*Pure & Appl. Chem.*, Vol. 65, No. 9, pp. 1875–1880, 1993.
Printed in Great Britain.


# An historical review of developments in cellular microcalorimetry

Richard B. Kemp

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

***Abstract***
The energy (enthalpy) balance method from the 1st Law of Thermodynamics was crucial to a thermochemical understanding of yeast and the metabolic physiology of animal muscle and nerve. Heat flow not explained by known chemical reactions led to a search for additional processes, both chemical and electrical in nature. This approach has been extended to other cell types and has been supplemented by simultaneous calorespirometry to study catabolic processes under different conditions. Work is reviewed which underlines the potential of heat measurements in understanding the interaction of cells with native and xenobiotic compounds as well as the thermodynamics of metabolic processes.

## INTRODUCTION

The Lavoisier Lectures are reflective celebrations of the extraordinary life of the founder of modern chemistry. Along the way, the eponym invented calorimetry and, to paraphrase his findings with Laplace (ref. 1), concluded that respiration of animals is a very slow combustion. This paper focuses attention on the role of modern calorimetry with its high accuracy and high time resolution, in studying the respiration and energy balance of animal eukaryotic cells.

After the Lavoisier-Laplace experiments on aerobic processes, the next milestone in the calorimetry of living matter was the massive experiment of Dubrunfaut (ref. 2) which involved sufficient yeast for a 4-day fermentation of 21.4 $m^3$ molasses solution containing 2559 kg cane sugar. The total enthalpy change was -1.42 GJ. From the metabolic enthalpy balance method derived from the 1st Law of Thermodynamics, if all 14964-mole equivalents of glucose were fermented to ethanol, the value for enthalpy change should be -2.07 GJ. The difference in terms of glucose equivalents can probably be ascribed to the yeast cells *growing* in the molasses, using as their carbon source some of the sucrose (ref. 3). This is a first inkling of the possible value of the enthalpy balance method in calorimetric studies but yeasts, while eukaryotic, are not animals and strictly not cells but organisms: so their 20th century story cannot be told here (but see ref. 3).

In calorimetric terms, the most intensively studied animal system is the muscle. Only ten years after Rubner (ref. 4) first showed that the Law of the Conservation of Energy applied to biology, Hill in 1912 published his classical paper on the thermogenesis of muscle using a differential calorimeter (ref. 5). He recognised that a knowledge of Gibbs energy changes is essential to an understanding of biological energetics and thus introduced the 2nd Law of Thermodynamics to the subject. Although a muscle fibre is a syncytium, not a cell, the understanding of muscle energetics consequent to the research of Hill and others is a vital prelude to undertaking thermochemical analyses of animal cells.

## MUSCLE AND NERVE

A vital feature of studies of muscle physiology has been to account for heat and work in terms of chemical reactions. Using an energy (enthalpy) balance equation (ref. 6), it was possible to explain that much of the heat is derived

from phosphocreatine splitting and the reactions which resynthesize it. This stimulated research into the sources of the unexplained energy, which was 21% in the case of that produced during isometric tetanic contractions at 0°C of *Rana temporaria* sartorius muscles (ref. 7). One of the contributors to this energy is the activity of muscle proteins in the myofibrils (ref. 8). Another source at the start of the contraction is the movement of $Ca^{++}$ ions from binding sites in the sarcoplasmic reticulum to binding sites on troponin C and parvalbumin (ref. 7). The reversal of this process when contraction is over involves ATP splitting (exothermic) by the calcium pump. This is part of the energetic cost of contraction, but not concurrent with it. The accompanying heat flow is less than expected because hydrolysis is coupled to an endothermic process which is thought, though not observed, to be the transport of two $Ca^{++}$ ions for each ATP split in the sarcoplasmic reticulum. At one time, it was considered that the active Na-K pump in the sarcolemma made a considerable contribution to muscle thermogenesis. To test this hypothesis, it was shown that resting rat soleus muscle had a rate of heat flow of $-157 \pm 3.7$ $mJ.g^{-1}$ wet $wt.min^{-1}$ (ref. 8). Ouabain at 10 mmol $dm^{-3}$ inhibited $^{22}Na^{+}$ efflux by 58% and $^{42}K^{+}$ influx by 30% and decreased heat flow by $-7.5 \pm 0.8$ $mJ.g^{-1}$ wet $wt.min^{-1}$. This means that the pump makes only a small contribution of 4.7%. ATP splitting is of course required for the coupled pumping of $Na^{+}$ and $K^{+}$ ions to restore the resting potential after generation of an action potential. It has been shown that the latter is accompanied by a biphasic change in heat flow, the first peak being exothermic and the second, endothermic. For a *Maia* (crab) non-myelinated nerve, the initial exothermic heat after a single impulse was found to be $-11.7$ $mJ.g^{-1}$, and the subsequent endothermic heat was $+10$ $mJ.g^{-1}$ (ref. 9). A similar experiment for rabbit non-myelinated fibres gave figures of 102.5 $\mu J.g^{-1}$ and 92.9 $\mu J.g^{-1}$ respectively (ref. 10). The mass-specific dissimilarity is probably owing to surface area differences. In both cases the nerves possessed residual heat, 1.7 $mJ.g^{-1}$ for the crab and 9.6 $\mu J.g^{-1}$ for the rabbit nerve. Deflections from the baseline continued for over 200 s compared with the biphasic peak of 500 ms.

