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Metabolic microcalorimetry and respirometry of aquatic animals

Introduction

Cellular energy transformations are accompanied by heat changes indicative of the physiological and metabolic activities of living organisms. The thermodynamic approach by metabolic (direct) calorimetry reveals the integrated sum of all enthalpy changes occurring within the experimental chamber, that is the living open system and its environment. The term microcalorimetry is loosely defined, indicating that heat flux is measured in the range of 1 to 1000 μW, equivalent to 0.0022 to 2.2 nmol O_2 s^{-1} or 8 to 8000 μmol O_2 h^{-1} for aerobic metabolism. Due to recent technological advancements, metabolic microcalorimetry has become a sensitive, non-invasive method for the study of respiration and complex metabolic processes in animals (Gnaiger, 1983*a*; Pamatmat, 1983; Shick, Gnaiger, Widdows, Bayne & de Zwaan, 1986; Widdows, 1987).

In physiological energetics, three main aspects of microcalorimetric investigations are distinguished: (i) Studies of environmentally induced transitions between different metabolic states, especially aerobic–hypoxic–anoxic transitions and aerobic recovery. (ii) Energetic studies of animal behaviour, associated with locomotion, gas exchange, and biological rhythms. (iii) Energy balance studies, combining metabolic calorimetry with simultaneous or parallel measurements of respiration and biochemical changes. The classic method of indirect calorimetry depends on the calculation of heat changes from measured oxygen consumption and theoretical oxycaloric equivalents. This indirect method is extended by including anaerobic metabolite changes in the calculation of theoretical heat changes. Comparison of direct and indirect calorimetry constitutes the thermodynamic energy balance method, the crucial test for a complete biochemical description of net processes under various metabolic states. Thermochemical interpretation of biochemical and calorimetric data is not only required in energy balance studies, it is important for understanding the functional significance of heat flux in physiological energetics (Gnaiger, 1983*a*). Specifically, calorespirometry – the simultaneous measurement of

Table 1. *The oxycaloric equivalent, $\Delta_k H_{O_2}$, as a function of the proportion of catabolic substrates, given as mass fractions; K – carbohydrate, L – lipid, P – protein; calculated from RQ [CO_2/O_2] and NQ [N/O_2]. The deviation of the oxycaloric equivalent [%] is relative to a generalized oxycaloric equivalent of −450 kJ. mol^{-1}. The last line shows an example of carbohydrate to lipid conversion, associated with a high respiratory quotient and oxycaloric equivalent. The last column lists the oxyenthalpic or combustion equivalent of oxygen, $\Delta_c H_{O_2}$, combining respiration and excretion, R + U, in terms of the bomb calorimetric enthalpy of combustion of catabolized substrates (after Gnaiger, 1983b).*

Substrate fractions			RQ	NQ	$\Delta_k H_{O_2}$	deviation	ΔHc_{O_2}
K	L	P			kJ/mol	%	kJ/mol
−1.00	–	–	1.00	0.00	−478	+6.1	−473
–	−1.00	–	0.72	0.00	−445	−1.1	−441
–	–	−1.00	0.97	0.27	−451	+0.2	−528
–	–	−1.00[a]	0.84	0.27	−443	−1.6	−528
−0.22	−0.51	−0.27	0.80	0.05	−450	–	−461
−0.11	−0.39	−0.50	9.83	0.10	−450	–	−475
−0.06	−0.25	−0.69	0.87	0.15	−450	–	−491
−0.02	−0.14	−0.84	0.91	0.20	−450	–	−506
−0.81	+0.18	−0.09	1.20	0.05	−497	+10.4	−507

[a] For urea as excretory product; all other values are for ammonia.

heat flux and oxygen flux in an open flow or perfusion system – enables the partitioning of total heat flux into aerobic and anaerobic components.

Aerobic energy balance

Oxycaloric equivalent and calorimetric-respirometric ratio

The heat dissipated in respiration or aerobic catabolism (k), ${}_k\dot{Q}$ [μW = μJ·s^{-1}], is calculated from the oxygen flux, $\dot{N}_{O_2}$ [nmol O_2·s^{-1}],

$$ {}_k\dot{Q} = \dot{N}_{O_2} \times \Delta_k H_{O_2} \qquad (1) $$

The conversion factor, $\Delta_k H_{O_2}$ [kJ·mol^{-1} O_2 = μW/(nmol O_2·s^{-1})], is the theoretical *oxycaloric equivalent*, the catabolic enthalpy change per mol O_2 (Table 1). This thermochemically derived conversion factor can be tested directly by calorespirometry, the simultaneous measurement of total heat flux, ${}_t\dot{Q}$ [μW], and oxygen flux, $\dot{N}_{O_2}$, which yields the *calorimetric–respirometric ratio* or CR ratio, [kJ·mol^{-1} O_2],

$$ \text{CR ratio} = \Delta_t Q_{O_2} = {}_t\dot{Q}/\dot{N}_{O_2} \qquad (2) $$

In metabolic calorimetry the animal is usually allowed to move freely within the calorimeter chamber. Any free energy temporarily conserved in locomotory work is ultimately dissipated within the chamber if the experimental design does not provide a mechanical energy transducer. Theoretically, the experimental CR ratio, $\Delta_t Q_{O_2}$, and the oxycaloric equivalent, $\Delta_x H_{O_2}$, should match. As a prerequisite for this aerobic energy balance, catabolism must be fully aerobic and dissipative (see below), the theoretical oxycaloric equivalent must be correct, and the two experimental terms in equation (2) must be accurate, not merely reproducible or precise.

Calculation of oxycaloric equivalents for aquatic animals involves assumptions on the relative proportions of catabolic substrates, or measurement of the respiratory quotient, RQ [mol $CO_2 \cdot mol^{-1}$ O_2], and nitrogen quotient, NQ [mol N excreted mol^{-1} O_2],

$$\Delta_k H_{O2} = -360 - 118\,RQ + 85\,NQ \qquad (3)$$

(Taken from equation 8b in Gnaiger, 1983*b*; here a larger sample of amino acid compositions was used; see Gnaiger & Bitterlich, 1984). If not only ammonia (equation 3) but also urea is excreted, the expression $+85\,NQ$ is replaced by $+(85 - 28\,x_{urea})\,NQ$. The nitrogen quotient accounts for the total nitrogen excreted in ammonia and urea, and X_{urea} is the fraction of nitrogen excreted as urea, ($x_{ammonia} + x_{urea} = 1$).

A general estimate of the oxycaloric equivalent in water in the biological temperature range is -450 kJ·mol^{-1} O_2, e.g. for $NQ = 0.05$ and $RQ = 0.80$ (corresponding to a proportional respiratory substrate loss of -22% carbohydrate, -51% lipid, and -27% protein; equation 2 in Gnaiger, 1983*b*; Table 1).

