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Studies of enthalpy balance and adenosine triphosphate turnover in cultured animal cells

R.B. Kemp

The Cell Biology Laboratory, The Department of Biological Sciences, The University College of Wales, Aberystwyth, Dyfed SY23 3DA, U.K.

Abstract

The measurement by microcalorimetry of heat flux in cultured animal cells can be allied to biochemical analyses of the catabolic process. This energy balance approach is exemplified by studies on muscle, human neutrophils and T-lymphoma cells using the heat flux/enthalpy flux ratio. The heat flux/reaction flux ratio is another form of this approach. An important example is the calorimetric-respirometric (CR) ratio, which was used to show that cultured LS-L929 mouse fibroblasts undergo intensive anaerobic glycolysis simultaneously with respiration. Once an account of energy balance was achieved, ATP turnover was calculated from heat flux measurements and the coupled flows and forces between catabolic reactions and the ATP cycle using mechanistic molar stoichiometries.

INTRODUCTION

Twenty five years ago, the construction (ref. 1) and commercial production of a differential heat conduction microcalorimeter (μW range) with a rapid response time (120 s) stimulated a substantial increase in studies of heat dissipation by erythroid (ref. 2) and non-erythroid cells (refs. 3,4).

Results from a calorimeter are expressed in power units [W] but, in a closed isothermal system, the measurement is actually of heat flow, $\phi = dQ/dt$ where Q = heat, not power, $P = dW/dt$, where W = work (refs. 5-7). Heat flow is a reflection of the metabolic activity of the cell, so it would be preferable to cite heat measurements in terms of "per unit volume" or biomass, scalar heat flux, J_Q [$W.m^{-3}$] (ref. 4). The enthalpy change, dH, of the closed isothermal system containing cells is due solely to the exchange of heat without any exchange of work. Within this limitation, scalar heat flux of cells equals the enthalpy flux of all the reactions in the calorimetric vessel, $J_{H,r}$ (ref. 7). Careful experimental design should eliminate or, by use of controls, estimate the heat flux of media interactions. Then, heat and enthalpy fluxes are related to metabolic flux by the molar enthalpy, $\Delta_r H_B$ [$J.mol^{-1}$] (ref. 7) of all the reactions.

$$J_Q = J_{H,r} = J_B \Delta_r H_B \qquad (1)$$

where subscript B indicates that any given reaction stoichiometry is divided by $|\nu_B|$ such that a stoichiometric form is obtained with $|\nu_B| = 1$. ν is the stoichiometric number.

There is a device from the 1st Law of Thermodynamics to ensure that full account has been taken of all the sources of enthalpy change in cells - the energy (enthalpy) balance method. The reactions in each metabolic pathway of the cell have known enthalpy changes and stoichiometries. Thus, analysis of substrate consumption and endproduct formation with appropriate calculations will give the overall molar enthalpy change. It should equal the total heat change. In formal

terms (ref. 7), this is the heat flux/enthalpy flux ratio (energy recovery), $Y_{Q/H}$,

$$Y_{Q/H} = J_Q/J_H \tag{2}$$

In seeking the sources of the "unexplained" heat, it was noted that oxidation of glucose and glutamine only accounted for 66% of the observed rate of oxygen consumption (ref. 13). It has been known since 1917 (ref. 17) that, in combustion of most organic compounds, reaction enthalpy is independent of the kind of carbon source when calculating the electron transfer from the substrate to oxidant. The oxycaloric equivalent is 110 $kJ.mol^{-1}$ electrons. Among others, Gnaiger and Kemp (ref. 18) have calculated from enthalpies of formation, the oxycaloric equivalents of some common catabolic substrates and suggest an average value of -450 $kJ.mol^{-1}O_2$ ± 5%. It was thus possible to calculate (ref. 13) that the oxidation of unidentified organic substrates by T-lymphoma cells would give a heat flow of 4.2 $pW.cell^{-1}$, increasing the theoretical enthalpy flux to 12.024 $pW.cell^{-1}$, $Y_{Q/H} = 1$. As stated earlier, the culture medium for growing T-lymphoma cells was complex, containing many amino and fatty acids as candidates for catabolic oxidation.

