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"Fire burn and cauldron bubble" (W. Shakespeare): what the calorimetric–respirometric (CR) ratio does for our understanding of cells?

R.B. Kemp*

Institute of Biological Sciences, University of Wales, Aberystwyth, Wales SY23 3DA, UK

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Abstract

In this paper, it has been shown that the existence of anaerobic pathways under aerobic conditions is not always due to poor environmental conditions. There are two sources of lactate in cultured cells, oxidative glutaminolysis and reductive glycolysis. Only the latter is measured in the CR ratio as a value more negative than the oxycaloric equivalent for the relevant catabolic substrate. The validity of the value for the CR ratio can be determined by Mayer's enthalpy balance method. Highly exothermic ratios are no reflection of thermodynamic efficiency but in many cases indicate the need for ATP not supplied by, or insufficiently available from, oxidative phosphorylation. For other types of cell grown in culture, a highly exothermic CR ratio is due to the fact that there are not sufficient quantities of the appropriate anabolic precursors in the culture medium. Then, biosynthetic precursors must be constructed from substrates using the catabolic pathways and this leads to the reduction of pyruvate in order to conserve NAD^+. An on-line measurement of the CR ratio would monitor cell growth and could be used to control fed-batch cultures.

Keywords: Calorimetric–respirometric ratio; Cultured cells; Anaerobic pathways; Lactate production; Glutaminolysis

1. Introduction

Over 220 years ago, Lavoisier with LaPlace performed the famous calorimetric experiment calculating from the latent heat of melting of ice the total heat produced by a guinea pig that was surrounded by ice [1]. Lavoisier also discovered that oxygen was required for animals to live from his experiment in which a bird in a bell jar expired at the same time as the light from a candle. He concluded from these studies that "respiration is a combustion, a slow one to be precise" [1].

For the majority of vertebrates, it was realised by early this century that there is the regularity between heat production and oxygen uptake rate (OUR) that came to be known as the oxycaloric equivalent [2]. Two major factors contribute to this constant relationship. First, all carbon compounds burn with approximately the same heat yield and, secondly, the most common anaerobic pathway in animals, the glycolytic production of lactate in muscle, does not contribute to the net metabolism because of the conversion of the lactate to glucose in the liver in the Cori cycle. There are, however, aquatic animals that live in

* Tel.: +44-1970-622333; fax: +44-1970-633350.
E-mail address: rbk@aber.ac.uk (R.B. Kemp)

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subnormoxic environments and these have a net contribution of anaerobic pathways to their overall metabolism. This causes an additional amount of heat production per mol O_2 [3]. It came as something of a surprise, however, to find that many animal cells growing in culture under fully controlled aerobic conditions also displayed the same phenomenon — there was the usual slow combustion but something else was occurring in their cauldron! The reasons behind this burning (highly exothermic) circumstance will be explained in this paper.

2. Theoretical

Providing there is no involvement of anaerobic processes in the catabolic process, it is sensible to regard the measurement of the OUR as the index of the metabolic rate. This can be made specific to the mass or the volume of the living system to give the oxygen flux, J_{O_2}. The use of a respirometer is often loosely called indirect calorimetry because, for exclusively (net) aerobic metabolism, the data can be converted to heat flux, J_Q, by applying the appropriate oxycaloric equivalent, $\Delta_k H_{O_2}$,

$$J_Q = \Delta_k H_{O_2} J_{O_2} \quad (1)$$

Oxycaloric equivalents are the theoretical values for the enthalpy changes of the catabolic part of metabolism (often called the catabolic half-cycle or half-reaction), for instance glucose to HCO_3^- and H^+, and they do not include any coupled process such as ATP production. Since no work is done, the net efficiency is zero. For pure substrates, the oxycaloric equivalent can be obtained directly by bomb (combustion) calorimetry [4]. This is further evidence that there is no work component in the oxycaloric equivalent because combustion in a bomb calorimeter is irreversible and thus totally inefficient, not being coupled to any energy conserving mechanism. If combustion data are not available, it is also possible to calculate the equivalents from the standard enthalpies of formation [5]. For a variety of substrates and conditions, Gnaiger and Kemp [5] have calculated that the theoretical oxycaloric equivalents range from -430 to -480 kJ mol^{-1} O_2; $\Delta_k H_{O_2} = -450$ kJ mol^{-1} $\pm$ 15% (see Table 1).