Carbon dioxide and ammonia in water

Side reactions of dissociation and buffering occur in the aqueous environment, and the corresponding heat effects must be accounted for. The gases are in the dissolved state, 81% of carbonic acid is dissociated as bicarbonate at pH 7 ($pK' = 6.37$, apparent dissociation constant, in pure water at 25 °C), and ammonia is in the form of ammonium ion. The proton quotient or production of protons per mol O_2 consumed, H^+/O_2, is approximately (Gnaiger, 1983*b*) in a closed system.

$$H^+/O_2 = RQ \frac{1}{1 + \exp(pK' - pH)} - NQ \qquad (4)$$

Again, $-$NQ in equation (4) is replaced by $-(x_{ammonia}\,NQ)$ if urea is excreted simultaneously. At steady state pH under constant physiological conditions all protons generated (equation 4) must be excreted. Natural environmental buffers have a low enthalpy of neutralization, usually not

more than -8 kJ·mol^{-1}H$^+$. This is incorporated in the oxycaloric equivalent (equation 3 and Table 1). For carbohydrate and protein (ammoniotelic) at pH 7, H^+/O_2 is 0.81 and 0.52, respectively. Aerobic buffer effects are small, -6.5 and -4.2 kJ·mol$^{-1}O_2$. If O_2 and CO_2 are exchanged between a gaseous and aqueous phase, then the oxycaloric equivalent for glycogen is -469, 2% less than -478 kJ·mol$^{-1}O_2$ (Table 1). Irrespective of the transport mechanism of ammonia in the form of NH_3(aq) or NH_4^+(aq) across respiratory surfaces, ammonium ion is the end-product in water under normal conditions. Endproducts but not transient transport forms have to be considered in thermochemical energy budget calculations. This was possibly misunderstood by Brafield (1985); his oxycaloric value is 5% too low for ammoniotelic protein catabolism in aquatic animals.

Metabolic and bomb calorimetry: direct and indirect

Another conceptual problem arises in the context of aerobic energy balance studies. The oxycaloric equivalent, $\Delta_k H_{O_2}$, is calculated for cellular and aqueous conditions to compare oxygen consumption and actually measured metabolic heat flux (equations 2 and 3). In ecophysiological energetics, however, indirect calorimetry is used to calculate the scope for growth,

$$P = C + F + R + U \qquad (5)$$

where the energy change in biomass, P, the energy input, C, and energy loss in faeces, F, are obtained by bomb calorimetry. Analogous to respirometers in indirect metabolic calorimetry, automatic CHN analyzers can be used in 'indirect bomb calorimetry' to obtain the combustion enthalpy of ash-free dry organic matter, $\Delta_c H$ [kJ/g $_{af}W$], from measured mass fractions of organic carbon, ω_C [g C/g $_{af}W$], and organic nitrogen, ω_N [g N/g $_{af}W$] (Gnaiger & Bitterlich, 1984).

$$\Delta_c h = 11.2 - 66.27\,\omega_C - 4.44\,\omega_N \qquad (6)$$

With careful homogenization, ashing and avoidance of moisture contamination of the dry material, reproducibility is better than 3%, and inaccuracies due to variable residual water contents are <2% (± 0.5 kJ·g^{-}1; Gnaiger & Bitterlich, 1984).

To account for the total catabolic loss in respiration and excretion, $R+U$, in terms of the combustion enthalpy, the *respiratory combustion equivalent*, $\Delta_c H_{O_2}$ has to be used (equation 7.1; Table 1). Alternatively, excretory loss, U, is calculated separately as a product of ammonia excretion, $\dot{N}_{NH_3}$ [nmol·s^{-1}], and the combustion equivalent of ammonia, $\Delta_c H_{NH_3}$ [kJ·mol^{-1} NH_3] (equation 7.2),

$$R + U = \dot{N}_{O_2} \times \Delta_c H_{O_2} \quad (7.1)$$

$$R + U = \dot{N}_{O_2} \times \Delta_k H_{O_2} + \dot{N}_{NH_3} \times \Delta_c H_{NH_3} \quad (7.2)$$

Based on the equality of the two expressions of equation (7), the combustion equivalents of oxygen consumption and ammonia are related as

$$\Delta_c H_{O_2} = \Delta_k H_{O_2} + NQ \times \Delta_c H_{NH_3} \quad (8)$$

Using the examples given in Table 1 and solving for the combustion equivalent for ammonia (equation 8), we obtain approximately $\Delta_c H_{NH_3} = -280\text{kJ mol}^{-1}NH_3$. Necessarily, this 'apparent' equivalent is different from the enthalpy of combustion of aqueous or gaseous ammonia (-380 and -345 kJmol^{-1}, respectively). The correction factors for the transition from the cellular state of R to the standard state of combustion are lumped in $\Delta_c H_{NH_3}$, to ensure consistency of thermodynamic state in the energy balance equation. For applications of these concepts see Hawkins *et al.* (1985) and Zamer & Shick (1987).

Calorespirometry: simultaneous heat and oxygen measurements

Errors in calculating oxycaloric equivalents have to be put into perspective relative to experimental accuracy. In 6 simultaneous calorespirometric experiments with the aquatic oligochaete *Lumbriculus variegatus*, the CR ratio was -451 ± 38 kJ·mol^{-1} O_2 (S.D.) (Gnaiger & Staudigl, 1987). If errors are $\pm 5\%$ for each measurement, then the error of the CR ratio is $\pm\sqrt{5^2+5^2} = \pm 7\%$ or ± 32 kJ·mol^{-1} O_2. This embraces the range of oxycaloric equivalents for different catabolic substrates (Table 1), emphasizing the need of high accuracy in calorimetric and respirometric energy balance studies.

In nonsimultaneous measurements of heat and oxygen flux, different states of activity of the animals in the calorimeter and respirometer may explain the large range of CR ratios, -420 ± 60 kJ mol^{-1} (S.D.), calculated from 11 mean values for different species. However, a significantly lower variability, -460 ± 13 kJ mol^{-1}, was calculated from simultaneous calorespirometric studies, again using 11 mean values reported for various aquatic euryoxic and stenoxic animals (Gnaiger & Staudigl, 1987). This difference underscores the need for simultaneous calorespirometry, thus eliminating any variability due to different metabolic states of animals in nonsimultaneous measurements. The important conclusion drawn from these results is that several anoxia-tolerant (oligochaetes, bivalves) and intolerant species (Crustacea, salmonid fish larvae) alike are fully aerobic under normoxic, stress-free conditions. The measured CR ratios and expected aerobic oxycaloric equivalents (Table 1) agree. Aerobic energy balance studies, therefore, provide a methodological baseline for calorespirometry

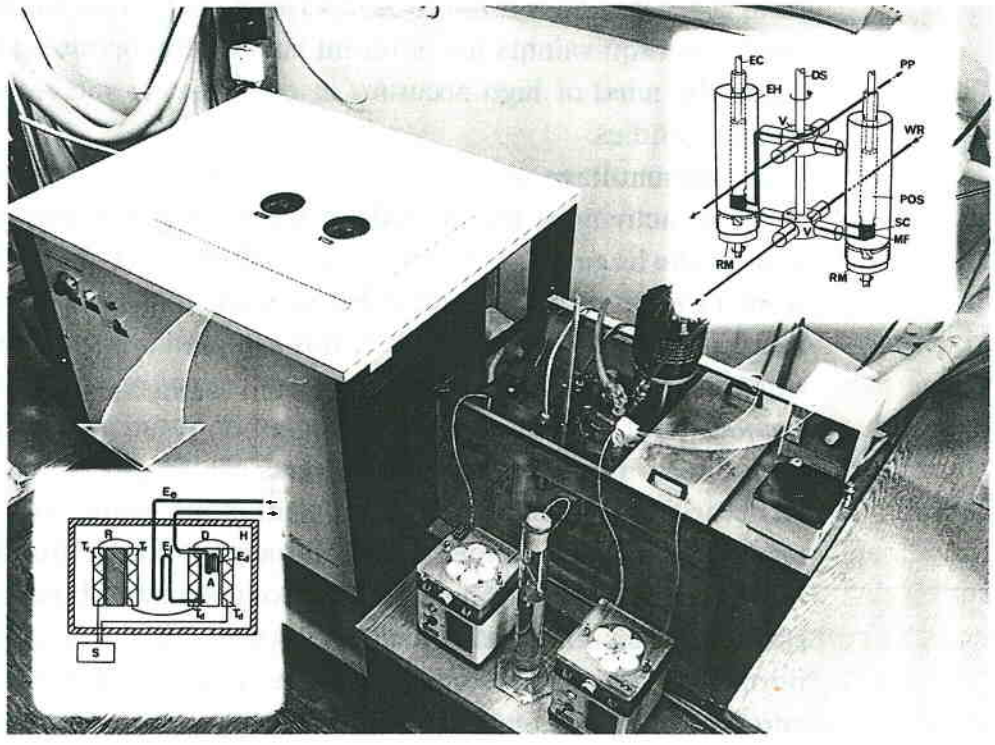
that can then be applied to studies of metabolically complex situations involving anoxia or net biosynthesis.