Ritchie (ref. 11) explained the total thermal profile in terms of changes in entropy accompanying the charging and discharging of a condenser. The change in enthalpy on discharging a condenser of capacity $C$ at a temperature $T$ may differ considerably from the Gibbs energy change by an amount $T\Delta S$, which can be shown to equal $\Delta G(T/C)(\delta C/\delta T)$. If $\delta C/\delta T$ is positive, heat will be released on discharge; and if $\delta C/\delta T$ is negative, the entropy change will lead to an absorption of heat. For any substance $\delta P/\delta T$ is zero near the absolute zero of temperature, $P$ being dielectric polarization. As the temperature increases $P$ also increases at first but starts to decrease after reaching a certain critical temperature, so that $\delta P/\delta T$ is positive in the range from zero temperature until the critical temperature is reached, and thereafter is negative. Below the critical temperature electrical polarization of the dielectric increases entropy; above this temperature, polarization decreases entropy. What happens in nerves thus depends on how the nerve membrane capacity varies with temperature. This is not known for crab and rabbit nerves but the squid giant axon has a positive temperature coefficient for dielectric polarization which, if also true of the nerves in question, means that entropy changes on discharge of the condenser would account for the bulk of the heat produced by the nerve. Ritchie (ref. 11) also speculated that the residual heat was due to activity of the sodium pump.

## NON-ERYTHROID CELLS

Little interest was shown in studying heat production of cells other than muscle and nerve until Wadsö's heat conduction flow microcalorimeter (ref. 12) was marketed and used to investigate thermal energy produced by blood cells (ref. 13). Over the last 20 years, Monti's group (see review in ref. 14) has explored the metabolism of the various cell types in whole blood and has emphasized the reliable and valuable role of biothermograms in diagnosing many clinical conditions such as Non-Hodgkin lymphoma, chronic lymphocytic leukaemia, thyroid dysfunction and acromelgaly.

Early studies on non-erythroid cells *in vitro* encompassed primary cultures of embryonic chick fibroblasts (ref. 15), established cell lines (ref. 16) and transformed cells (ref. 17) which gave heat flows, of 3, 25 and 58 $pW.cell^{-1}$, respectively. There were early difficulties in matching calorimetric vessels to the precise requirements of delicate cells from solid tissues or anchorage-dependent cultures (ref. 18) but a catalogue has now been written (refs. 19-21) showing heat flows for cells from bovine sperm (1.3 $pW.cell^{-1}$), through horse lymphocytes (8 $pW.cell^{-1}$), rat white adipocytes (40 $pW.cell^{-1}$) and human keratinocytes (83 $pW.cell^{-1}$), to hamster brown adipocytes (110 $pW.cell^{-1}$) and rat hepatocytes (329 $pW.cell^{-1}$). It is obvious that one of the reasons for these great differences is size, so it would be helpful if heat measurements could be stated in terms of scalar heat flux, ${}_tJ_Q$ ($W.m^{-3}$). Data would then relate more closely to metabolic (mostly catabolic) flux and reveal interesting diversity between cells. For instance, a human foreskin fibroblast (40 $pW.cell^{-1}$) is a quarter the volume of a rat hepatocyte but only produces an eighth of the heat. Care must be taken in such comparisons to eliminate the possibility of differences in the mass-volume ratio, $\rho = m/V$, because protein mass may be the decisive factor (ref. 22). It has been shown (ref. 20) for both Vero cells (ref. 23) and 2C11-12 mouse macrophage hybridoma cells (ref. 24) that there is a very marked influence of cell size on heat flux expressed per unit protein content. This is reminiscent of Kleiber's finding for whole animals (ref. 24) that mass-specific oxygen flux decreases with increasing size. It should also be remembered that volume alters during the cell cycle and this would affect metabolic flux if plasma membrane substrate receptors with high flux control coefficients are limited during cell growth (ref. 22).