The reason why the oxycaloric equivalents for all substrates are similar is because of the regularity for the heat evolved per equivalent of oxygen. The fact that the data are alike for a wide range of carbon compounds was first reported by Thornton [6] and the most widely accepted average value is -115 kJ per degree of reductance (per equivalent of the available electrons) [7].

Table 1
The oxycaloric equivalents of aerobic respiration for various substrates in aqueous solution at pH 7[a,b]

Substrate	CO_2/O_2	$\Delta_k H^0_{O_2}$	$\Delta_k H'_{O_2}$	$\Delta_k H_{O_2}$
Glucose	1.0	−469	−476	−469
Glycogen	1.0	−469	−477	−469
Palmitic acid	0.70	−431	−435	−434
Triacyglycerols	0.72	−439	−444	−442
Protein → urea	0.84	−436	−442	−438
Protein → NH_4^+	0.97	−443	−450	−443

[a] $\Delta_k H^0_{O_2}$ and $\Delta_k H'_{O_2}$ (kJ/mol O_2) are calculated with enthalpies of neutralisation of 0 and -9 kJ/mol H^+, respectively, and with all reactants dissolved in water. $\Delta_k H_{O_2}$ refers to the same conditions as $\Delta_k H'_{O_2}$, except that O_2 and CO_2 are exchanged with the gas phase. The CO_2/O_2 ratio is the molar gas exchange ratio or respiratory quotient. Values for triacylglycerols, $(C_{18.8}H_{33}O_2)_3$, and protein, $(C_{4.79}H_{7.51}O_{1.49}N_{1.34}S_{0.032})_n$, are calculated from average fatty acid and amino-acid compositions of organisms, with aqueous urea or ammonium ion as nitrogenous end-product.

[b] Reproduced from [5] with permission.

It is worth noting that Thornton's rule also gives an indirect approach to determining the enthalpy of combustion, $\Delta_c H_i$, for the species, i, from the known approximation of the heat evolved per equivalent of oxygen, Q_0, [7] using the relationship [8],

$$\Delta_c H_i = Q_0 \gamma_i \quad (2)$$

where γ_i is the degree of reductance for any compound i of the generalised C-molar formula $CH_{e_{i_1}}O_{e_{i_2}}N_{e_{i_3}}$ defined by

$$\gamma_i = 4 + e_{i_1} - 2e_{i_2} - 3e_{i_3} \quad (3)$$

Eq. (3) makes γ_i four times the number of moles of oxygen required to oxidise one C-mole of compound i to CO_2, H_2O and N_2. This approach is most useful for calculating the enthalpy of biomass for which the direct approach of combustion calorimetry is difficult because of the large amounts of medium-free, dried biomass required for a reliable estimation — 1.5 g [9]. There is still a problem, however, in that this approach requires knowledge of the degree of reductance calculated from the elemental analysis of the biomass.

'Empirical' formulae for the elemental composition of biomass are beginning to appear in the literature and Bushell et al. [10] have given the formula for the murine hybridoma, PQXB1/2, as $CH_{1.7}N_{0.25}O_{0.25}$. From Eq. (3), the degree of reductance of the biomass (γ_b) is 4.45. More recently, there have been determinations of the biomass formula for the SP2/0-Ag 14 myeloma cell line at $CH_{1.78}N_{0.25}O_{0.43}$ [11] that gives γ_b=4.17 and for the *Zac*3 hybridoma at $CH_{1.64}N_{0.24}O_{0.36}$ [12] to give γ_b=4.2. Erickson [7] regards 4.291 as an acceptable generalised degree of reductance when the elemental formula is unknown. Using the generalised value for Thornton's regularity (above) and applying the calculated degrees of reductance to Eq. (2), the enthalpy of combustion for biomass appears to lie in the range −480 to −512 kJ mol^{-1} O_2 with the likelihood of it being towards the lower side. The average value of −450 kJ mol^{-1}±15% [5] calculated from the enthalpies of formation of carbohydrates, fats and proteins is only slightly lower than this range and certainly within the experimental error.