The most versatile applications of calorespirometry are offered by microcalorimeters with perfusion (open-flow) chambers, connected to an open-flow respirometer (Fig. 1).

Perfusion microcalorimeters

Instrument design. Principles of design and the range of micro-calorimeters used in 'studies of biochemical processes and of cellular

Fig. 1. An open-flow calorespirometer: Combination of the LKB-2107 flow sorption microcalorimeter (left) and the Cyclobios Twin-Flow respirometer (right). Left inset; operation principle of the heat flow calorimeter: A, 0.5 cm^3 pyrex animal chamber; D, heat detector; E_d detector heat exchanger; E_e; external heat exchanger; E_i, internal heat exchanger; H, heat sink; R, reference heat detector; S, signal amplifier; T_d, detector thermopile; T_r, reference thermopile. Right inset; Twin-Flow principle; the inflow and outflow of the perfusion medium through gold or stainless steel capillaries connecting to the calorimeter is indicated by arrows: DS, drive shaft for simultaneous switching of the 4-way valves; EC, electrode cable of the POS; EH, stainless steel POS sleeve; MF, magnetic stirrer; POS, polarographic oxygen sensor; PP, peristaltic pump; RM, rotating magnet; SC, right stirring chamber in calibration position; V, 4-way valve; WR, water reservoir (after Gnaiger, 1983*c*; Foto: P. Flöry, Cyclobios).

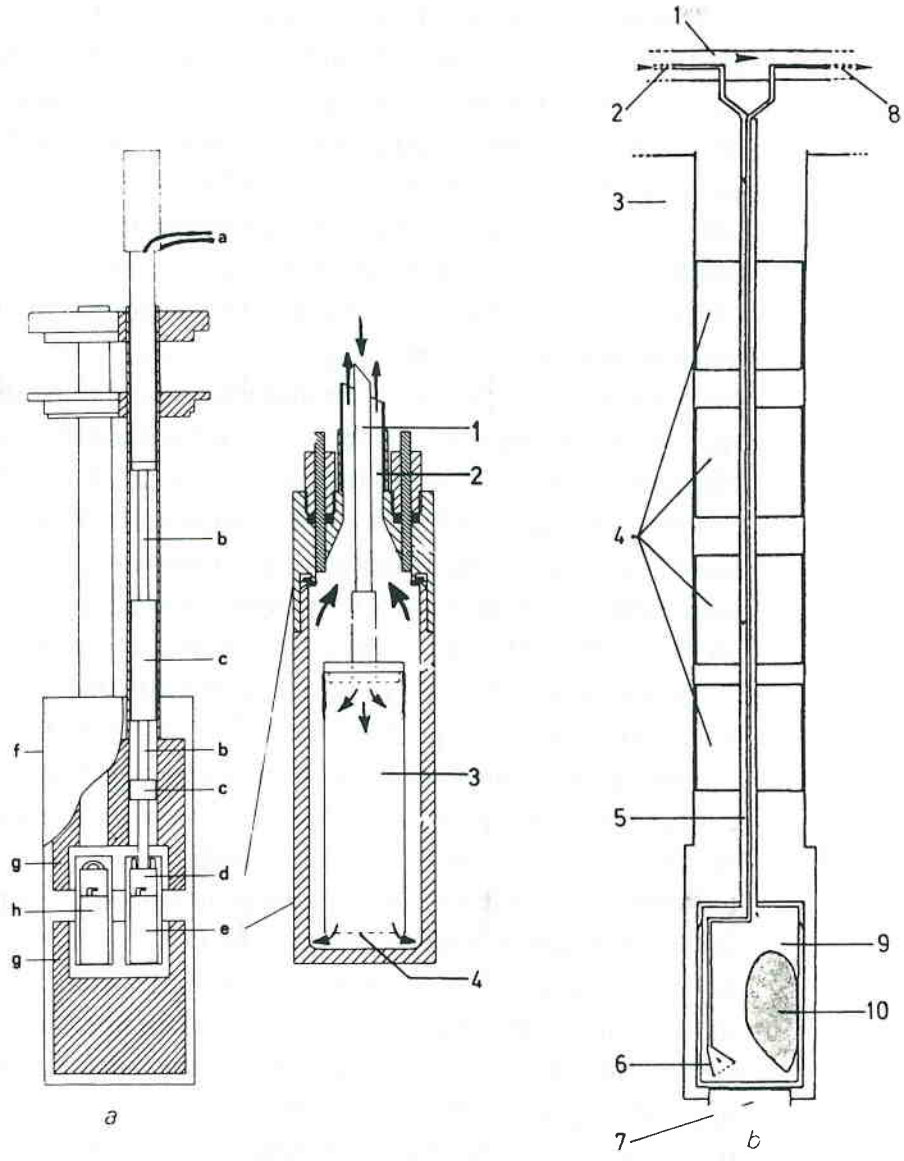


systems such as micro organisms and tissue cells, etc.', has been reviewed recently by Wadsö (1987). Here we are exclusively concerned with studies of 'etc.', living animals. Adiabatic calorimeters ideally exchange no heat with the surroundings, and the observed temperature increase is directly proportional to the heat flux within the reaction chamber. An adiabatic calorimeter with continuous aeration for studies of fish up to 100 g is described by Smith, Rumsey & Scott (1978). The chamber must be closed to avoid heat exchange and disturbances in sensitive adiabatic microcalorimeters, which excludes an open-flow mode. By simultaneously measuring the decline in P_{O_2} with a polarographic oxygen sensor, an initial phase of progressive hypoxia can be distinguished from anoxia when depletion of dissolved oxygen is completed (Hammen, 1983).

This strictly unidirectional experimental regime is avoided by continuous perfusion of the animal chamber. High-precision thermistors are used to measure the temperature difference between water flowing through a thermally insulated animal chamber and an identical twin chamber serving as a reference (Lock & Ford, 1983). An entirely different principle is employed in heat conduction calorimeters where the temperatures of outflow and inflow water are essentially identical. This is achieved either by efficient heat exchangers adjacent to the animal chamber (Fig. 1) or by a counter current principle (Fig. 2). In contrast to adiabatic instruments, a heat conduction calorimeter maintains nearly constant temperatures within the reaction chamber, which is surrounded by semiconductor Peltier elements. These thermopiles provide good thermal contact between the reaction chamber and a constant-temperature heat sink, for rapid conduction of heat at minute temperature differences, ΔT, between chamber and heat sink. In heat conduction microcalorimeters, ΔT is in the order of 10^{-6} °C μW^{-1}, that is <0.0001 °C in a typical physiological experiment. The recorded signal, however, is not temperature but the thermopile voltage which, in turn, is proportional to ΔT (Calvet & Prat, 1963).

