

BBABIO 43195

Anaerobic metabolism in aerobic mammalian cells: information from the ratio of calorimetric heat flux and respirometric oxygen flux

Erich Gnaiger¹ and Richard B. Kemp²¹ Department of Zoology, University of Innsbruck, Innsbruck (Austria) and ² Department of Zoology, University College of Wales, Aberystwyth (U.K.)

(Received 8 May 1989)

(Revised manuscript received 12 October 1989)

Key words: Calorimetry; Respirometry; Oxycaloric equivalent; Anaerobic metabolism; Thermodynamic efficiency; Metabolism; (Human cell culture)

Calorimetric and respirometric studies of cultured cells show that both neoplastic and non-neoplastic cell types maintain an anaerobic contribution to their total heat flux. In many mammalian cells this can be explained quantitatively by lactate production observed under fully aerobic conditions. Uncoupling and enhanced futile substrate cycling increase the ratio of heat flux to oxygen flux, the calorimetric-respirometric (CR) ratio. The interpretation of calorimetric and respirometric measurements requires an energy balance approach in which experimentally measured CR ratios are compared with thermochemically derived oxycaloric equivalents. The oxycaloric equivalent is the enthalpy change per mole of oxygen consumed, and equals -470 kJ/mol O₂ in the aerobic catabolism of glucose, assuming that catabolism is 100% dissipative (the net efficiency of metabolic heat transformation is zero). CR ratios more negative than -470 kJ/mol O₂ have been reported in well-oxygenated cell cultures and are discussed in terms of integrated aerobic and anaerobic metabolism.

Introduction

Futile substrate cycling is one of the mechanisms regulating metabolic flux [1,2]. Futile cycling decouples substrate level phosphorylation. For example, in the glucose/glucose 6-phosphate cycle, ATP is consumed in the phosphorylation of sugar, but ATP is not regenerated in the dephosphorylation step which completes the futile cycle. The quantitative significance of decoupling in ATP turnover still requires resolution. Uncoupling is a different control mechanism to decoupling, because uncoupling of electron transport decreases the formation of ATP. Uncoupling is responsible for enhanced oxygen uptake in brown adipose tissue, which results in a calorogenic response [3–5].

It is frequently observed that both decoupling and uncoupling not only elevate oxygen flux but disproportionately increase heat flux [6–9], resulting in an increased ratio of calorimetric heat flux to respirometric oxygen flux (CR ratio) [10,11]. It has been argued that

the higher heat loss per unit oxygen uptake reflects the lower efficiency of ATP production associated with the wasteful operation of futile cycles (e.g., Ref. 7). By reducing the catabolic ATP output, more energy is lost as heat. This intriguing hypothesis demands a more

List of symbols

Symbol	Unit	Meaning
CR ratio	kJ/mol O ₂	calorimetric-respirometric ratio, heat flux divided by oxygen flux
J_{O_2}	mol O ₂ ·s ⁻¹ ·g ⁻¹	oxygen flux, per unit biomass
Lac/O ₂	mol·mol ⁻¹	lactate/oxygen ratio
$\Delta_k H_{O_2}$	kJ/mol O ₂	oxycaloric equivalent, catabolic enthalpy change per mol O ₂
$\Delta_k H_{Lac}$	kJ/mol Lac	catabolic enthalpy change per mol lactate
$\Delta_k Q_{O_2}$	kJ/mol O ₂	heat change per mol O ₂ calculated for simultaneous aerobic and anaerobic catabolism
$\Delta_t Q$	J	total observed heat change over a period of time, Δt
subscript k		catabolic, half cycle reaction excluding net ATP production
subscript t		total, experimentally observed

Correspondence: E. Gnaiger, Department of Zoology, University of Innsbruck, Technikerstr. 25. A-6020 Innsbruck, Austria.

detailed thermodynamic analysis of coupled, decoupled and uncoupled metabolism. This analysis indicates that such highly negative CR ratios are the result of anaerobic processes and are not related to un- or decoupling as such. Emphasis is placed on the integration of respiration and glycolysis in isolated and cultured cells. Importantly, aerobic glycolysis is not restricted to specific cell types such as tumour cells [11]. Aerobic lactate production explains CR ratios more negative than $-470 \text{ kJ} \cdot \text{mol}^{-1}$ in several isolated mammalian, including human, cells.