The theoretical oxycaloric equivalent is the expected ratio of the measured calorimetric heat flux and the respirometric oxygen flux, the CR ratio,

$$\text{CR ratio} = \frac{J_Q}{J_{O_2}} \tag{4}$$

In healthy vertebrates, the most common anaerobic product is lactate most commonly in muscle as a result of localised anoxia. However, homeostatic mechanisms ensure that the CR ratio is the same as the above generalised oxycaloric equivalent [2] with the Cori cycle in the liver converting to glucose any lactate formed in the tissues and subsequently excreted into the circulating blood. As noted some years ago [13,14], it is rare indeed for tissue cells grown in culture to have a CR ratio close to the generalised oxycaloric equivalent. In fact, the only authenticated report is for hamster mature brown adipocytes that have a CR ratio of –490 kJ mol^{-1} O_2 when treated with noradrenaline [15]. This hormone stimulates thermogenesis in brown fat by acting on the 33 kDa uncoupling protein (UCP; also known as thermogenin) in the inner mitochondrial membrane to open channels between the cytosol and the mitochondrial matrix [16]. Thus, it causes the dissipation of the protonmotive force, meaning that the electron transport from NADH to O_2 now proceeds at a maximal rate not coupled to the demand for ATP. There is a proportionate increase in both heat production and OUR as a result of the relatively uncontrolled catabolic flux. This is analogous to the situation in the bomb calorimeter (see above) because uncoupling results in a totally inefficient process that permits no energy conservation, i.e. there is no oxidative phosphorylation to produce ATP [17].

For most cells, the CR ratio is highly negative and there have been reports of values as highly exothermic as that reported for human T-lymphoma cells, CCRF-CEM, at −1100 kJ mol^{-1} [18]. In common with the whole animals from which they originate, the usual anaerobic product for cells in culture is lactate [19]. Net production of lactate from glucose is accompanied by a dissipative catabolic enthalpy change, $\Delta_k H_{Lac}$, of −80 kJ mol^{-1} when the acid is buffered in the cytosol. The plasma membrane is very permeable to lactate (and pyruvate) and then the enthalpy change depends on the nature of the buffer in the medium [5]. It is −63 kJ mol^{-1} when excreted into a bicarbonate buffer, −59 kJ mol^{-1} into a phosphate buffer and −77 kJ mol^{-1} into 20 mM HEPES buffer. The molar amount of lactate produced per unit amount of oxygen consumed (Lac/O_2) indicates the relative extent of aerobic glycolysis. The catabolic (k) heat change per mol O_2, $\Delta_k H_{(ox+anox)}$ (CR ratio), is then calculated as,

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

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, $\sum_p/O_2 \times \Delta_k H_p$. Eq. (5) is a form of enthalpy recovery in which the experimental CR ratio is matched against the calculated data from known enthalpy changes and material flows. This is a specialised form of the enthalpy balance method, first advocated as a general approach by Mayer [20]. He developed a physiological theory of combustion in which there is a general balance between the amount of matter consumed and the evolution of heat, 'so as to occasion growth and the renewal of worn-out parts'. In this way, he first introduced the concept of the enthalpy balance method in terms of 'receipts and expenditure' that has proved so useful in determining several mechanisms in cells. Of course, its validity is enshrined in his Conservation

Law of Thermodynamics (the First Law of Thermodynamics).

3. Reasons for anaerobic pathways in mammalian cells

It might be assumed that the sole reason for anaerobic processes in cultured cells is anoxic environmental conditions. While this is, of course, the cause in some cases, there are perfectly plausible biochemical explanations in many instances in which there are fully aerobic physiological conditions. Nevertheless, it should be remembered that the use of ratios considerably increases experimental error so it is vital to ensure that the conditions for both measurements are as similar as possible. All polarographic measurements of OUR are done for stirred cell suspensions whereas there is only a limited availability of stirred calorimetric vessels. Without stirring, cells sediment to form a 'crowded' pile or 'heap' (the Uriah effect) with limited access to the (slightly) dissolved oxygen and this phenomenon can result in increased glycolysis due to the Pasteur effect. Also, an unstirred layer at the electrode surface of the respirometer causes an incorrect reflection of the OUR in an unstirred vessel. In many cases, the calorimetric vessel has a gaseous headspace that allows recruitment of oxygen, whereas the OUR is always measured without one.

It should be remembered that microcalorimeters have extremely low detection limits that can only be matched by some of the better respirometers, for instance the high resolution Oroboros Oxygraph with large diameter Orbisphere electrodes (see [17,21]).