The thermocouples of the thermopiles surrounding one chamber are connected in series, but in opposition to the thermopiles of an identical twin or reference chamber. The twin principle (Fig. 1) serves to cancel out any uniform temperature disturbances of the heat sink, provided that the sensitivities and response times of the twin halfs are identical. If possible, it is advantageous to monitor both, the differential twin-signal and the single mode signal of the reference chamber; then abnormal baseline disturbances can be detected and corrected by calibrated mathematical models (Kaufmann & Gnaiger, 1981). In order to achieve a stable experimental baseline it is advisable to site the calorimeter in a dry, temperature controlled room

Fig. 2. Perfusion vessels of the LKB-Thermometrics microcalorimeter, (*a*) Module with 3.5 cm^3 chambers; twin system (after Görman Nordmark *et al.*, 1984): a, stainless steel capillaries connecting to the respirometer; b, outer stainless steel tube; c, heat exchanger brass bolts; d, e, animal chamber, the magnification shows the 'nested chamber' and flow directions; f, steel cylinder, immersed in the thermostated water bath; g, heat sinks; h, reference chamber; 1, inflow; 2, outflow as counter current heat exchanger; 3, nested animal chamber; 4, 100 μm mesh bottom. (*b*) Module with a single 25 cm^3 perfusion chamber (after Widdows, 1987): 1, temperature controlled water jacket; 2, inflow from water reservoir; 3, heat sink; 4, thermostat; 5, counter current heat exchanger; 6, outflow 150 μm filter; 7, thermopile; 8, outflow to polarographic oxygen sensor and peristaltic pump; 9, 25 cm^3 stainless steel chamber; 10, experimental animal.



(within ±1 °C), to avoid direct sunlight and to provide efficient air circulation with additional fans.

The choice of a particular calorimeter primarily depends on the size of the experimental animals and of the budget. The lower limit of animal size is set by the sensitivity and long-term stability of the instrument, availability of large numbers (e.g. of meiofauna), and tolerable crowding effects. With a stability in the order of ±0.5 μW, 0.1 mg $_dW$ would represent the minimum dry biomass, but under anoxia the minimum is rather 1 mg due to 70–95% reduced anoxic heat flux (Gnaiger, 1983d). Long-term (>24 h) stabilities better than 2 μW, required at the lower size limit, can only be achieved in heat conduction microcalorimeters with small reaction chambers in a twin arrangement (Figs. 1 and 2*a*) and at low perfusion rates (Suurkuusk & Wadsö, 1982). The upper limit of animal size depends on the geometry and dimensions of the calorimeter chamber, and on the feasability of high perfusion rates sufficient for maintaining aerobic conditions. With increasing size of the chamber (Fig. 2*b*) the twin configuration becomes less efficient due to temperature inhomogeneities in space, but with larger biomass, less rigorous standards are set with respect to sensitivity and stability, hence single mode long-term stabilities of 3 μW are sufficient (Gnaiger, 1983d; Widdows, 1987).

Static and dynamic calibration. Electrical power dissipated across a resistor is instantaneously and completely transformed into an equivalent rate of heat dissipation, and can be accurately regulated, <±0.1%. Therefore, electrical calibration is most convenient, although for some applications cross-calibration is required by chemical methods using the high enthalpies of some buffer reactions (Chen & Wadsö, 1982). Commercial microcalorimeters are equipped with built-in precision resistance heaters (e.g. 50Ω). It is important to place the calibration resistor into a representative position in thermal contact or better within the animal chamber, especially at high perfusion rates and for dynamic calibration. Electric wires leading from an external power source into the chamber may cause thermal leaks. The most elegant solution is achieved by an internal power source sealed into a calibration capsule containing an electronic circuit for switching on and off a known power at fixed time intervals, e.g. two hours (Widdows, 1987). The internal calibration capsule is powered by three 1.5 V silver oxide microbatteries (RM47) which produce a background heat flux of ca. −6 μW due to the timer circuit and the enthalpy changes associated with the chemical reaction in the battery. Comparison of the built-in external calibration in the ThermoMetric microcalorimeter and the internal calibration capsule agree within <1%.

At steady state, a constant rate of heat dissipation is balanced by an equal rate of heat flow. Then the steady deflection of the voltage across the thermopiles is directly proportional to the heat flux, resulting in a strictly linear calibration. The inverse of the sensitivity is the static calibration constant, typically between 3.3 μW/μV (Suurkuusk & Wadsö, 1982) and 25 μW/μV (Pamatmat, 1983). When the calibration current is switched on or off, the output signal follows exponentially from an initial to a final steady-state, due to the inertia of the system. If the average signal during time intervals Δt [s], is $\dot{Q}$, following a change of the apparent signal by ΔQ, then the total heat flux is calculated as

$$_{t}\dot{Q} = \dot{Q} + \tau(\Delta Q / \Delta t) \quad (9)$$

τ [s] is the exponential time constant, the ratio of heat capacity to thermal conductivity of the calorimeter chamber and thermopiles (Calvet & Prat, 1963). With increasing size of biomass and calorimeter chamber, the heat capacity increases and heat conduction decreases, the latter due to longer thermal diffusion paths and a decreased surface to volume ratio. Therefore, the time constant of heat conduction calorimeters increases with increasing size of the animal chamber, from ca. 120–150 s for the 0.5 and 3.5 cm^3 chamber filled with water (Fig. 1 and 2), to 600 s for the 150 cm^3 thin-walled glass chamber of the Pamatmat calorimeter (Fig. 6). The time constant obtained from dynamic calibrations should be tested for accurate reconstruction of entire transition periods. With increasing chamber size and decreasing time intervals, e.g. <600 s, second-order exponential time constants become increasingly significant for accurate time corrections (Randzio & Suurkuusk, 1980; Suurkuusk & Wadsö, 1982). In studies with active animals, however, these second-order effects are frequently obscured by variable heat exchange due to changing locomotion and ventilation.

An entirely different dynamic aspect predominates the initial period of an experiment, after placing the animal in the calorimeter chamber. Thermal disturbances of the heat sink cannot be avoided during insertion of the chamber, but can be minimized by thermal pre-equilibration and always very slowly lowering the chamber into the calorimeter. A standardized procedure of filling the chamber must be imitated without actually adding an animal, to determine the stabilization time in blank runs, e.g. 1 h for the 3.5 cm^3 perfusion chamber (Fig. 2*a*), but 4 to 5 h in a calorimeter with a 150 cm^3 chamber (Pamatmat, 1983).

Perfusion rate. High perfusion or flow rates exert disturbing effects on the baseline and distort the sensitivity or calibration constant. Both effects are

minimized by a counter-current principle achieved by two concentric stainless steel tubes (Fig. 2). Since a larger volume of water is contained in the outer than the inner capillary, the retention and equilibration time is longer if the inflow is directed through the outer capillary. Reversed flow can be used initially to eliminate air bubbles through the outer capillary (Görman Nordmark *et al.*, 1984). With reversed flow one obtains the option of a 'nested chamber' design with improved water exchange characteristics (Fig. 2*a*). This is particularly important for homogeneous perfusion of sedimenting particles, such as batches of *Artemia* embryos (Hand & Gnaiger, 1988).

