In: *Techniques in comparative respiratory physiology: An experimental approach.*
Bridges C.R., Butler P.J. (eds.)
Soc. Exp. Biol. Seminar Series, Cambridge Univ. Press: pp. 113-135 (1989)

E. GNAIGER, J. M. SHICK and J. WIDDOWS

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

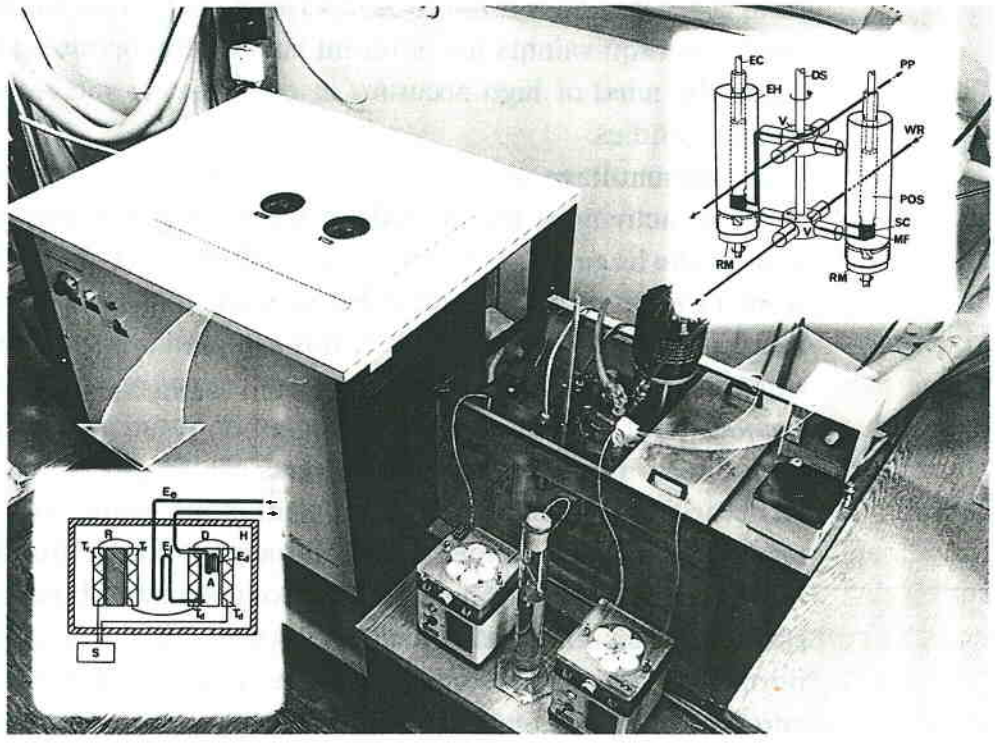
that can then be applied to studies of metabolically complex situations involving anoxia or net biosynthesis.

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).

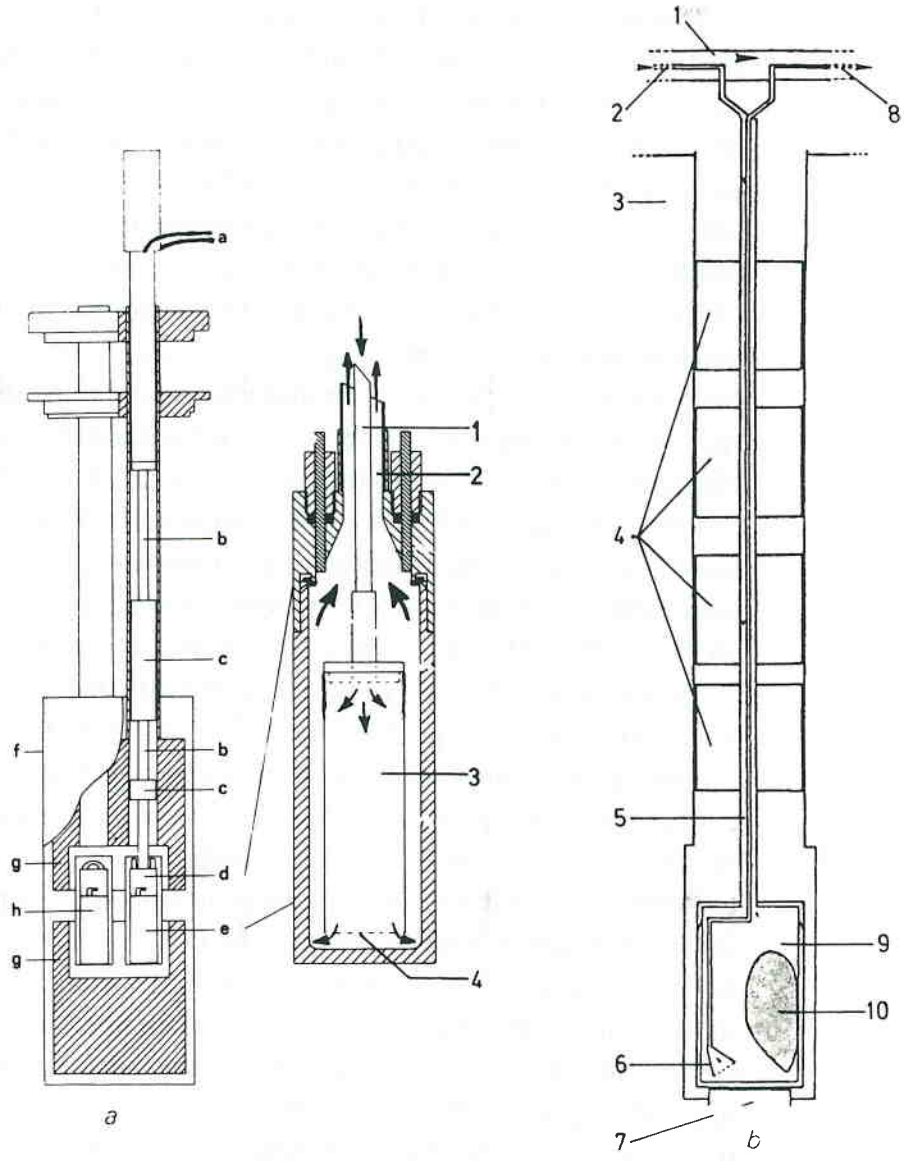


systems such as micro organisms and tissue cells, etc.', has been reviewed recently by Wadsö (1987). Here we are exclusively concerned with studies of 'etc.', living animals. Adiabatic calorimeters ideally exchange no heat with the surroundings, and the observed temperature increase is directly proportional 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