Thermochemical calculations

From the principle of the First Law of Thermodynamics, the energy (enthalpy) balance method is used to interpret simultaneous measurements of biochemical processes and heat changes occurring in living systems. The energy balance method has been rigorously employed in studies of muscle energetics [12,13], and investigations of aerobic and anoxic metabolism of whole animals [14] and microorganisms [15]. At constant barometric pressure, the total heat change, ΔQ , observed over a period of time Δt , equals the enthalpy change of a closed system, minus the total work performed by the system. In calorimetric studies of cellular suspensions [16,17], instrumental design allows no exchange of energy other than heat through the walls of the experimental chamber. Therefore, the heat change equals the enthalpy change of all the metabolic reactions occurring in the chamber of the calorimeter.

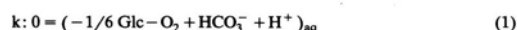
In the following section we illustrate the energy balance approach by analyzing the enthalpy changes associated with the catabolism of glucose under conditions in vivo. Enthalpy changes of biochemical reactions are calculated on the basis of balanced reaction stoichiometries and enthalpies of formation for compounds in dilute aqueous solution at 25°C (Table I). General thermodynamic symbols conform with IUPAC recommendations [18]. Negative signs denote energy losses from the system. Subscripts preceding the symbol for a thermodynamic quantity (Q , H , etc.) denote the process, trailing subscripts refer to the substance under consideration.

Standard reaction enthalpies refer to reactions buffered at pH 7.0 without any change in pH [19]. The enthalpy of neutralization of the bicarbonate buffer is -9 and -5 kJ/mol H^+ at 25°C [20] and 37°C [21]. The enthalpies of neutralization (binding of protons) of the imidazole groups of intracellular buffers are -30 kJ/mol H^+ [22]. Since bicarbonate and phosphate groups contribute to intracellular buffering capacity, the enthalpy of neutralization of the intracellular buffer system is estimated at -25 kJ/mol H^+ [14].

Standard reaction enthalpies refer to reactions buffered at pH 7.0 without any change in pH [19]. The enthalpy of neutralization of the bicarbonate buffer is -9 and -5 kJ/mol H^+ at 25°C [20] and 37°C [21]. The enthalpies of neutralization (binding of protons) of the imidazole groups of intracellular buffers are -30 kJ/mol H^+ [22]. Since bicarbonate and phosphate groups contribute to intracellular buffering capacity, the enthalpy of neutralization of the intracellular buffer system is estimated at -25 kJ/mol H^+ [14].

Oxycaloric equivalents and calorimetric-respirometric ratios

The calorimetric-respirometric ratio (CR ratio, kJ/mol O_2) is obtained from the experimental measurement of the total heat flux and oxygen flux. The theoretical counterpart of the CR ratio is the 'oxycaloric equivalent', that is, the calculated enthalpy change of respiratory oxygen uptake, expressed per unit amount of oxygen [23]. For example, the oxycaloric equivalent for the catabolism of glucose can be calculated from Eqn. 1,



The symbol k (catabolic) refers to the catabolic half cycle which accounts for the stoichiometric conversion of reduced organic substrates to endproducts (e.g., glucose to $\text{HCO}_3^- + \text{H}^+$). The oxycaloric equivalent, $\Delta_k H_{\text{O}_2}$, is the enthalpy change of the catabolic half-cycle reaction, and does not include any coupled process such as ATP production, anabolism or mechanical work [14,23].

The enthalpy change of the catabolic reaction shown in Eqn. 1 is -467 and -472 kJ/mol O_2 at 25 and 37°C , respectively (from Table I). Since carbonic acid is not fully dissociated at pH 7, the standard oxycaloric equivalent, $\Delta_k H_{\text{O}_2}^\circ$, for glucose at 25°C is -469 instead of -467 kJ/mol O_2 . If the reaction is buffered at an enthalpy of neutralization of -9 kJ/mol H^+ , the oxycaloric equivalent, $\Delta_k H_{\text{O}_2}$, for glucose is -476 kJ/mol O_2 (Table II). In media containing buffer systems with a high enthalpy of neutralization, -25 kJ/mol H^+ , the oxycaloric equivalent changes by less than 3% to -489 kJ/mol O_2 .