The change between inflow and outflow concentration of oxygen, ΔC_{O_2} [mmol $O_2 \cdot dm^{-3}$ = nmol $\cdot mm^{-3}$], is a function of biomass, ${}_dW$ [g dry weight], weight specific oxygen flux, $\dot{N}_{O_2}$ (nmol $O_2 \cdot g^{-1} \cdot s^{-1}$], and perfusion rate, $\dot{V}_W$ [$mm^3 \cdot s^{-1}$],

$$\Delta C_{O2} = {}_dW \times \dot{n}_{O_2} / \dot{V}_W \qquad (10)$$

The perfusion rate must be adjusted to the metabolic requirements, to maintain outflow oxygen sufficiently high relative to the inflow oxygen concentration, Cin_{O2},

$$\dot{V}_W = ({}_dW \times \dot{n}_{O_2}) / (Cin_{O_2} \times R_{O_2}) \qquad (11)$$

R_{O_2} is the oxygen reduction ratio, that is the signal change relative to the calibration value (Gnaiger, 1983*c*),

$$R_{O_2} = \Delta C_{O_2} / Cin_{O_2} = \text{signal change / inflow signal} \qquad (12)$$

When aerobic heat flux is converted to oxygen flux (Table 1), the calorimetric signal can serve, in conjunction with equation (11), as a control for appropriate perfusion rates. At air saturation concentrations between 0.41 and 0.21 nmol $O_2 \cdot mm^{-3}$ (4 and 25 °C fresh water and sea water, respectively), every µW heat flux must be multiplied by 0.04–0.07 $mm^3 \cdot s^{-1}$ or 0.13–0.25 $ml.h^{-1}$ to obtain the perfusion rate required for maintaining R_{O_2} at 0.15 (outflow concentration at 85% of inflow).

For example, calculate the perfusion rate required to set R_{O_2} at 0.15 in an experiment with 0.014 g oligochaetes at 20 °C (Gnaiger & Staudigl, 1987): At a total heat flux of 76 µW, $\dot{n}_{O_2}$ is 12 nmol $O_2 \cdot g^{-1} \cdot s^{-1}$, Cin_{O_2} is 0.280 nmol O_2 mm^{-3} in air-saturated fresh water near sea level (101 kPa), then the flow rate must be 4.0 $mm^3 \cdot s^{-1}$ or 14 $ml \cdot h^{-1}$. At 10 °C the perfusion rate can be reduced to 1.5 mm^3 s^{-1} or 5.3 ml h^{-1}, assuming $Q_{10} = 2.2$. With a 1 g marine mussel at a respiratory activity of 3 nmol s^{-1} at 15 °C (Fig. 5), a perfusion rate of 40 $mm^3 \cdot s^{-1}$ or 145 $ml \cdot h^{-1}$ yields a R_{O_2} of 0.30.

Simultaneous respirometry: the Twin-Flow respirometer

The above calculations apply equally for designing open-flow respirometric experiments, where polarographic oxygen sensors (POS) are

employed for continuous monitoring of inflow and outflow oxygen concentrations. As the oxygen reduction ratio, R_{O_2}, is a relative value (equation 12), it can be calculated from the experimental signal recorded arbitrarily as mm or percentage chart recorder deflection, voltage or current of the POS. Oxygen flux, $\dot{N}_{O_2}$ [nmol·s^{-1}], at steady state is (equations 11 and 12),

$$\dot{N}_{O_2} = R_{O_2} \times Cin_{O_2} \times \dot{V}_W = \Delta C_{O_2} \times \dot{V}_W \quad (13)$$

The Twin-Flow respirometer and its applications in calorespirometry (Fig. 1) were described in detail (Gnaiger, 1983*c*; Cyclobios, 1985). Cin_{O_2} is kept constant by continuous equilibration of water with air (or a gas mixture). The water reservoir and the entire respirometer are simply immersed into a water bath regulated at constant temperature (± 0.05 °C) accurately at or slightly above experimental temperature of the calorimeter. Otherwise supersaturation of heated water results in gas bubble formation in the perfusion system. Moreover, the water reservoir should be located at the highest level of the perfusion system to avoid negative pressure and hence bubble formation.

Accurate calibration of the oxygen sensors is essential in a perfusion respirometer. If the variability of oxygen measurements in the inflow and outflow amounts to only 0.5%, then the error of oxygen consumption is 4.4% at R_{O_2} of 0.15. Frequent calibrations of the POS in a fixed position improve its accuracy to better than 0.5%, which is possible with the Twin-Flow principle (Gnaiger, 1983c). Calibration with inflow water at Cin_{O_2} is made when the POS is intersected between the water reservoir and animal chamber. By a system of two 4-way valves the sensor is switched into measuring position at the outflow. The difference in the signal is directly proportional to R_{O_2} and $\dot{N}_{O_2}$ (equation 13). During calibration the record of oxygen flux is interrupted, but this loss of signal is avoided by using two POS switched at intervals into alternate positions (Fig. 1, inset). As a prerequisite for simultaneous calorimetry, the direction of flow remains unchanged despite the switching between measuring and calibration positions of the POS.

A peristaltic pump is connected to the outflow for regulating perfusion at a constant rate (±0.5%). Frequent measurements of the perfusion rate, $\dot{V}_W$, are required to correct for gradual changes due to ageing of the tubings. The measurements are easily made by using a calibrated 1 or 2 ml pipette and a stop watch. Inaccuracies affect the respirometric results and the CR ratio, as the calorimetric signal is independent of the accurate value of $\dot{V}_W$. The response time of the respirometer is an inverse function of the perfusion rate and may be very different from that of the calorimeter.

Therefore, the accuracy of instantaneous CR ratios depends on the quality of dynamic corrections of both systems (Gnaiger, 1983*c*). In cases of changing ventilatory and locomotory patterns, the apparent fluctuations of oxygen consumption may not correlate with changes in metabolic flux but merely reflect external oxygen exchange.

Artefacts of oxygen consumption and CR ratios can also arise from oxygen diffusion or microbial and chemical oxygen consumption in the flow lines. All flow lines and connections from the water reservoir to the outflow POS must be essentially leak tight and diffusion free. Teflon or silicone tubings are not feasible. Gold capillaries with 1 mm inner diameter are flexible and inhibit bacterial growth at the surface. Where glass capillaries are too fragile, stainless-steel capillaries can be applied. Non-biological blank oxygen consumption commonly occurs when using stainless steel. The problem of corrosion can be minimized by coating the inner surfaces with an organosilane concentrate (Prosil–28). Blank oxygen uptake is determined after removing the animals from the system and replacing the chamber without cleaning precautions. Microbial blank oxygen uptake reaches steady state after several hours of contact with the animal (Dalla Via, 1983). The blank flux varies with P_{O_2} (Gnaiger, 1983*c*) and must be subtracted from the measured flux. Heat dissipation in the empty animal chamber is automatically indicated in the experimental baseline of the calorimeter hence no further corrections are required.