**pH and oxygen**

Among all the possible errors in cellular heat measurements, two - bulk phase pH and dissolved oxygen - have been highlighted by recent studies. When T-lymphoma cells, CCRF-CEM, were suspended in a balanced salts solution with 20 $mmol.dm^{-3}$ HEPES at pH range 6.7-7.6, there was a linear relationship between rate of heat flow and pH of 21% per pH unit (ref. 25). The suspension medium was inadequate to support growth but cells incubated short-term (20 min) with growth medium showed a more acute pH dependency of 61% (ref. 26). In long-term growth culture (28 h), the optimum pH of the bulk phase was 7.2, lower and higher values causing a decrease in growth rate (ref. 27). It was also found that pH decreased with increasing total heat produced per $cm^3$ of cell suspension, which is a reflection of the high rate of aerobic glycolysis in the cells with lactate being excreted into the bulk phase (medium). Changes in heat flow with pH may be due to pH sensitivity of the glycolytic rate (ref. 27) but the exact influence of bulk phase pH on intracellular pH is not certain.

The solubility of oxygen in water is only $2.102 \times 10^{-7}$ $mol.cm^{-3}$ at 101.325 kPa and 37°C (ref. 22) and even less in culture media because of the salting-out effect. If cellular oxygen flux exceeds availability, then obviously the cells will be stressed in these hypoxic conditions and resort to greater participation of glycolysis, leading to more accumulation and excretion of lactate. This, in turn, would lower bulk phase pH which, as stated earlier, can act as a negative feedback to the glycolytic pathway. Such can be so of cells at high concentrations in suspension but is more generally evident when cells are allowed to sediment under batch conditions. For instance, murine macrophages at 30 and $60 \times 10^6$ $cells.cm^{-3}$ manifested sharp decreases in heat flow with time (ref. 28). This was ascribed to the "crowding effect", a phrase originally coined to describe density-dependent inhibition of oxygen consumption (see ref. 27). Although oxygen dissolved in the bulk phase culture medium may be adequate, the microenvironment surrounding "crowded" cells rapidly becomes depleted, leading to hypoxia. This reason could be ascribed to the finding that the heat flow of mouse lymphocyte hybridoma cells decreased with increasing cell concentration (ref. 29). It has been shown (ref. 27) that a $pO_2$ of 2 kPa ($2 \times 10^{-8}$ $mol.cm^{-3}$) is the critical level for T-lymphoma cells.

### Oxycaloric equivalents

It has ofgen been convenient in studies of intermediary metabolism by cells *in vitro* only to take indirect calorimetric measurements (oxygen) and neglect direct (heat) ones, relying on an assumption of singular aerobic glycolysis. In this case, oxygen flux ($_kJ_{O_2}$) is related to heat (flux ($J_Q$) by appropriate oxycaloric equivalents, $\Delta_kH_{O_2}$ (ref. 30). These are the enthalpy changes of the catabolic half-cycle (e.g. glucose to $HCO_3^-$ and $H^+$) and do not include any coupled process such as ATP production; that is, no work is done so the net efficiency is zero. They are calculated from enthalpies of formation, $\Delta_fH$ (ref. 30), and, for given substrates, are the same as values obtained by bomb calorimetry, $\Delta_cH$, but adjusted to aqueous solution. For aerobic glucose catabolism at pH 7 the equivalent is -469 $kJ.mol^{-1}$ $O_2$.