If O_2 and CO_2 are exchanged with the gas phase, $\Delta_k H_{\text{O}_2}$ is $-469 \text{ kJ} \cdot \text{mol}^{-1}$ (Table II), which differs from

TABLE I

Enthalpies of formation for calculating the enthalpy changes in catabolism of glucose

$\Delta_f H$ ($\text{kJ} \cdot \text{mol}^{-1}$) at 25°C (see Ref.; 4.184 J/cal) and 37°C [17]. g and aq refer to the gaseous and aqueous state; eq,b refers to the aqueous state of a dissociated substance in equilibrium at pH 7 [19].

Substance	Formula	State	25°C	37°C	Ref.
Oxygen	O_2	g	0.0	0.0	def.
		aq	-12.1	-9.6	44
Hydrogen ion	H^+	aq	0.0	0.0	def.
Water	H_2O	l	-285.8	-285.5	19
Carbon dioxide	CO_2	g	-393.5	-393.5	20
		aq	-413.3	-411.1	20
		eq,b	-405.8		
Carbonic acid	H_2CO_3	aq	-699.1	-696.5	20
		eq,b	-691.6		
Bicarbonate ion	HCO_3^-	aq	-689.9	-691.9	20
α, β -D-Glucose	$\text{C}_6\text{H}_{12}\text{O}_6$	aq	-1263.8	-1263.9	19
Lactate ion	$\text{C}_3\text{H}_5\text{O}_3^-$	aq	-686.6	-686.3	19

TABLE II

Oxycaloric equivalents of aerobic respiration of various substrates in aqueous solution at pH 7

$\Delta_k H_{O_2}^0$ and $\Delta_k H_{O_2}'$ (kJ/mol O_2) are calculated with enthalpies of neutralization 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 [23,45].

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

the combustion equivalent of oxygen, -467 kJ/mol O_2 , due only to the enthalpies of solution, dilution and mutarotation of glucose. A change of aqueous CO_2 at pH 7 to gaseous CO_2 (Table I; eq.b to g) is accompanied by an enthalpy change of 12.3 kJ \cdot mol $^{-1}$, and the enthalpy change of oxygen from the gaseous to the aqueous state is -12.1 kJ \cdot mol $^{-1}$ (Table I). Therefore, to obtain the standard oxycaloric equivalent in solution

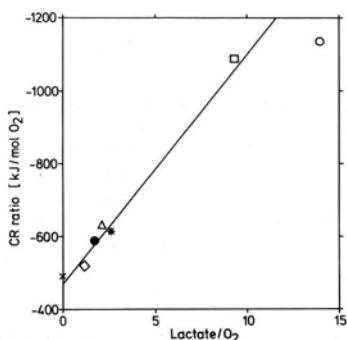


Fig. 1. Calorimetric-respirometric ratio, CR ratio, as a function of the molar lactate/ O_2 ratio in cultured or isolated mammalian cells. Hamster brown adipocytes (\times) [37], zero lactate production assumed; mouse macrophage hybridoma 2C11-12 (\circ) [46]; human neutrophils, activated for oxidative burst (\bullet) and resting (\circ) [30]; human T-lymphoma cells, CCRF-CEM, during growth, untreated, average lactate flux during the first hour (\blacktriangle) [47]; LS-L929 fibroblasts (Δ) [46]; human T-lymphoma cells, CCRF-CEM, under non-growing conditions (\square) [17]. The full line is the theoretical relation, $\Delta_k Q_{O_2}$ (Eqn. 2), with glucose as a substrate and the gases exchanged with the gas phase. The intercept with the X-axis is the oxycaloric equivalent, $\Delta_k H_{O_2} = -469$ kJ/mol O_2 ; the slope is the catabolic enthalpy change per mol lactate, $\Delta_k H_{Lac} = -63$ kJ/mol lactate.

when O_2 and CO_2 are exchanged with the gas phase, (12.3 $CO_2/O_2 - 12.1$) kJ/mol O_2 is added to $\Delta_k H_{O_2}^0$ (Table II). CO_2/O_2 is the molar amount of CO_2 produced per unit oxygen consumed, which is 1.0 for the respiration of carbohydrate. Therefore, the opposite enthalpies cancel out when oxygen and carbon dioxide are exchanged with the gas phase.