Closed-system calorimetry and respirometry: *parallel* heat and oxygen measurements

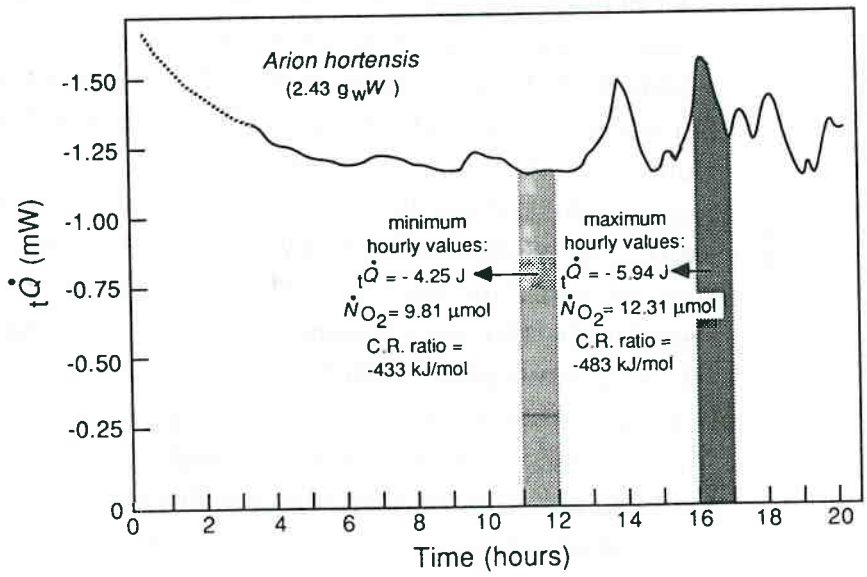
The continuous depletion of the small amount of oxygen dissolved in water limits the usefulness of closed 'batch-type' calorimeters in the study of aerobic aquatic animals. In 150 cm^3 air-saturated water a 40 mm long specimen of *Mytilus edulis* is already experiencing hypoxia by the time thermal equilibrium of the calorimeter is re-established. Prolonged aerobic conditions in water can be maintained by including a gas phase in the closed chamber and stirring the water for equilibration (Görman Nordmark *et al.*, 1984). The ventilatory activity of some animals such as *Mytilus edulis* provides sufficient stirring, >80% air saturation in 40 cm^3 of seawater placed in the 150 cm^3 calorimeter chamber (Fig. 6).

Use of a two-phase system has permitted measurement of heat flux by isolated ovaries at oxygen levels that approximate those prevailing *in vivo* (Bookbinder & Shick, 1986). Parallel measurements of oxygen flux in gently agitated Gilson respirometer flasks indicate that the majority of the energy metabolism in these massive, unperfused organs is anaerobic. The

unstirred boundary layer adjacent to the ovaries in the static calorimeter chamber may be larger than *in vivo*, and open-flow calorimetry seems better suited for such studies. A large anaerobic component was postulated from parallel microcalorimetric and respirometric measurements of the isolated snail heart (Herold, 1977). However, the average CR ratio was *lower* than the oxycaloric equivalent (less heat instead of additional anaerobic heat was observed), but calculation of the linear slope between calorimetric and respirometric rates at various P_{O_2} yields a regression-CR ratio of -459 kJ·mol^{-1} ($r = 0.98$). This agreement of the regression-CR ratio with the oxycaloric equivalent, in combination with an intercept different from zero, usually indicates a problem with the baseline position.

The 20 to 50-fold higher concentration of oxygen in air compared to water, makes closed-chamber calorimeters more suitable for studying air-breathing animals, e.g. insects (Coenen-Stass, Schaarschmidt & Lamprecht, 1980; Dunkel, Wensman & Lovrien, 1979; Peaking, 1973). Prolonged measurement of heat flux by, e.g. the terrestrial slug *Arion hortensis*, does not subject the animal to hypoxia in a closed 150 cm^3 chamber (Fig. 3). Such long-term measurements are likely to detect all phases of activity and associated metabolism (Pamatmat, 1983), and are far less tedious than the manual manipulations involved in traditional volumetric measurements of oxygen. In the present example, oxygen consumption by *A. hortensis* was measured every 0.25 h for six hours in a Gilson constant pressure

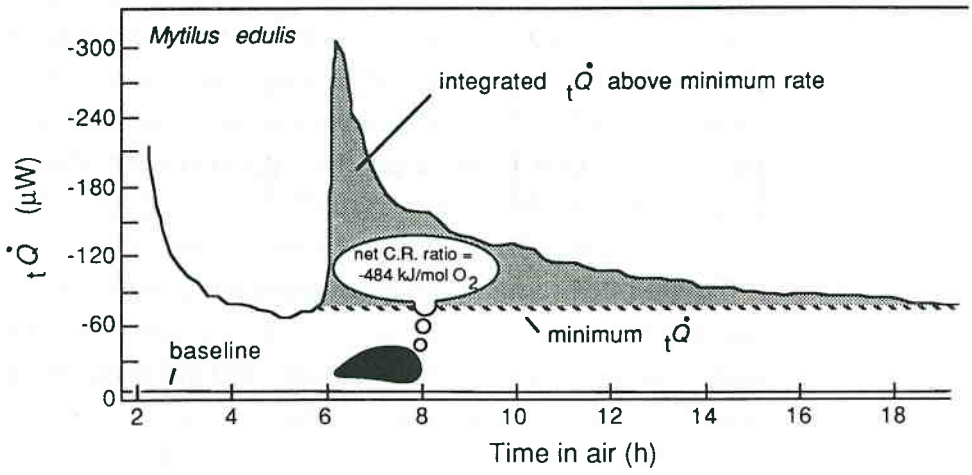
Fig. 3. Continuous recording of heat flux in air by a terrestrial slug, *Arion hortensis* at 15 °C. Minimum and maximum hourly values of heat flux from this recording and minimum and maximum hourly oxygen flux for the same specimen measured on the day following the calorimetric experiment yield estimates of the CR ratio.



respirometer. Comparison of the minimum and maximum hourly values of $\dot{N}_{O_2}$ measured periodically, with those for ${}_t\dot{Q}$ measured continuously reveal CR ratios similar to the theoretical oxycaloric equivalent (Fig. 3). Direct calorimetry thus seems particularly well suited to monitor daily patterns of activity and energy flux in such animals.

Intertidal animals experience intermittently terrestrial conditions and then rely on aerobic and anaerobic energy metabolism to varying extents. Although total heat flux can be measured under simulated intertidal conditions, the method does not distinguish between aerobic and anaerobic sources of heat, and parallel or sequential indirect measurements are required to do so. Neither form of indirect calorimetry (respirometry, or biochemical measurement of anaerobic end-products) is sufficient to quantify total metabolism. Studies on *Mytilus edulis*, which generally closes its shell valves during exposure to air, indicate that 0 to 45% of the heat dissipated during exposure derives from aerobic respiration (Shick *et al.*, 1986), whereas *Cardium edule*, which gapes and breathes air when exposed, remains fully aerobic (Widdows & Shick, 1985), having a mean CR ratio of -433 kJ·mol^{-1} O_2. Likewise, intertidal specimens of the sea anemone *Anthopleura elegantissima* have a mean CR ratio of -473 kJ·mol^{-1} O_2 during 12 hours of aerial exposure (Shick & Dykens, 1984). Subtidally acclimatized specimens of the sea anemone *Actinia equina* become active and have high heat flux during acute intertidal exposure, although their oxygen flux declines over the same period; this indicates an increasing

Fig. 4. Heat flux of *Mytilus edulis* (0.4 g ${}_dW$; 15 °C) during aerial exposure, measured in the 28 cm^3 closed ampoule of the LKB-ThermoMetrics microcalorimeter. On reimmersion the mussel released a gas bubble that was measured volumetrically. Consumption of all the O_2 in this bubble and the heat flux integrated above the minimum rate yield the net CR ratio, indicating a fully aerobic peak due to air gaping (after Shick *et al.*, 1986).



reliance on anaerobic metabolism as their hydrostatically supported body collapses, which increases diffusion distances and reduces gas exchange surface area (Shick, 1981).

