutamine was completed deaminated and utilized in catabolism. The importance of this amino acid in mammalian cells as an energy source has long been stated [53, 54], but the calculations show that less than half the glutamine in the medium was oxidized to lactate and carbon dioxide (glutaminolysis [42, 55]) in the formation of biosynthetic precursors. It is assumed that the remainder of the glutamine was transaminated, or acted as an amino donor to form amino sugars from fructose-6-phosphate or was incorporated into purines and pyrimidines. It should be recalled that the target protein for these recombinant

cells is IFN-γ, a heavily glycosylated protein (24 kDa in total; 8 kDa for two oligosaccharide chains) with many amino sugars.

A very high proportion of the glucose is converted to lactate by glycolysis with only 10% of it being respired in the mitochondria or as a result of the pentose phosphate pathway. In this respect, a recent report indicates that the majority of glucose oxidized in rapidly-proliferating murine hybridoma cells was channelled through the latter pathway [56], presumably because of the requirements for reductive biosyntheses. High lactate flux is a consequence of the demand for biosynthetic precursors by growing cells [42]. It is not a reflection of adverse oxygen tension or the Uriah crowding effect. As with most studies of intermediary metabolism in cultured animal cells these days, the CHO 320 cells were growing in a controlled, fully aerobic environment.

Although only a small fraction of glucose was completely oxidized, it is calculated from Eq. (12) on the basis of an ATP stoichiometric coupling coefficient (ν_{ATP/O_2}) of 6, that respiration provided 3.6 mol ATP whereas the glycolytic pathway ($\nu_{ATP/Lac}=1$) gave 1.8 and glutaminolysis ($\nu_{ATP/Gln}=8.5$), 0.71. This means that glucose respiration provided 59% of the energy requirement and glutamine oxidation only 12%. Unpublished data for the production of ^{14}carbon dioxide by cells incubated with D-[U-^{14}C]glucose and D-[U-^{14}C]glutamine showed that 9.7% of the former (10.7% in Eq. (12)) was respired and 33.5% of the latter was oxidized by glutaminolysis [57]. Of course, the amounts of energy from the two substrates may well change as the proportion of them alters with their different consumption rates. In fact, a reciprocal regulation of glucose and glutamine has been shown for some cultured cells [58]. This is one of the reasons that catabolic reactions of the type shown in Eq. (12) should be calculated for different stages of batch growth [44].

Guan and Kemp [44] have shown it is possible to extend analysis of the data beyond the half-reaction to the growth reaction shown in Eq. (11). For the period 51–76 h as shown in Figs 2 and 3, this was,



$$C_6H_{12}O_6 + 0.377C_5H_{10}N_2O_3 + 0.44O_2 = 3.52CH_{2.0}O_{0.82}N_{0.18} + 1.3C_3H_6O_3 + 0.46CO_2 + 0.12NH_3 + 0.29H_2O \quad (13)$$

The biomass and IFN-γ terms were combined to give the first formula on the right hand side. An elemental analysis of the biomass has not yet been completed for these cells (but see [59] for a murine hybridoma PQXB1/2) and, although an elemental analysis could be calculated for "model" IFN-γ, the heterologous protein produced in culture suffers from macro- (number of oligosaccharide chains) and microheterogeneity [60]. The most immediately useful consequence of writing the growth equation [44] was to show that the demand for glucose and glutamine was in the ratio 2.7:1 rather than that existing in the medium which was 5.5:1. This infers that cells would be deprived of glutamine well before all the glucose was consumed: this supposition is borne out by the findings for consumption rates revealed in Fig. 3. The cells continued to grow beyond the point of glutamine exhaustion

probably because cells that had passed S phase of the cell cycle – the point when DNA is synthesized with the peak demand for glutamine. They could divide using energy from glucose but then stopped in G_1 phase (G_0). It is not advocated that the amount of glutamine be increased because this would result in more of the toxic ammonia [61] in the medium as a result of chemical decomposition and cellular deamination. It would also result in greater lactate accumulation by glutaminolysis and could be harmful to cells as well [62]. It would be preferable if a fed-batch approach were used (see, for instance [63]) in which the cells were supplied the two substrates in the correct ratio, ideally in response to a signal indicating need.

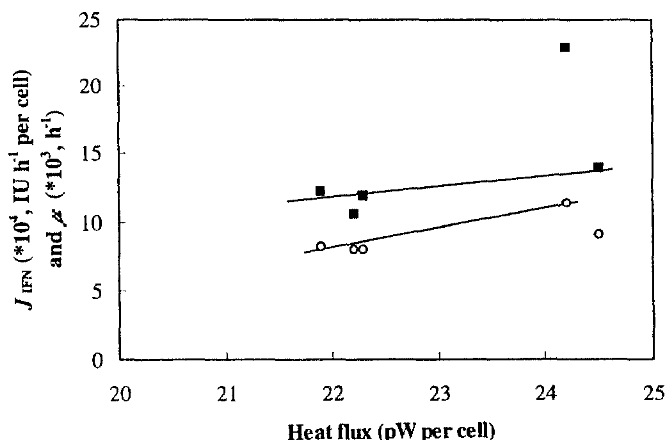


Fig. 4 A tentative plot of specific growth rate of CHO 320 cells (μ) and specific IFN-γ production rate (IFN flux J_{IFN}) *vs.* heat flux. Note that the heat flux is an instant value whereas μ and J_{IFN} are averaged over a period of 12 h

It is evident from Fig. 3 that the signal decreased when the metabolic fluxes had lower values. This relationship is emphasized in Fig. 4 which demonstrates a trend that heat flux monotonically decreased with the metabolic flux of IFN-γ and the specific growth rate [64]. The reason will be seen by reference to the growth reaction [Eq. (11)] in which the enthalpy change can be regarded as a unique kind of stoichiometric coefficient relating energy (heat) to the material balance and thereby allowing its incorporation into the growth reaction. If the reason for a change in heat flux is understood, then action can be taken, for instance by feeding the cells an appropriate mixture of nutrients. For this reason, among others, it would be advantageous to study the anabolic half-reaction. This is revealed by subtracting the catabolic half-reaction from the growth reaction.

* * *

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