3.1. Oxygen tension

Oxygen is a hydrophobic gas with a consequentially low solubility in water (210.2 μmol dm^{-3} in air-saturated pure water [21]). It is even less soluble in physiological solutions because the salting out effect reduces solubility considerably and sometimes by more than 10%, depending on the complexity of the medium [17]. This is particularly important in closed vessels and especially in those without an air space. It is also a contributory cause of hypoxia and the progression to anoxia in excised tissues and cell suspensions. There is a decrease in specific heat production with increasing sample size that is known in both

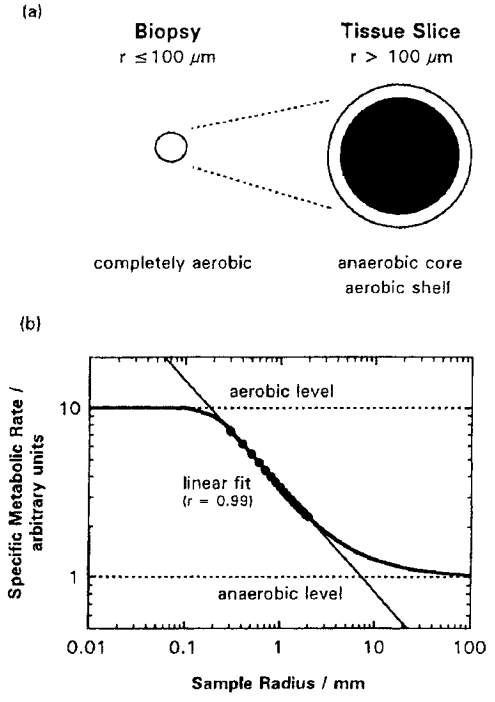

Fig. 1. Influence of the sample size on the metabolic rate (crowding effect). (a) Whereas a small biopsy is completely aerobic, a large tissue slice consists of an an aerobic shell and an anaerobic core. (b) Thus, with increasing sample radius, the heat output per unit of volume shows a sigmoidal decrease from the higher aerobic to a lower anaerobic level. When plotted on log scales, the steep portion of this transition fits a linear regression. Both the critical depth of tissue aerobiosis (100 μm) and the relationship of anaerobic to aerobic metabolism (1:10) are rough assumptions (reproduced with permission from [22]).

direct and indirect biocalorimetry as the 'crowding effect'. This has been mathematically modelled for tissue biopsies by Singer et al. [22] — see Fig. 1. The same phenomenon occurs in unstirred layers of cells (see for instance [23]) in which the physiological conditions in the interstices between them are so poor in terms of low oxygen tension, an inappropriate pH, high ammonia concentration, etc., that they cause a decrease in the metabolic activity [24].

3.2. Low thermodynamic efficiency

It is often thought that the highly exothermic CR ratios reported for cells that have been decoupled or uncoupled in terms of oxidative phosphorylation to form ATP is due to inefficiency. To quote an example