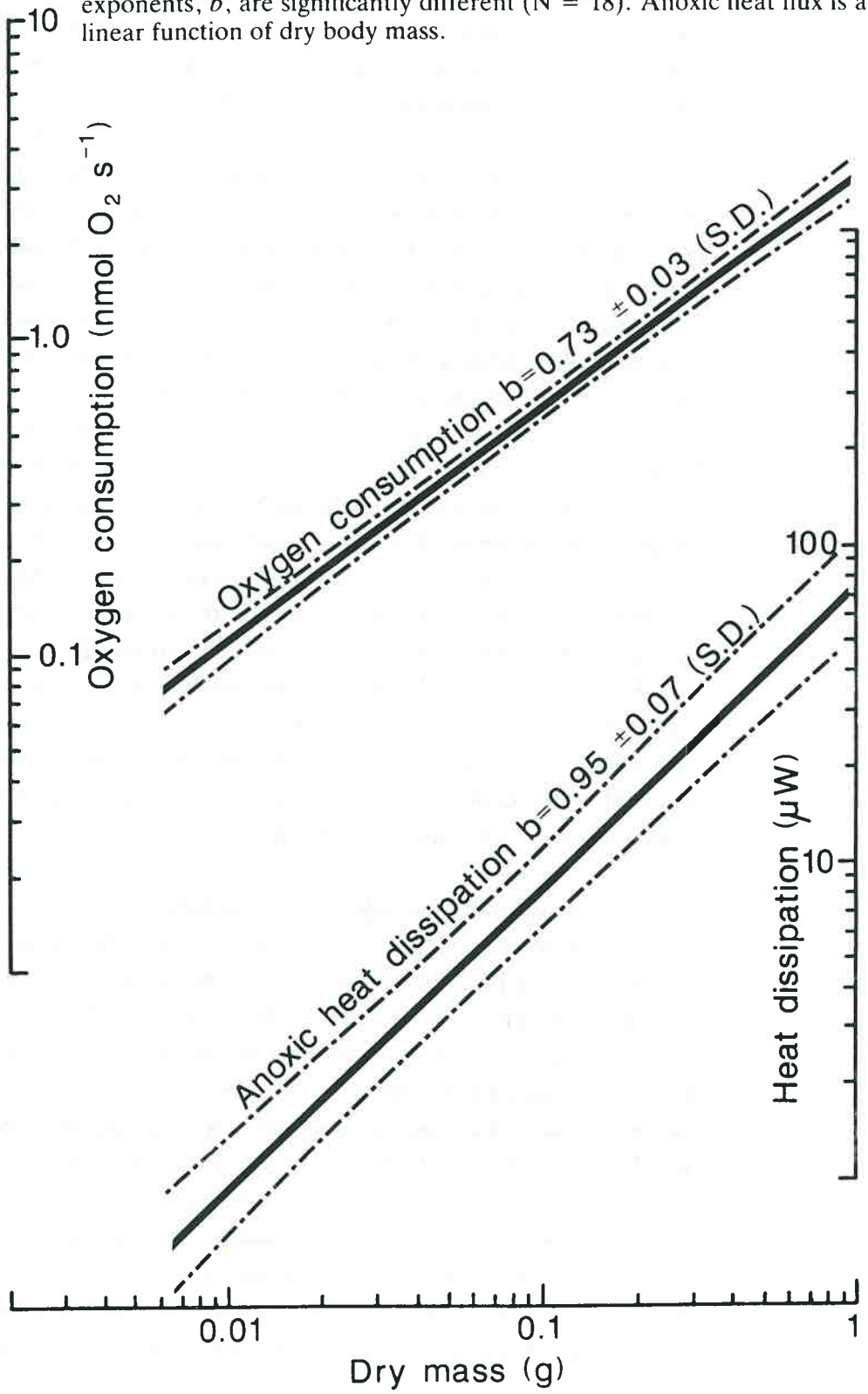
In intertidal bivalves, air-gaping in particular can cause an individual's heat flux to increase 5 to 8-fold, as metabolism switches from being predominantly anaerobic to largely aerobic during periods of high rate (Fig. 4). Parallel oxygen and heat measurements confirm the largely aerobic nature of such peaks; the net CR ratio for maximum flux in air-exposed *M. edulis* is -467 kJ·mol^{-1} O_2 (Shick *et al.*, 1986). Reassuringly, we also observed agreement in the rates of aerial heat flux of *M. edulis* measured in the ThermoMetric calorimeter (-0.305 ± 0.065 mW g^{-1}; N=12) and the Pamatmat calorimeter (-0.288 ± 0.047 mW·g^{-1}; N=9) (mean ± S.E.; Shick *et al.*, 1986).

Microcalorimetry, and fundamental aspects of aerobic and anoxic metabolism

Quantification of the extent of 'metabolic shutdown' following aerobic–anoxic transitions require highly sensitive methods. Using the modular 4-channel ThermoMetric microcalorimeter, we studied the anoxic rate of *Mytilus edulis* in the size range of 0.016 to 1.0 g dry weight (Fig. 5), with 4 or 3 specimens (18 or 25 mm long) in 5 cm^3 ampoules, and 3 or 1 specimens (35 or 45 mm) in 28 cm^3 ampoules. Confirming Pamatmat's preliminary result, the anoxic mass exponent is not different from 1.0. This linear function of anoxic heat flux and body mass is fundamentally different from the exponential or power function of aerobic metabolism (Fig. 5; see also Widdows, 1987). According to a dimensional analysis of the metabolic power function by Heusner (1985), the $\dot{N}_{O_2}/\text{mass}^{2/3}$ ratio is the 'mass-independent metabolism'. Consequently, the deviation of the aerobic mass exponent of 0.73 (Fig. 5) from the value of 2/3 = 0.67 would indicate deviation from biological similitude with increasing size of *M. edulis*. This conclusion cannot be drawn from the direct calorimetric measurements, as the constant ${}_t\dot{Q}$/mass ratio indicates strict linear mass-independence under anoxia. Clearly, the functional analysis of aerobic and anoxic biochemical mechanisms can make a positive contribution towards a general physiological theory, but further studies are required before contrasting the Aerobic Surface Law with an 'Anoxic Mass Law'.

Furthermore, the meaning of metabolic rate must be clarified for relating distinct mechanisms such as heat flux, turnover of carbon chains, oxidative metabolism, glycolysis to pyruvate and subsequent operation of pyruvate oxidases, succinate-propionate-acetate formation, and accumulation or excretion of these anaerobic end-products. The term direct

Fig. 5. Anoxic heat flux in N_2 gas and oxygen flux in aerobic seawater as a function of body size of *Mytilus edulis* (March, 12 °C), plotted on equivalent scales (-450 μW nmol^{-1} O_2/s^{-1}]. The exponential mass exponents, *b*, are significantly different (N = 18). Anoxic heat flux is a linear function of dry body mass.



calorimetry has led to the contention, that this method provides the general approach to measure metabolic rate 'directly', without further explanation of the functional significance of heat flux. It must be pointed out that the calorimetric and the biochemical measures of metabolic flux, that is heat flux and ATP turnover respectively, are not related by a constant coefficient: the heat dissipated per unit ATP turnover is ca. -80 kJ·mol^{-1} for fully coupled aerobic metabolism, but less than -40 kJ·mol^{-1} for the propionate-acetate pathway (Gnaiger, 1983*a*). The direct calorimetric measure of heat flux is identical to catabolic power (Gibbs energy change per unit time) under aerobic conditions, but a large difference exists between catabolic power and heat flux in anoxic metabolism due to significant isothermal entropy changes of glycolytic reactions. Therefore, metabolic flux measured by direct calorimetry is not a general indication of the rate of entropy production (Gnaiger, 1983*a*).