For a variety of substrates and conditions the theoretical oxycaloric equivalents range from -430 to -480 kJ/mol O_2 ; $\Delta_k H_{O_2} = -450$ kJ \cdot mol $^{-1} \pm 5\%$ (Table II). Even without detailed information about external or internal catabolic substrates, simultaneous calorimetry and respirometry (calorespirometry) provides an accurate method for studies of aerobic heat and enthalpy balance. Recently determined CR ratios are -460 ± 13 kJ \cdot mol $^{-1}$ for various aquatic animals [24], -448 to -468 kJ \cdot mol $^{-1}$ for muscle tissue without net performance of work [25-27], -450 ± 13 kJ \cdot mol $^{-1}$ for brown adipose tissue [25,26], and -440 ± 33 kJ \cdot mol $^{-1}$ for aerobic microbial growth [28]. Such agreement of the experimental CR ratios and the oxycaloric equivalent indicates a balanced aerobic energy (enthalpy) budget. In contrast, most CR ratios obtained with isolated or cultured mammalian cells are in the range of -490 to -800 kJ/mol O_2 (Fig. 1). The metabolic basis for this discrepancy is addressed below, by a discussion of simultaneous aerobic and anaerobic catabolism.

Simultaneous aerobic and anaerobic catabolism

The involvement of glycolytic reactions under aerobic conditions, with accumulation or excretion of anaerobic endproducts, is well documented for such cells as sperm [29], hepatocytes after addition of fructose [7], neutrophils [30], T-lymphoma cells [17] and L-929 cells [31] (for a review on aerobic glycolysis of cancer cells see Ref. 32). The most common anaerobic end-product in mammalian cells is lactate.

Net production of lactate from glucose is accompanied by a dissipative catabolic enthalpy change, $\Delta_k H_{Lac}$, of -80 kJ/mol Lac when the acid is buffered intracellularly, and -63 kJ \cdot mol $^{-1}$ if it is excreted into a bicarbonate buffer [14]. Total molar heat changes in living cells are -64 kJ/mol Lac measured in anaerobic bacteria [33], and -70 kJ/mol Lac in erythrocytes [34].

Comparable to the respiratory quotient (CO_2/O_2 ratio [35]), the molar amount of lactate produced per unit amount of oxygen consumed (Lac/ O_2 ratio) indicates the relative extent of aerobic glycolysis. This ratio is constant for hours in some aerobic cultures of cardiomyocytes [36]. The catabolic (k, as opposed to total measured) heat change per mol O_2 , $\Delta_k Q_{O_2}$, is then calculated as (full line in Fig. 1),

$$\Delta_k Q_{O_2} = \Delta_k H_{O_2} + Lac/O_2 \times \Delta_k H_{Lac} \quad (2)$$

The least negative CR ratio observed in isolated mammalian cells is $-490 \text{ kJ} \cdot \text{mol}^{-1}$ [37], indicating fully aerobic metabolism. CR ratios ranging from -550 to -1100 kJ/mol O_2 are explained by observed Lac/ O_2 ratios ranging from 1 to 10 (Fig. 1; Eqn. 2). One point seems to be off the line (open circle). However, the CR ratio of -1130 kJ/mol O_2 , and Lac/ O_2 ratio of 14 in resting neutrophils are thermochemically consistent within experimental error [30]. During oxidative bursts at high oxygen and heat flux, the CR ratio is -590 kJ/mol O_2 and the Lac/ O_2 ratio decreases consistently to 1.7 (Fig. 1). CR ratios significantly more negative than $-500 \text{ kJ} \cdot \text{mol}^{-1}$ indicate metabolic states with intensive participation of anaerobic processes. Sometimes this may be due to crowding and poor oxygenation of the medium [11,38].