CALORIMETRIC-RESPIROMETRIC RATIO

As intimated above, oxygen flux ($_rJ_{O_2}$) is related to heat flux ($_rJ_Q$) by appropriate oxycaloric equivalents, $\Delta_rH_{O_2}$ (ref. 18),

$$_rJ_Q = \Delta_rH_{O_2} \times {}_rJ_{O_2} \tag{3}$$

This equivalent is the expected ratio of calorimetric measured heat flux and respirometric oxygen flux, CR ratio,

$$\text{CR ratio} = Y_{Q/B} = {}_rJ_Q/{}_rJ_{O_2} \tag{4}$$

In contrast to the heat flux/enthalpy flux ratio, this is a heat flux/reaction flux ratio, $Y_{Q/B}$ (ref. 7),

$$Y_{Q/B} = J_Q/J_B = J.mol^{-1} \tag{5}$$

For cells to possess fully aerobic catabolism, the CR ratio must be close to the oxycaloric equivalent (-450 $kJ.mol^{-1}$ O_2 ± 5%). A number of cell types of different origin have now been studied (see refs. 4, 18-20) and only one, the hamster brown adipocyte, had fully aerobic catabolism (-490 $kJ.mol^{-1}$ O_2 - ref. 21). It has been shown (ref. 18) that CR ratios more negative than about -500 $kJ.mol^{-1}$ O_2 must be due to simultaneous anaerobic and aerobic catabolism because the calculations have been made on the assumption of zero net efficiency. Therefore, alternative explanations, such as increased futile cycling and uncoupling cannot be the cause.

It used to be thought that intensive glycolysis under aerobic conditions *in vitro* only occurred in neoplastic cells (refs. 12,22) or when normal cells became so crowded that dissolved oxygen was not physically available in the microenvironment. This is a plausible explanation for the highly exothermic CR ratio of mouse lymphocyte hybridoma cells at confluency (ref. 23). It is now becoming clear that most growing normal cells have more negative CR ratios than possible from respiration alone (see refs. 24,25). While it is true that glycolysis in growing cells can provide much of their energy requirements (ref. 26), it has been proposed that the only essential feature of glycolysis is the provision of intermediates for biosynthesis (refs. 27,28).

In simultaneous aerobic and anaerobic metabolism, the catabolic heat change per mol O_2, $\Delta_rH_{(ox + anox)}$, is calculated using the enthalpy balance method by adding to the appropriate oxycaloric equivalent, the molar amounts of glycolytic end-products (p) formed per unit amount of oxygen consumed (p/O_2 ratio) with the

requisite dissipative enthalpy changes of endproduct ($\Delta_r H_p$)

$$\Delta_r H_{(ox + anox)} = \Delta_r H_{O_2} + \sum_p p/O_2 \times \Delta_r H_p \quad (6)$$

and, if the energy recovery is 1, then a full account has been made of all reactions and side reactions in a complex reaction system, which do not exchange energy in the form of work. If there is "unexplained" heat, $Y_{Q/H} > 1$, then the chemical analysis has failed fully to account for the measured heat flux. On the other hand, if endothermic reactions remain undetected, the ratio will be less than 1.

STRIATED MUSCLE ENERGETICS

Using the energy balance method, Woledge (ref. 8) showed that 79% of the heat dissipated in an isometric tetanus of frog sartorius muscle at 0°C was due to the splitting of phosphocreatine, $Y_{Q/H} = 1.26$. In the search for the sources of "unexplained" heat, it was found that the activity of muscle proteins in the myofibrils made a contribution (ref. 9). In terms of molar enthalpy change, this remains the situation because the remainder of the heat flux was due to the movement of Ca^{++} ions from binding sites in the sarcoplasmic reticulum to binding sites on troponin C and parvalbumin (ref. 10). The reversal of this process at the end of contraction involved the exothermic splitting of adenosine triphosphate (ATP) by the calcium pump ATPase. This was part of the energetic cost of contraction even though it was not concurrent with it. The accompanying heat flow was less than expected probably because hydrolysis of ATP was coupled to the endothermal process of transporting two Ca^{++} ions for each ATP split in the sarcoplasmic reticulum.