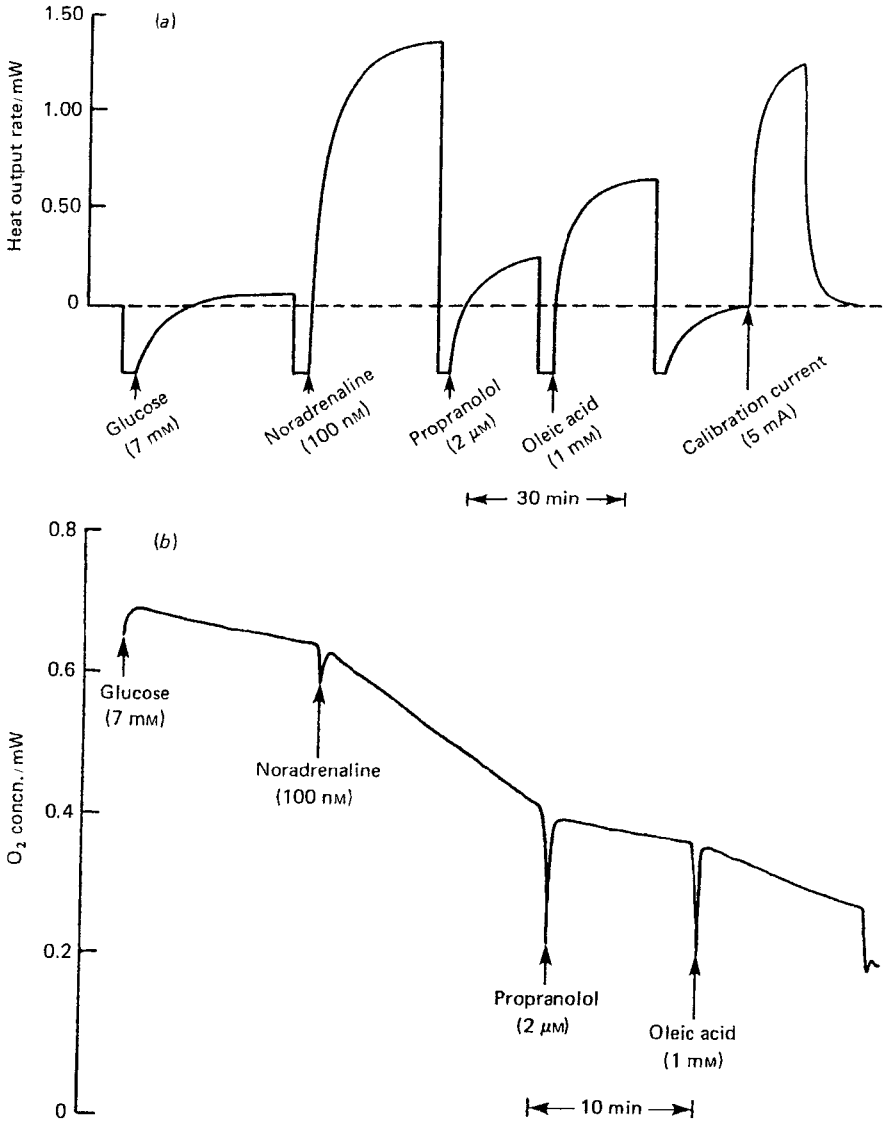

Fig. 2. Representative traces of heat output (a) and O_2 consumption (b) rates by isolated brown adipocytes from cafeteria-fed rats after the sequential addition of glucose, noradrenaline, propranolol and oleic acid. Isolated brown adipocytes (2.65×10^5 cells/cm^3) were prepared from two cafeteria-fed rats. Noradrenaline, propranolol and oleic acid were prepared in Krebs–Henseleit bicarbonate-buffered saline, equilibrated at 37°C for 3.0 min, and added in a concentrated form at the times indicated (reproduced with permission from [25]).

of this possibility, Clark et al. [25] studied the hypertrophy of brown fat in rats fed on a highly palatable food as a mechanism to dissipate the additional energy by increased thermogenesis. They measured the heat flow rate in a rotating LKB batch calorimeter and the OUR in a stirred respirometer. The results for the dissociated brown adipocytes shown in Fig. 2 demonstrate that the injection of noradrenaline stimulated the heat flow rate more than the OUR to give a considerably exothermic CR ratio of -1477 kJ mol^{-1} O_2. This result was interpreted to mean that the necessary ATP production had a reduced efficiency (described later).

In a series of papers using the same combination of direct and indirect calorimetry, Clark's group also studied isolated rat hepatocytes (see reviews in [13,17]). Judging from calculations later undertaken

by Kemp [10], the research showed that dihydroxyacetone and fructose both caused considerably more negative CR ratios than the appropriate oxycakoric equivalents; between -542 kJ mol^{-1} O_2 and approximately -650 kJ mol^{-1} O_2 [26]. These findings were attributed to the increased rates of substrate cycling between metabolites of glucose/glucose 6-phosphate, fructose 6-phosphate/fructose 1,6-bisphosphate and pyruvate/phosphoenolpyruvate to decouple the catabolic process from ATP production and lower the efficiency of ATP production.