The theoretical oxycaloric equivalent is the *expected* ratio of calorimetric measured heat flux and respirometric oxygen flux, CR ratio. Hamster brown adipocytes suspended in glucose-containing saline gave a ratio of -490 $kJ.mol^{-1}$ $O_2$ (ref. 31) indicating fully aerobic metabolism: but many cell types in completely aerated culture media have ratios more exothermic than this, and as high as -1100 $kJ.mol^{-1}$ $O_2$ (ref. 30); see reviews, refs. 20), 21, 32). In some cases, for instance rat hepatocytes cultures in saline containing 25 $mmol.dm^{-3}$ fructose (ref. 33), highly negative CR ratios have been ascribed to increasing futile cycling which would consume ATP and result in decreased ATP production for other more "useful" requirements. A similar explanation has been invoked for other examples of highly negative CR ratios, for instance resting human neutrophils (ref. 34) in which the catabolic half-cycle is uncoupled from ATP production, reducing the efficiency of the latter. Since oxycaloric equivalents are calculated on the assumption of zero net efficiency, irrespective of intermittent efficiencies of ATP production in the phosphorylation reaction (ref. 35), increased futile cycling and uncoupling cannot be the reasons for highly exothermic CR ratios.

The correct cause is the activation of anaerobic catabolism (ref. 30), the most common endproduct of which in mammalian cells is lactate. In simultaneous aerobic and anaerobic glycolysis, the catabolic heat change per mol $O_2$, $\Delta_kH_{(ox+anox)}$, is calculated by adding to $\Delta_kH_{O_2}$ the molar amount of lactate produced per unit amount of oxygen consumed (Lac/$O_2$ ratio) multiplied by the appropriate dissipative catabolic enthalpy change per lactate, $\Delta_kH_{lac}$. Aerobic glycolysis to lactate has thus been shown to be the reason for highly exothermic CR ratios in many cells types (refs. 20, 21). For others, e.g. 2C11-12 mouse macrophage hybridoma cells (ref. 22), the calculation for lactate failed to achieve a complete reconcilation. Analyses of other intermediates such as succinate and pyruvate, however, completed the picture. It is already recognised that tumour cells (ref. 30) and lines derived from them, for instance T-lymphoma (refs. 25-27), undergo intensive aerobic glycolysis. It is now increasingly clear that many normal cells freshly obtained from animals or established *in vitro* as a line, conduct simultaneous aerobic and anaerobic catabolism even in fully aerobic conditions.

### Energy (enthalpy) balances

Neutrophils freshly isolated from human blood produced 111 fmol lactate. $h^{-1}$. $cell^{-1}$, had a CR ratio of -1132 $kJ.mol^{-1}$ and a Lac/$O_2$ ratio of 14 (ref. 34), which accounted for the highly negative CR ratio. Phorbol-12-myristate-13-acetate (PMA) generally causes a respiratory burst in phagocyte cells. The burst is required for the production of NADPH in the reduction and excitation of oxygen to the superoxide anion and hydrogen peroxide. PMA reduced the CR ratio to -588 $kJ.mol^{-1}$ $O_2$ compatible with the measured Lac/$O_2$ ratio of 1.7. Resting netrophils produced 2.5 $pW.cell^{-1}$ as heat and the energy (enthalpy) balance method ascribed 1.8 $pW.cell^{-1}$ to glycolysis and 1.0 $pW.cell^{-1}$ to the pentose phosphate pathway (PPP). The total heat produced in the PMA-activated burst was 23.5 $pW.cell^{-1}$, the enthalpy balance method showing that the majority was from stimulation of PPP (19.52 $pW.cell^{-1}$) and the residue by glycolysis (4.03 $pW.cell^{-1}$), giving a calculated total of 23.55 $pW.cell^{-1}$.