ENTHALPY BALANCE OF ANIMAL CELLS

With the classical studies of muscle physiology as a background, Eftimiadi and Rialdi (ref. 11) used well-established biochemical procedures to estimate the molar enthalpy changes of pathways in resting human neutrophils cultured in physiological saline containing glucose. The changes were calculated from enthalpies of formation and the relative proportions of each catabolic pathway in the complex reaction system was determined from assays of oxygen consumption and lactate production, as well as a radiocarbon trace for the pentose phosphate pathway (PPP). It was thus calculated that the molar enthalpy change for glycolysis amounted to 1.8 $pW.cell^{-1}$ with a relatively minor contribution by glycolysis (4.03 $nW.cell^{-1}$). The calculated total enthalpy change was 23.55 $nW.cell^{-1}$ which was almost identical to the measured heat flow of 23.5 $nW.cell^{-1}$ - an energy recovery of 1. This showed that account had been made of all the reactions contributing to the metabolic burst by PMA-activated human neutrophils. Most of the NADPH required for oxygen reduction to hydrogen peroxide and other reactive metabolites was supplied by the pentose phosphate pathway.

Bäckman (refs. 12,13) sought to discover the contribution of glucose and glutamine to the total energy turnover during growth of a human acute lymphocytic leukaemia cell line of the T-cell type (CCRF-CEM;T-lymphoma) cultured in the complex RPMI 1640 medium supplemented with 10% foetal calf serum and 300 $mg.dm^{-3}$ glutamine and buffered to pH 7.2 ± 0.1 with 20 $mmol.dm^{-3}$ HEPES. Radiocarbon assays showed that glucose was partly oxidized to CO_2 (21%) but mostly broken down to lactate (79%). Glutamine was partly oxidized to CO_2 with formation of lactate and pyruvate (glutaminolysis - ref. 14) and there was also an accumulation of glutamate, amounting to 20% of glutamine utilization. The greatest danger in this type of study is isotope exchange but reaction rates were obtained from rates of accumulation of lactate, pyruvate, glutamate and CO_2. Molar enthalpy changes with HEPES as buffer were calculated from published enthalpies of formation (refs. 15,16) and so it was possible to derive enthalpy flux values in terms of $pW.cell^{-1}$. The reactions involved in converting glucose to lactate gave a theoretical 4.5 $pW.cell^{-1}$; glucose oxidized to CO_2, 1.2 $pW.cell^{-1}$; glutamine to glutamate, 0.024 $pW.cell^{-1}$ and glutaminolysis, 2.1 $pW.cell^{-1}$. The total "explained" heat was thus 7.824 $pW.cell^{-1}$. Since the observed heat flow was 12.0 $pW.cell^{-1}$, there was an energy recovery or heat/enthalpy flux ratio, $Y_{Q/H}$, of 1.53.

This could be normalized to the oxycaloric equivalent, $\Delta_r H_{O_2}$, to give the anaerobic quotient, AQ,

$$AQ = \Delta_r H_{O_2} / \Delta_r H_{(ox + anox)} \quad (7)$$

An AQ of 1 would indicate fully aerobic catabolism and the greater the decrease from 1, the more intensive the glycolytic process.