It is important to examine the claims that CR ratios more negative than the corresponding oxycaloric equivalent, are due to decreased efficiency. As established by Gnaiger [27], an enthalpy of 258 kJ (i.e. 6×43) is metabolically conserved for each mole of oxygen utilised in the respiration of glucose. Therefore, the highest thermodynamic efficiency for ATP production is 0.55 (i.e. 258/469), using the oxycaloric equivalent of -469 kJ mol^{-1} O_2 [5]. The enthalpy change per mol O_2 of the fully coupled reaction is -211 ($-469+6\times43$) kJ mol^{-1} O_2. The ATP/O_2 ratio decreases with uncoupling. So, the thermodynamic efficiency is lowered, and the enthalpy change per mol O_2 decreases from -211 to a maximum of -469 kJ mol^{-1} for fully uncoupled respiration. Since it has been established above that this value represents zero efficiency, then uncoupling and decoupling to give highly exothermic CR ratios cannot be due to the lower efficiency of ATP production [27]. The reasons for these CR ratios is that, because ATP cannot be synthesised by oxidative phosphorylation, the necessary requirements for it must be fulfilled by the reduction of pyruvate to lactate with the conservation of NAD^+. Substrate phosphorylation in the glycolytic pathway only produces stoichiometrically a net 2 ATP per glucose. The heat produced in this process is additional to that dissipated in the uncoupled flow of electrons across the mitochondrial membrane of the brown adipocytes. Similarly, the decreased availability of ATP in uncoupled hepatocytes means that the demand for it can only be satisfied by increased glycolytic flux.

3.3. Glutaminolysis

As indicated above and in [5,13,14,17,19,24], in many cases of highly exothermic CR ratios the enthalpy balance approach in Eq. (5) proved that aerobic glycolysis to produce lactate was the cause. This is not always the case, however, because many cell types have a special growth requirement for glutamine that can participate in catabolic processes as well as in the biosynthesis of biomass. This amino acid is present in media primarily for purine and pyrimidine synthesis in the anabolism of nucleic acids but it also as an amino donor in the de novo formation of other amino acids and amino sugars. In some tissue cells, however, glutamine is completely oxidised to provide energy. More commonly and highly relevant to the present discussion, it also can be partially oxidised by a process called glutaminolysis to lactate [28,29], with a ATP stoichiometric coefficient of 6ATP/O_2 — see [30]. This pathway causes an overestimate of aerobic glycolysis because the oxycaloric equivalent for glutamine is within the normal range and does not give an additional flow of heat per mole of oxygen. For the HEPES-buffered medium of the type used for 2C11-12 mouse macrophage hybridoma cells [31], the metabolic reaction and corresponding enthalpy change is [32],

$$\begin{aligned} &{}^{+}H_3N(CHCH_2CH_2CONH_2)COO^{-}(aq) \\ &\quad + \tfrac{3}{2}O_2(g) + 3H_2O(l) + HEPES(aq) \\ &\quad \rightarrow CH_3CH(OH)COO^{-}(aq) + 2HCO_3{}^{-}(aq) \\ &\quad + 2NH_4{}^{+}(aq) + HEPES - H^{+}(aq) \quad - 695\,kJ \end{aligned} \tag{6}$$

From this it can be seen that the enthalpy change for the partial oxidation of glutamine is -463 kJ mol^{-1} O_2. The overestimate in terms of the amount of lactate produced in glycolysis can only be addressed by radioactive experiments of the type undertaken with 2C11-12 cells that showed the proportion of total lactate formed in glutaminolysis was 38% [31].

3.4. Lactate excretion resulting from the need for biosynthetic precursors

The reason that normal mammalian cells in culture produce lactate in many cases may be poor medium design [33]. Culture media used to be formulated on the basis of a buffered physiological saline with glucose, the essential amino acids and serum. They were not customised for the actual needs of particular

cell types. For this reason, glucose and glutamine are used by cells as biosynthetic precursors for compounds not available, or not in the correct quantities, in the medium [24]. In the glycolytic pathway, amino sugars arise from fructose 6-phosphate and the amino donor, glutamine; the amino acids serine (for fatty acids) and alanine from 3-phosphoglycerate; the heterocyclic acids, phenylalanine, tyrosine and tryptophan from phosphoenolpyruvate; and oxaloacetate from pyruvate and carbon dioxide (the anaplerotic reaction) [17]. These reactions mostly require three-carbon units to be produced at a rapid rate. As a result, a large quantity of pyruvate is available that is surplus to energy requirements and is reduced to lactate to pay back NAD^+. The partial oxidation of glutamine to lactate catalysed by some of the enzymes of the Krebs' cycle and followed by transamination, enables other amino acids to be formed from α-ketoglutarate and oxaloacetate.

In order to optimise cell growth and minimise the production of toxic lactate, Xie and Wang [34] undertook an exhaustive stoichiometric analysis of all the metabolic requirements for growth of a particular hybridoma cell and this reduced their lactate production by 90%. A more empirical approach monitored by the heat flux probe [33] was taken to improve the culture medium of recombinant CHO 320 cells. The redesigned medium improved the specific growth rate and the flux of IFN-γ while decreasing the catabolic flux, especially of glucose and lactate.