The frequently observed reduction of anoxic heat flux to 10% of aerobic values would be equivalent to a reduction of anoxic ATP turnover to 20% of aerobic values calculated on the basis of propionate-acetate excretion and oxygen consumption. Biochemical estimates of metabolic flux under anoxia are frequently lower (de Zwaan & Wijsman, 1976), indicating that either unknown metabolic pathways contribute to the total anoxic flux, or that oxidative processes based on residual oxygen under 'anaerobic' conditions (0.5 to 2% air saturation) are detected by the non-specific calorimetric method, but not by biochemical analyses (Gnaiger & Staudigl, 1987). Therefore, careful energy balance studies, based on enthalpy changes of all metabolic reactions, are required for extending the concept of indirect calorimetry into the anoxic domain.

Anoxic and anaerobic energy balance

Shick, de Zwaan and de Bont (1983) measured, simultaneously, the anoxic heat flux and the accumulation of metabolites in *Mytilus edulis*, testing Gnaiger's (1980) claim that direct and indirect (biochemical) measurements of anoxic energy flux in euryoxic invertebrates do not agree. Thermochemical interpretation (Gnaiger, 1983*a*) of their results revealed the expected exothermic 'anoxic gap', as a significant fraction of the anoxic heat flux could not be explained by known anoxic pathways. This result is in line with a gap in the anoxic proton balance observed by intracellular pH measurements and metabolite changes (Graham & Ellington, 1985). Additional support for the contention that previous biochemical estimates of anoxic catabolic flux are incomplete, stems from the observation of anoxic ammonia accumulation in *Mytilus edulis*, contrary to assumptions on

ammonia fixation to account for the accumulated alanin (Bitterlich, Gnaiger & Widdows, unpublished).

Departures from the anoxic metabolic arrest of invertebrates include the cockle *Cardium edule* (40% anaerobic/aerobic $_{t}\dot{Q}$; Pamatmat, 1980), which is a rather aerobically-poised bivalve that does not tolerate anoxia well. The clam, *Mulinia lateralis* maintains anoxic heat flux from 5–30% (Gnaiger & Shick, unpubl.) to 97% of aerobic values (Shumway, Scott & Shick, 1983). The large 'anaerobic'/aerobic ratio (40%) in the oligochaete, *Lumbriculus variegatus* (Gnaiger, 1980), was obtained under perfusion conditions without simultaneous proof of complete oxygen removal, and the strictly 'anoxic'/aerobic ratio is lower (16%; Gnaiger & Staudigl, 1987). The problem of oxygen leakage and diffusion is obviously enhanced in perfusion systems, and cannot be neglected when using closed chambers sealed with inappropriate materials (e.g. silicone stoppers). Even an ideal oxygen conformer maintains at 1–2% air saturation an aerobic rate of 1–2% of the normoxic value. This condition is frequently referred to as 'anaerobic'. At a measured anaerobic/aerobic ratio of 20%, therefore, 5–10% of the 'anaerobic' rate would be supported by oxygen consumption, which is not only important for energy flux considerations (Gnaiger & Staudigl, 1987), but critical for anaerobic redox balance calculations and estimations of total 'anaerobic' ATP turnover.

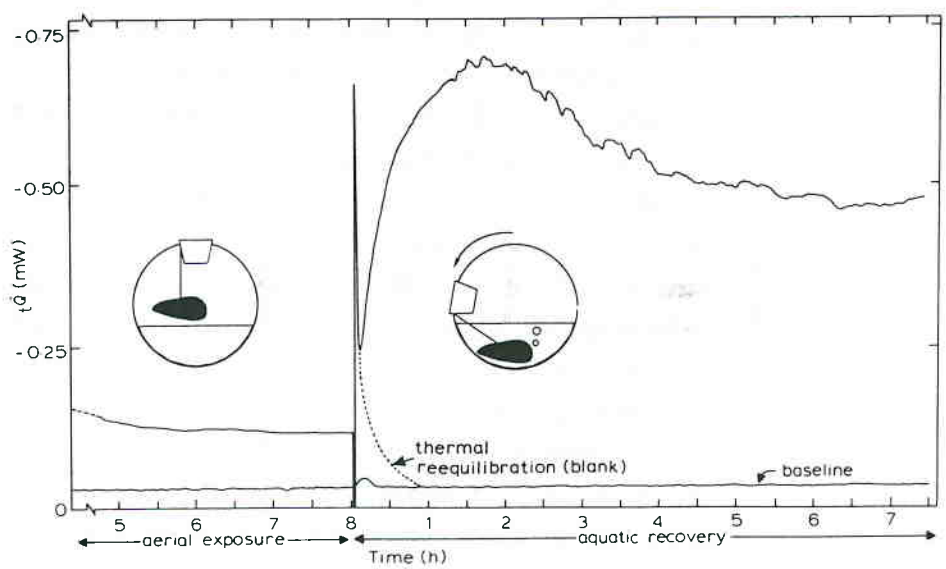
Aerobic recovery from hypoxia and efficiency of biosynthesis

During aerobic recovery from anoxia the conservation of heat in glyconeogenic processes results in CR ratios lower than the oxycaloric equivalent for dissipative metabolism, indicating the internal thermodynamic efficiency of coupled metabolism. Therefore, the 'overshoot' of heat flux during aerobic recovery (Fig. 6) is less than the corresponding oxygen flux (Famme & Knudsen, 1983; Shick *et al.*, 1986). The relatively large chamber of the Pamatmat calorimeter has proved useful in studying the hypoxic–aerobic transition when aerially-exposed 'intertidal' mussels are reimmersed. The experimental mussel is suspended in air above the water by a nylon monofilament cemented to its shell (Fig. 6). The glass chamber is then sealed inside a larger epoxy-coated tin canister, to which a second monofilament is attached. The entire vessel is finally placed between the thermopiles of one half of the double-twin calorimeter. The mussel is reimmersed at simulated 'high tide' by rotating the chamber 90° using the second monofilament (Fig.6). This minimum contact with the vessel during the momentary opening of the calorimeter presents a minimum of thermal disturbance. Re-equilibration time as determined in blank experiments is consistently 0.75 to 0.83 hours, and the reproducible blank re-equilibration

curve allows calculation of aquatic heat flux for all but the initial 0.25 h of recovery.

A quantitative understanding of the efficiency of anabolism and of maintenance is a prerequisite for modern analyses of energy allocation and growth efficiency (Hawkins, Bayne & Day, 1986). Anabolism during recovery from anoxia and hypoxia, as studied by direct calorimetry and biochemistry, can serve as a model for thermodynamic efficiencies in anabolic ATP utilization and growth. Today, students of ecological and physiological energetics are increasingly prepared to ponder over the complexities involved in this thermodynamic approach that is fundamental in physiological energetics, comparable to the aspects of efficiency of catabolic ATP production and metabolic regulation (Gnaiger, 1987). An experimental and theoretical analysis, comparing the biochemical and thermodynamic efficiency of aerobic recovery metabolism after accumulation of succinate and lactate, is available (E. Gnaiger, M. Shick & J. Widdows, in preparation).

Fig. 6. Heat flux of *Mytilus edulis* (0.32 g $_dW$; 15 °C) during aerial exposure and aquatic recovery, measured in the 150 cm^3 closed chamber (insets) of the Pamatmat calorimeter. The sharp deflection at the start of recovery and exponential re-equilibration as measured without an animal results from opening the calorimeter to rotate the sample chamber and immerse the mussel. Parallel measurement of oxygen flux yields low CR ratios corrected for re-oxygenation of body fluids and mantle cavity water, indicating the high thermodynamic efficiency of recovery metabolism (after Shick *et al.*, 1986).



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