The most common glycolytic endproduct is lactate. Its actual molar enthalpy change is dependent (ref. 18) on whether it is buffered within the cell (-80 $kJ.mol^{-1}$) or excreted into a medium buffered with bicarbonate (-63 $kJ.mol^{-1}$), HEPES (-77 $kJ.mol^{-1}$) or phosphate (-59 $kJ.mol^{-1}$). A calculation from the lactate produced by PMA-activated human neutrophils (103 $fmol.h^{-1}.cell^{-1}$, ref. 11), gives a catabolic molar enthalpy change, $\Delta_r H_{(ox + anox)}$, of -577 $kJ.mol^{-1}$, very close to the observed CR ratio of -588 $kJ.mol^{-1}$ O_2. Incidentally, a similar calculation for resting neutrophils reveals a problem of analysis because $\Delta_r H_{(ox + anox)}$ is -1348 $kJ.mol^{-1}$ on the basis of a rate of lactate production of 111 $fmol.h^{-1}$ $cell^{-1}$, whereas the CR ratio is -1132 $kJ.mol^{-1}$ O_2. Leaving aside this discrepancy, the enthalpy balance approach has revealed in several cases the need to analyse for endproducts additional to lactate. T-lymphoma cells possessed a CR ratio of -747 $kJ.mol^{-1}$ O_2 (analysed from ref. 13) but $\Delta_r H_{(ox + anox)}$ is only -715 $kJ.mol^{-1}$. A Pyr/O_2 ratio of 0.07 accounts for -10 $kJ.mol^{-1}$ but, clearly, a further endproduct succinate, needs to be found to achieve an energy balance. Analysis of results for resting 2C11-12 mouse macrophage hybridoma cells (ref. 25) gave a catabolic enthalpy change, $\Delta_r H_{(ox + succ)}$, of -803 $kJ.mol^{-1}$. Therefore, other endproducts, such as pyruvate, require measurement. The same could be stated for the thermochemical analysis of L929-derived LS mouse fibroblasts which were shown to have a CR ratio of -633 $kJ.mol^{-1}$ O_2 and a calculated molar enthalpic change, $\Delta_r H_{(ox + lac)}$, of -601 $kJ.mol^{-1}$.

The CR ratio is a valuable device for indicating the existence of anaerobic processes in cells maintained under aerobic conditions and for constructing an enthalpy balance to account for all the components in the process. It conveys information on the relative intensity of glycolysis. For instance, glycolysis was responsible for 64% of the heat flow from resting human neutrophils (CR ratio = -1132 $kJ.mol^{-1}$ O_2) and only 17% of that from PMA-activated cells (CR ratio = -588 $kJ.mol^{-1}$ O_2) but it is not the nature of a ratio to convey absolute intensity of aerobic and anaerobic processes or the actual carbon flux.

ADENOSINE TRIPHOSPHATE TURNOVER

Under steady state conditions, ATP generation (supply) is tightly coupled to its utilization (demand), giving a constant ATP/ADP ratio. Therefore, the accurate quantification of ATP flux is a major experimental problem. The catabolic process, however, is tightly coupled to ATP supply and ATP demand is tightly coupled to anabolic processes, which have a net enthalpy change close to zero (ref. 30). In developing this theme, ATP generation is not regarded as anabolic but as a process separate to catabolism and anabolism - Gnaiger (ref. 31) has coined the word "ergobolism" for the ATP cycle. At any rate, at steady state, the endothermic enthalpy of ADP phosphorylation is balanced by the exothermic enthalpy of ATP hydrolysis. So, the turnover of ATP is reflected in the catabolic flux - the Gibbs energy changes of the catabolic process coupled to ATP supply, the molar reaction Gibbs energy, $\Delta_r G_B$. For aerobic catabolism only, the small entropy change for oxidation of sugars, amino acids and fatty acids (ref. 32) means that the molar reaction Gibbs energy, $\Delta_r G_{O_2}$, is close to the oxycaloric equivalents. For example, the Gibbs energy change for glucose, $\Delta_r G_{glc}$, is -2903 $kJ.mol^{-1}$ (ref. 33). Since 6 mol O_2 are consumed per mol glucose in aerobic catabolism, $\Delta_r G_{O_2}$ is -2903/6 = -484 $kJ.mol^{-1}$ O_2: the oxycaloric equivalent for glucose being -469 $kJ.mol^{-1}$ O_2 (ref. 18). Thus, the ATP yield per J in glucose oxidation is 12.8 $\mu mol\ ATP.J^{-1}$. In the breakdown of glucose, the net production of lactate coincidently has only a small entropy change. Taking the enthalpy change, $\Delta_r H_{lac}$, for lactate excreted into a bicarbonate buffer (ref. 18), the ATP yield per J is 15.9 $\mu mol\ ATP.J^{-1}$.