In another study using the enthalpy balance method, T-lymphoma cells were shown to have a heat flow of 12.2 pW.cell$^{-1}$, a CR ratio of 747 kJ.mol$^{-1}$ $O_2$, a Lac/$O_2$ ratio of 3.9, a Pyr/$O_2$ ratio of 0.07 and a calculated $\Delta_r H_{(ox+lac+pyr)}$ of 725 kJ.mol$^{-1}$ $O_2$, very close to the CR ratio (ref. 27). These cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum and containing 30 mg.dm$^{-3}$ L-glutamine and 5.5 mmol.dm$^{-3}$ glucose. The medium was buffered to pH 7.17 ± 0.03 with 20 mmol.dm$^{-3}$ HEPES. It was calculated by the enthalpy balance method that 60% of the heat flow was from oxidative processes but that the remaining 40% represented 97% of the carbon flux (ref. 36). Radioisotope studies showed that practically all the glucose carbon accumulated in lactate with only 1.4% in $CO_2$ (ref. 37). L-glutamine carbon was retrieved as L-glutamine (23%) and $CO_2$ (23%) and there was evidence that L-glutamine was metabolized via Krebs' cycle to pyruvate and lactate (glutaminolysis). The enthalpy balance showed that 47% of the total energy turnover was from glucose catabolism and 17% from glutamine catabolism. The remainder was owing to other undetermined oxidative processes, as estimated from the rate of oxygen uptake (ref. 37). Serum would be a rich source of carbon catalysable goodies!

### Toxicological aspects

Calorimetry is of increasing application to the study of drugs and other bioactive agents on cells (ref. 21). It has been shown that measuring cellular heat flow is a reliable assay for the action of anthralin (anti-psoriasis drug) and derivatives on human keratinocytes (ref. 38). The isolectins of phytohaemagglutinin can be distinguished by their different effects on heat flow of T-lymphoma cells (ref. 39) while β-adrenoceptor blockers reduced heat production by human muscle biopsies (ref. 40). A noval calorimetric cytotoxicity test for the effect of metal and non-metal particles on rabbit alveolar macrophages has been published (ref. 41), as has a method for estimating the acute cytotoxicity of heavy metals using heat flux of HeLa cells (ref. 42).

Anchorage-independent L929-derived LS mouse fibroblasts have been defined in terms of thermochemistry (refs. 43, 44), a CR ratio of 673 kJ.mol$^{-1}$ indicating intensive anaerobic glycolysis even under fully aerobic conditions. At steady state, the ATP/ADP ratio was 4.46, the adenylate energy charge 0.8 and the phosphorylation potential 1405 M$^{-1}$ (ref. 45). This latter value translates to an actual Gibbs energy change of ADP phosphorylation, $\Delta_e G$, of 49.2 kJ.mol$^{-1}$, from which can be calculated an aerobic catabolic force efficiency (ref. 46) of 63%. In the search for *in vitro* alternatives to animals in toxicological procedures, the above parameters have been employed to some effect. The ranking order of acetaminophen (paracetamol), aspirin, diazepam, digoxin and ethanol for acute lethal toxicity to humans was the same as those for decreases in ATP/ADP ratio to 1 (ref. 45) and phosphorylation potential by 50%. CR ratios for cells in the presence of each of the reagents at their 50% cell death ($CD_{50}$) value were more exothermic than the controls. The ranking order was the same as that for humans, except that aspirin and acetaminophen had equal ranking rather than the slightly more toxic figure for the former than the latter in humans. The technical ease of obtaining CR ratios has much to commend itself over the difficulties in arriving at reliable values for phosphorylation potential.

## CONCLUSION

This synopsis of some of the more important contributions of calorimetry to cellular physiology can only give a flavour of the potential of heat measurements. While it is Gibbs energy changes that are central to an understanding of bioenergetics and physiological calorimetry cannot of itself provide this understanding, measurement of enthalpy changes do valuably complement our knowledge of the thermodynamics of cellular metabolism.

## REFERENCES

1. A. Lavoisier and P.S. Laplace, Mem. Acad. Sci. 3, 355-374 (1783).
2. M. Dubrunfaut, C.R. Hebd. Seances Acad. Sci. 42, 945-948 (1856).
3. E.H. Battley, Energetics of Microbial Growth, p.97, Wiley, New York (1987).