An illustration of the relationship between highly negative CR ratios and growth can be seen in Fig. 3 from a study of the growth of recombinant CHO 320 cells [35]. The growth of these cells was monitored on-line by a dielectric spectrometer that measures the volume fraction of viable cells. It can be seen that the oxygen flux of the viable cells remained constant over the whole culture period even when there was no net cell growth, as indicated by the plateau in the capacitance curve. On the other hand, the CR ratio was highly exothermic (ca. $-700\ kJ\ mol^{-1}\ O_2$) during growth, only reducing to a level ($-443\ kJ\ mol^{-1}\ O_2$) indicative of oxidative metabolism when there was no increase in cell numbers. Guan et al. [35] stated there was some evidence that the amount of excreted lactate decreased after glucose and glutamine were fully exhausted in the medium. Since there was still an oxygen flux (see Fig. 3), it is possible that the lactate was oxidised as a source of energy. The mechanism for the oxidation of lactate to pyruvate requires the isozyme H_4 of the lactate dehydrogenase complex and a low $NADH/NAD^+$ ratio. These conditions occur in liver cells for the operation of the Cori cycle but it is not clear that other cells can operate in this way. It seems probable that pyruvate carboxylase (PYC) is more important to the continuation of this oxidative

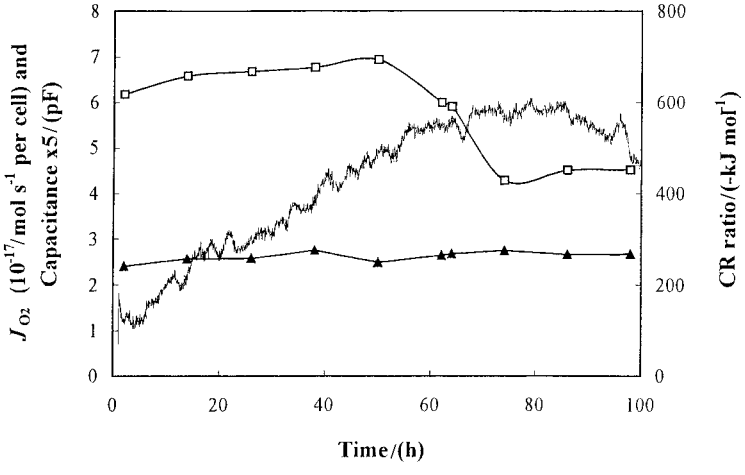

Fig. 3. Comparison of the viable cell concentration measured by the on-line capacitance signal with oxygen flux and the calorimetric–respirometric (CR) ratio in CHO 320 cell batch culture buffered by 20 mM HEPES and 4 mM bicarbonate. The curves are ▲——▲ for the averaged oxygen flux in terms of an individual viable cell on average, and □——□ for the CR ratio. The curve — stands for the capacitance signal with the value for the cell-free medium automatically 'backed off' by the software program (reproduced with permission from [17]).

pathway than the dehydrogenase and there is evidence that the transfection of BHK cells with a PYC construct dramatically decreased lactate production and improved growth [36]. In any case, it would seem advantageous to the control of cell metabolism in bioreactors if it were possible to measure the OUR of the cells in their typically dilute suspensions [37]. This is not as easy as it may appear because conventionally it requires high resolution, polarographic respirometry [38]. Since closed system respirometry cannot be used in bioreactors, it is likely that the OUR would be most sensitively measured in batch cultures by the method of the stationary liquid phase balance [37].

4. The on-line application of the CR ratio to cells growing in bioreactors

It is clear from the foregoing sections that combining the on-line measurement of OUR with the heat flux probe developed by us [35] is a key for the future success in the biotechnology industry of strategies to optimise cell cultures for the most efficient production of high quality target proteins. Yet it has remained difficult to measure consumption for the relatively dilute cell number concentrations in the typical bioreactor culture. This is due to the small oxygen flux compounded by the low solubility of the hydrophobic gas in medium that exhibits the salting out effect which lowers solubility in pure water by as much as 10% [21].