Lac/ O_2 ratios may be too low to account completely for high CR ratios [7]. This may be due to the formation of other glycolytic endproducts, such as pyruvate, acetate or succinate [14]. However, low efficiency of net ATP production (see Introduction) cannot explain CR ratios more negative than -470 kJ/mol O_2 . Importantly, for a thermodynamic interpretation of CR ratios, futile cycling is not different from any other dissipative maintenance process with a net efficiency of zero. The increase in CR ratios from -550 in untreated cells to more exothermic values than -600 kJ/mol O_2 observed after uncoupling or decoupling [7,9,39] are due to an induction of anaerobic catabolism. After uncoupling the respiratory chain, anaerobic metabolism compensates for a low ATP/ O_2 ratio by generating ATP by substrate-level phosphorylation [40]. Similarly, when decoupling is induced by the addition of fructose to isolated rat hepatocytes [7], integrated aerobic and anaerobic processes must be considered.

At high fructose concentrations, respiration is inhibited, while glycolytic end-products accumulate, a phenomenon known as the Crabtree effect. It is commonly believed that this effect is restricted to microbial and tumour cells with uniquely high glycolytic capacities [41]. However, inhibition of respiration and increase of lactate production are observed under aerobic conditions in beating rat heart cell cultures [42] and in isolated rat lung cells [43]. Thus, the same general mechanisms responsible for the integration of respiration and glycolysis in tumour cells [41] appear to be operating to some extent in several isolated mammalian cells.

It is concluded that low efficiency cannot cause per se an increase of the ratio of heat and oxygen flux (CR ratio) above the theoretical enthalpy equivalent of oxygen consumption. Anaerobic mechanisms explain disproportionately increased heat changes. Simultaneous calorimetry and respirometry, in conjunction with a thermodynamic interpretation of experimental CR ratios, provide a unique method to quantify oxidative

and anaerobic components of complex metabolic systems.

Acknowledgements

We thank Drs. L. Gustafsson, C.J. Langdon, A. Schön and I. Wadsö for helpful comments. This work was supported by an award from The Nuffield Foundation at the University College of Wales. E.G. was also supported by the FWF Austria, project P7162-BIO.

References

- 1 Newsholme, E.A. and Start, C. (1973) *Regulation in Metabolism*, Wiley, Chichester.
- 2 Hue, L. (1982) in *Metabolic Compartmentation* (Sies, H., ed.), pp. 71–97, Academic Press, London.
- 3 Nedergaard, J. and Lindberg, O. (1982) *Int. Rev. Cytol.* 74, 187–286.
- 4 Nilsson-Ehle, P. and Nordin, G. (1985) *Int. J. Obesity*, 9, Suppl. 1, 169–172.
- 5 Clark, D.G., Brinkman, M. and Neville S.D. (1986) *Biochem. J.* 235, 337–342.
- 6 Reis, A. and Büger, P. (1967) *Elektromedizin* 12, 183–187.
- 7 Clark, D.G., Filsell, O.H., Topping, D.L. (1979) *Biochem. J.* 184, 501–507.
- 8 Schaarschmidt, B., Lamprecht, I. and Simonis, M. (1979) *Experientia Suppl.* 37, 176–183.
- 9 Hoffner, S.E.S., Meredith, R.W.J. and Kemp, R.B. (1985) *Cytobios* 42, 71–80.
- 10 Kemp, R.B. (1987) in *Thermal and Energetic Studies of Cellular Biological Systems* (James, A.M., ed.), pp. 147–164, John Wright, Bristol.
- 11 Kemp, R.B. and Gnaiger, E. (1989) in *Energy Transformations in Cells and Animals* (Wieser, W. and Gnaiger, E., eds.), pp. 91–97, Thieme Verlag, Stuttgart.
- 12 Kushmerick, M.J. (1983) in *Handbook of Physiology, Skeletal Muscle*, pp. 189–236, Am. Physiol. Soc., Bethesda.
- 13 Woledge, R.C., Curtin, N.A. and Homsher, E. (1985) *Energetic Aspects of Muscle Contraction*, pp. 357, Academic Press, London.
- 14 Gnaiger, E. (1983) *J. Exp. Zool.* 228, 471–490.
- 15 Battley, E.H. (1987) *Energetics of Microbial Growth*, Wiley, New York.
- 16 Kemp, R.B. (1980) in *Biological Microcalorimetry* (Beezer, A.E., ed.), pp. 113–130, Academic Press, New York.
- 17 Schön, A. and Wadsö, I. (1986) *Cytobios* 48, 195–205.
- 18 Mills, I., Cvitas, T., Homann, K., Kallay, N. and Kuchitsu, K. (1988) *IUPAC, Quantities, Units and Symbols in Physical Chemistry*, Blackwell, Oxford.
- 19 Wilhoit, I. (1969) in *Biochemical Microcalorimetry* (Brown, H.D., ed.), pp. 305–317, Academic Press, New York.
- 20 Berg, R.L., Vanderzee, C.E. (1978) *J. Chem. Thermodynam.* 10, 1113–1136.
- 21 Roughton, F.J.W. (1941) *J. Am. Chem. Soc.* 63, 2930–2934.
- 22 Gnaiger, E. (1980) *Thermochim. Acta* 40, 195–223.
- 23 Gnaiger, E. (1983) in *Polarographic Oxygen Sensors. Aquatic and Physiological Applications* (Gnaiger, E. and Forstner, H., eds.), pp. 337–345, Springer, Berlin.
- 24 Gnaiger, E. and Staudigl, I. (1987) *Physiol. Zool.* 60, 659–677.
- 25 Paul, R.J. (1983) *J. Gen. Physiol.* 81, 337–354.
- 26 Chinet, A., Clausen, T. and Girardiet, L. (1977) *J. Physiol.* 265, 43–61.
- 27 Chinet, A.E. (1988) in *Physiological and Biochemical Microcalorimetry* (Gnaiger, E., ed.), *Veröffentl. Univ. Innsbruck*, 168, pp. 20–21.