It has been found that LS-L929 mouse fibroblasts had a heat flux of 60 $mW.g^{-1}$ protein, an oxygen flux of 95 $nmol.s^{-1}.g^{-1}$ protein, a pyruvate flux of 16 $nmol.s^{-1}.g^{-1}$ protein and a lactate flux of 200 $nmol.s^{-1}.g^{-1}$ protein (ref. 24). Using ATP yields calculated above, it was estimated that the cells had an ATP turnover of 0.834 $\mu mol.s^{-1}.g^{-1}$ protein. The ATP content of the cells was 7.1 $\mu mol.g^{-1}$ protein and, thus, the turnover time was 8.5s.

There is a second, non-calorimetric means to determine ATP turnover based on similar assumptions to those used above, originating in irreversible thermodynamics (refs. 20,34). The supposition is that total cellular energy flow is functionally related to the coupling of input to output flows and input forces to compensatory forces (ref. 29). In the overall process, the high driving force of the catabolic reactions is coupled to the ATP cycle which is in turn coupled to anabolic reactions (ref. 31). Each has a force and a flow and the product is power. The force in the ATP cycle (e) is ADP phosphorylation to ATP - the actual Gibbs energy change, $\Delta_e G_{ATP}$. The partner to this force is the flow of the cycle expressed as the rate of ATP production, $_e\dot{N}$ ($\mu mol\ ATP.h^{-1}$).

The catabolic flow, $_k\dot{N}_i$, can be measured as catabolic substrate consumption, $_k\dot{N}_S$; as the rate of oxygen consumption, $_k\dot{N}_{O_2}$; or as the rate of accumulation and excretion of endproducts, $_k\dot{N}_p$ (ref. 34). The catabolic input force is expressed as the Gibbs energy change per mol substrate, $\Delta_k G_i$. The link between ATP cycle and catabolic flows and forces is provided by the mechanistic molar stoichiometry, $\nu_{ATP/i}$, where i is the catabolic reactant. The catabolic force efficiency, $_kf$, and flow efficiency $_kj$, are defined as the ATP cycle/catabolic flow and force ratios, respectively, normalized for the ATP stoichiometry (ref. 34):

$$_kf = \frac{\Delta_e G}{(\Delta_k G_i / \nu_{ATP/i})} \qquad (8.1)$$

$$_kj = \frac{_e\dot{N}}{(_k\dot{N}_i \times \nu_{ATP/i})} \qquad (8.2)$$

In eq. (8), the two expressions in brackets are the catabolic coupling force and flow, respectively, normalized for the mechanistic ATP-coupling stoichiometry (ref. 31):

$$\Delta_k G = \Delta_k G_i / \nu_{ATP/i} \qquad (9.1)$$

$$_k\dot{N} = _k\dot{N}_i \times \nu_{ATP/i} \qquad (9.2)$$

From eqs.(8.2) and (9.2), it is seen that, for the fully coupled process ($_kj = 1$), the rate of ATP production, $_e\dot{N}$, numerically equals $_k\dot{N}_i$ which is then the rate of ATP turnover. Taking the data available for LS-L929 mouse fibroblasts (ref. 24) and using the ATP stoichiometric coefficient for oxygen of 36/6 = 6.0 ATP/O_2 and, for glycolysis, the mechanistic stoichiometries of 1.0 ATP/Lac and 1.0 ATP/Pyr, ATP turnover rate is (95 x 6) + (200) + (16) = 0.786 $\mu mol.s^{-1}.g^{-1}$ protein. By this estimate, turnover time was 9.0 s, which is in reasonably good agreement with that calculated from heat flux measurements (8.5 s).

CONCLUSIONS

Microcalorimetric measurements of cultured animal cells is a reliable and non-destructive technique to ensure that all catabolic pathways involved in energy production are drawn into account by biochemical analysis. A balanced energy budget is achieved when the heat flux/enthalpy flux ratio $Y_{Q/H}$ is 1. One form of another enthalpy budget method, the heat flux/reaction flux ratio, $Y_{Q/B}$, is valuable in studying the involvement of anaerobic glycolysis in the aerobic catabolic process. It is the calorimetric-respirometric ratio (CR ratio), in which substance B is the oxygen consumed in respiration.

ATP turnover is technically difficult to estimate but it is shown to be possible directly from heat flux measurements and also from the coupled flows and forces between catabolism and the ATP cycle using mechanistic molar stoichiometrics.

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