4. M. Rubner, Die Gesetze des Energieverbrauchs bei der Ernahrung, Deuticke, Leipzig (1902).
5. A.V. Hill, J. Physiol. 44, 466-513 (1912).
6. R.C. Woledge, Prog. Biophys. Molec. Biol. 22, 37-74 (1971).
7. R.C. Woledge, In Energy Transformations in Cells and Organisms (eds. W. Wieser and E. Gnaiger ), pp. 36-45, Thieme, Stuttgart (1989).
8. A. Chinet, T. Clausen and L. Girardier, J. Physiol. 265, 43-61 (1977).
9. B.C. Abbot, A.V. Hill and J.W. Howarth, Proc. Roy. Soc. B148, 149-187, (1958).
10. J.W. Howarth, R.D. Keynes and J.M. Richie, J. Physiol. 194, 745-793 (1968).
11. J.M. Richie, Prog. Biophys. Molec. Biol. 26, 147-188 (1974).
12. P. Monk and I. Wadsö, Acta Chem. Scand. 22, 1842-1852 (1968).
13. K. Levin and A. Boyo, Scand. J. Clin. Lab. Invert. Suppl. 118, 55-65 (1971).
14. M. Monti, In Thermal and Energetic Studies of Cellular Biological Systems, (ed. A.M. James) pp. 131-146, Wright, Bristol (1987).
15. R.B. Kemp, Pestic. Sci. 6, 311-325 (1975).
16. D.P. Cerretti, K.J. Dorsey and D.W. Bolen, Biochim. Biophys. Acta 462, 748-758 (1977).
17. G.C. Kresheck, Cancer Biochem. Biophys. 1, 39-42 (1974).
18. R.B. Kemp, In Biological Microcalorimetry (ed. A.E. Beezer), pp. 113-130, Academic Press, New York.
19. R.B. Kemp, In Thermal and Energetic Studies of Cellular Biological Systems (ed. A.M. James), pp. 147-166, Wright, Bristol (1987).
20. R.B. Kemp and E. Gnaiger, In Energy Transformations in Cells and Organisms (eds. W. Wieser and E. Gnaiger), pp. 91-97, Thieme, Stuttgart (1989).
21. R.B. Kemp, Thermochim. Acta 193, 253-267 (1991).
22. R.B. Kemp, Thermochim. Acta 172, 61-74 (1990).
23. A. Schön and I. Wadsö, J. Biochim. Biophys. Meth. 13, 135-143 (1986).
24. M. Kleiber, The Fire of Life; an Introduction to Animal Energetics, p. 254, Wiley, New York (1961).
25. A. Schön and I. Wadsö, Cytobios 48, 195-205 (1986).
26. P. Bäckman, Thermochim. Acta 172, 123-130 (1990).
27. P. Bäckman, T. Kimura, A. Schon and I. Wadso, J. Cell. Physiol. 150, 99-130 (1992).
28. J.D. Loike, S.C. Silverstein and J.M. Sturtevant, Proc. Natl. Acad. Sci. USA 78, 5958-5962 (1981).
29. L. Nässberger, L. Truettson and M. Monti, Biol. Cell 62, 33-37 (1988).
30. E. Gnaiger and R.B. Kemp, Biochim. Biophys. Acta 1016, 328-332 (1990).
31. J. Nedergaard, R. Cannon and O. Lindberg, Nature (London) 269, 518-520 (1979).
32. R.B. Kemp, Thermochim. Acta (in the press).
33. I.G. Jarrett, D.G. Clark, O.H. Filsell, J.W. Harvey and M.G. Clark, Biochem. J. 180, 631-638 (1979).
34. E. Eftimiadi and G. Rialdi, Cell Biophys. 4, 231-244 (1982).
35. E. Gneiger, Thermochim. Acta 172, 31-52 (1990).
36. P. Bäckman and I. Wadsö, J. Biochim. Biophys. Meth. (in the press).
37. P. Bäckman, Biochim. Biophys. Acta (in the press).
38. M. Pätel, Thermochim. Acta 49, 123-129 (1981).
39. C.A.K. Borrebaeck and A. Schon, Cancer Res. 47, 4345-4350 (1987).
40. B. Fagher, H. Liedholm, M. Monti and U. Moritz, Clin. Sci. 70, 435-441 (1986).
41. S.A. Thóren, J. Toxicol. Environ. Health (in the press).
42. S.M. McGuinness, D.A. Roess and B.G. Barisas, Thermochim. Acta 172, 131-145 (1990).
43. S.E.S. Hoffner, R.W.J. Meredith and R.B. Kemp, Cytobios 42, 71-80 (1985).
44. R.B. Kemp, Ind. J. Technol. (in the press).
45. R.B. Kemp, A. Anton, M. Carlsson and P.M. Evans, ATLA (in the press).
46. E. Gnaiger, In Evolutionary Physiological Ecology (ed. P. Calow), pp. 7-36, C.U.P., London (1981).