- 28 Birou, B., Marison, I.W. and Von Stockar, U. (1987) *Biotechnol. Bioeng.* 30, 650-660.
- 29 Inskip, P.B. and Hammerstedt, R.H. (1983) *J. Biochem. Biophys. Methods* 7, 199-210.
- 30 Eftimiadi, C. and Rialdi, G. (1982) *Cell Biophys.* 4, 231-244.
- 31 Clegg, J.S. and Jackson, S.A. (1988) *Biochem. J.* 255, 335-344.
- 32 Pedersen, P.L. (1978) *Prog. Exp. Tumor Res.* 22, 190-274.
- 33 Forrest, W.W., Walker, D.J. and Hopgood, M.F. (1961) *J. Bacteriol.* 82, 685-690.
- 34 Minakami, S. and De Verdier, C.H. (1976) *Eur. J. Biochem.* 65, 451-460.
- 35 Blomberg, A., Larsson, C. and Gustafsson, L. (1988) *J. Bacteriol.* 170, 4562-4568.
- 36 Jacobson, S.L. and Piper, H.M. (1986) *J. Mol. Cell Cardiol.* 18, 661-678.
- 37 Nedergaard, J., Cannon, B. and Lindberg, O. (1977) *Nature* 267, 518-520.
- 38 Nässberger, L., Truetsson, L. and Monti, M. (1988) *Biol. Cell* 62, 33-37.
- 39 Jarrett, I.G., Clark, D.G., Filsell, O.H., Harvey, J.W. and Clark, M.G. (1979) *Biochem. J.* 180, 631-638.
- 40 Gnaiger, E. (1981) *Thermochim. Acta* 49, 75-85.
- 41 Sussman, I., Erecinska, M. and Wilson, D.F. (1980) *Biochim. Biophys. Acta* 591, 209-223.
- 42 Frelin, C., Pinson, A., Moalic, J.M. and Padieu, P. (1974) *Biochim.* 56, 1597-1602.
- 43 Ayuso-Parrilla, M.S., Perez-Diaz, J., Marin, A. and Parrilla, R. (1978) *Biochim.* 60, 823-826.
- 44 Gill, S.J., Wadsö, I. (1982) *J. Chem. Thermodynamics* 14, 905-919.
- 45 Gnaiger, E. and Bitterlich, G. (1984) *Oecologia* 62, 289-298.
- 46 Kemp, R.B. and Meredith, R.W.J. (1990) *Cytobios*, in press.
- 47 Borrebaeck, C.A.K. and Schön, A. (1987) *Cancer Res.* 47, 4345-4350.