These limitations have pointed researchers towards an instrumental requirement of using very expensive mass spectrometers to obtain the required accuracy [39,40]. One dedicated to each bioreactor in an industrial plant to fulfil the need for the continuous measurement of the variable for control purposes might seem prohibitive on the grounds of cost. For this type of measurement, Eyer et al. [39] and Oeggerlli et al. [40] among others wrote the necessary oxygen mass balance for the whole bioreactor, the so-called global balance [37]. It may be possible to envisage a more pragmatic solution in terms of cost if the balance were based on the liquid phase of the culture, particularly with a constant dissolved oxygen concentration (DO) [37]. This is called the method of the stationary liquid phase balance. Some groups taking this approach have still used a mass spectrometer to measure the molar fraction of oxygen (for instance, [39,40]) but others have found much cheaper alternatives [41,42]. It is, of course, possible to be too simple and then OUR can only be approximate [41]. Ramírez and Mutharasan [42], however, have found a combination of equipment in which the molar fraction of oxygen is calculated very accurately [37] from the set point of each gas flow rate that is fixed automatically to control the DO. The problem with all methods involving only the liquid phase is obtaining the volumetric mass transfer coefficient, $k_L a$, but a good solution to that problem lies in the choice of hydrophobic silicone tubing for aeration [37].

For bench scale systems the bioreactor is aerated through a metered mixture of oxygen, nitrogen and carbon dioxide (initially 5%) delivered through a predetermined length of silicone tubing. This type of tubing has a very high oxygen diffusion coefficient so that the oxygen concentration on the surface in contact with the medium is constant and solely dependent on the oxygen molar fraction in the tubing [37]. Of course, it is important to avoid any gas bubbles. In order to maintain a constant $k_L a$, the DO must be measured with a high precision, low drift, sterilisable oxygen electrode A/D interfaced to the bioreactor controller and thence to suitable software. The DO is maintained constant throughout the experiments at a percentage saturation to be determined by experimentation. The total flow rate of the gases must also remain constant and relatively high but, again, the actual rate must be determined for the particular cell system. This requires very high quality mass flow controllers. The DO control is made automatically through the software using an on/off control program in which the DO must be maintained to within 1% of the set point. The $k_L a$ at the gas–liquid interface of the silicone tubing is small, so the $k_L a$ value will depend on the length of tubing [37].

Since the dissolved oxygen concentration, C_L is maintained constant, the oxygen transfer rate (OTR) must be equal to the OUR. Therefore, an oxygen balance in the liquid phase yields

$$\mathrm{OUR} = k_L a[C_L^* - C_L] \tag{7}$$

where C_L^* is the dissolved oxygen concentration in equilibrium with the gaseous phase in the silicone tubing. The gas flow in the tubing will be maintained

at a very high rate so that only a very small percentage of the oxygen (<0.5%) will be consumed and thus it can be assumed that there is an oxygen equilibrium between the gas and liquid phases [42]. So, C_L^* can be calculated as

$$C_L^* = B\frac{Pn_M F_{O_2}}{HF_T} \quad (8)$$

where F_{O_2} and F_T are the oxygen and total gas flow rates, respectively, H is the apparent Henry's constant for oxygen in medium, P is the total pressure, n_M is the molar concentration of the medium (assuming water), and B is a conversion factor equal to 1000 when C_L^* is expressed in mmol. Henry's constant can be readily obtained assuming that oxygen concentration in medium saturated with air is 0.194 mM [41].

5. Conclusions

It has been shown that the CR ratio indicates the intensity of anaerobic pathways operating simultaneously with aerobic reactions. The most common end product is lactate but caution is necessary in interpreting its production for enthalpy balance purposes because it is also found in the partial oxidation of glutamine (glutaminolysis). When the medium is normoxic, the highly exothermic CR ratio can be due to one of two causes: (i) the ATP demand exceeds the mitochondrial capacity, resulting in the reduction of pyruvate with conservation of NAD^+; or (ii) the need for biosynthetic precursors from catabolic substrates because the medium is, or becomes, deficient in them. This can be monitored on-line by a combination of the heat flux probe [35] and OUR measured by the stationary liquid phase balance [37,42]. The prospect for the next millennium is that the ratio becomes established as the control variable in the growth of cells in batch culture to give considerably higher specific production of higher fidelity target proteins. To quote three separate lines from the Witches in Act 4, sc. 1 of Shakespeare's *Macbeth*,

"Fire burn and cauldron bubble
Like a hell-broth boil and bubble
Make the gruel thick and slab"

Then, the efficiency of fed-batch culture will be excellent!

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