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ERICH GNAIGER

Animal energetics at very low oxygen: information from calorimetry and respirometry

Introduction: 'facultative' anaerobiosis?

For more than two decades the topic of anaerobiosis has been the subject of intensive investigation in invertebrate physiology and biochemistry (Hammen, 1969; Hochachka & Mustafa, 1972; Saz, 1971; Simpson & Awapara, 1966; De Zwaan & Zandee, 1972; see also von Brand, 1946). Still, mammalian biochemists are insufficiently aware of the importance of anaerobic mitochondrial energy transformation in metazoans, although ATP formation coupled to anaerobic mitochondrial electron transport occurs not only in invertebrates (Saz, 1981) but in mammalian myocyte mitochondria (Wiesner *et al.*, 1988).

The term invertebrate facultative anaerobiosis, adopted from the microbiological literature, implies the 'unsubstantiated assumption that there are facultative anaerobes among metazoans' (Hammen, 1976). It has been applied with reference to animals that do not tolerate anoxia for more than several days. Growth is arrested in such euryoxic animals as oxygen is depleted and other energy-demanding activities are greatly reduced, whereas by definition facultative microorganisms grow under anoxia. The most tolerant animals enter a state of dormancy for long-term anoxic survival, and metabolic heat flux is lowered to as little as 2% of the aerobic level (Hand & Gnaiger, 1988). To date it is not known if any animal is capable of completing its life cycle under strict anoxia, with the possible exception of meiofauna living in the anoxic sulphide system (Meyers *et al.*, 1987; Powell *et al.*, 1980; Wieser *et al.*, 1974). A reported example of facultative anaerobiosis among aquatic oligochaetes has been critically discussed by Gnaiger & Staudigl (1987).

The previous critique of the term invertebrate facultative anaerobiosis focused on the duration of anoxia. An additional, important, yet much neglected aspect is discussed here, namely the extent and quantification of the 'anaerobic' condition. The actual measurement and control of oxygen is not simple under conditions when oxygen is supposedly not available to

A
B
C
30 min
A_c
A_m
B_c
B_m
C_m
C_e
0.8
0.4
0.0
P_{O_2} (kPa)
60
40
20
0
diffusion (pmol O_2 s^{-1})

Figure 1

the animal. Special methods are required to detect oxygen uptake at very low oxygen concentrations. Whereas specific anaerobic aspects of intermediary metabolism are quantified by biochemical analysis of accumulated and excreted organic end products, direct calorimetry detects indiscriminately both aerobic and anaerobic sources of metabolic heat at any oxygen regime (Pamatmat, 1978; Gnaiger, 1983*a*). Therefore, direct calorimetry should complement respirometric and biochemical approaches in the study of energy metabolism at very low oxygen.

Anaerobic metabolism

In zoophysiology, 'anaerobic' (without air) is rarely defined in terms of controlled measurements of the actual extent of anaerobic conditions. By comparison, oxygen exclusion techniques are highly advanced in anaerobic microbiology (Holland *et al.*, 1987) and in studies of isolated microxic cells and mitochondria (see below). Despite careful precautions to exclude molecular oxygen, this is not always achieved completely owing to insufficient oxygen removal and diffusion of oxygen through the materials of experimental vessels, seals and tubes (Figure 1). If no measurements of the actual oxygen level in 'anaerobic' media are available, caution is required when interpreting the biological results. When strictly *anoxic* conditions are not achieved, *anaerobic* metabolism proceeds simultaneously with oxygen consumption (Gnaiger & Staudigl, 1987).

For instance, in one of the few biochemical publications where the upper level of oxygen in 'anaerobic' experiments is reported, the value 'less than

Figure 1. Oxygen diffusion through silicone tubing connecting two stainless steel capillaries (1.1 mm inner diameter) in an open-flow calorespirometry system. A, Viton tubing without detectable diffusion; B and C, silicone tubing with narrow spacing and a 3 mm gap between the inner steel capillaries, respectively, resulting in elevated oxygen of (B) 0.24 kPa (1.8 mmHg; 3.0 μmol dm^{-3}; 1.2% air saturation) and (C) 0.80 kPa (6.0 mmHg; 10.0 μmol dm^{-3}; 3.9% air saturation). Recorder traces are shown of the two polarographic oxygen sensors (POS) of the Twin-Flow respirometer (Cyclobios, Austria). Water is equilibrated with nitrogen in a glass reservoir, connected to the Twin-Flow valve system (Gnaiger, 1983*b*) by a stainless steel capillary. While one POS is in *calibration* position directly connected to the water reservoir (subscript c) the other POS is in *measuring* position after the water has passed the capillary connection (subscript m). The trace in the upper panel shows, from right to left: B_c, the calibration at 0 kPa; C_m, the switch to measuring position while the capillary gap is simultaneously increased to 3 mm; B_m, the response to a narrowing of the gap; and B_c, back to calibration. Perfusion with deionized water is kept constant at 5.56 mm^3 s^{-1} (20.0 cm^3 h^{-1}) at 20 °C.

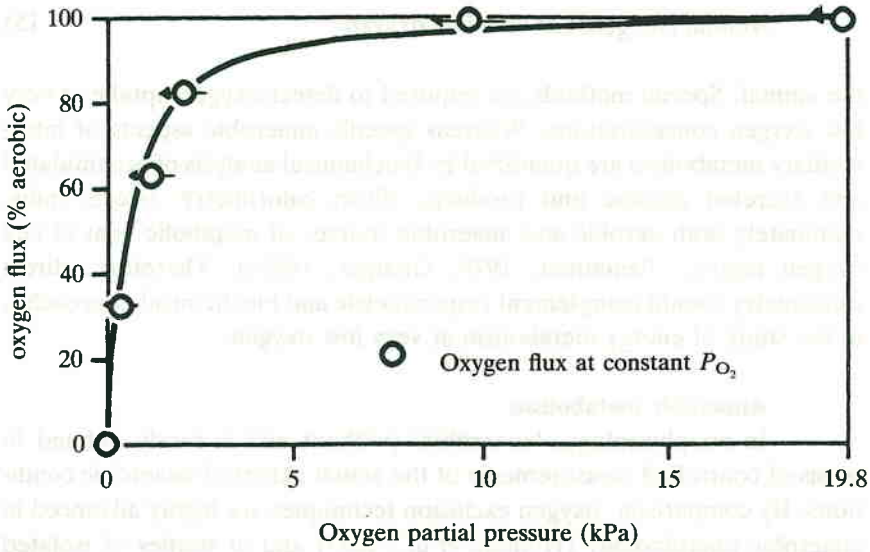


Figure 2. Oxygen flux–pressure relation in the planktonic copepod *Cyclops abyssorum*. Oxygen flux was measured at 6 °C in a 0.5 cm^3 glass chamber of a Cyclobios Twin-Flow respirometer at 5 steady-state P_{O_2} levels. The P_{O_2} of the inflow and outflow water is shown by arrows (after Gnaiger 1983*b*).

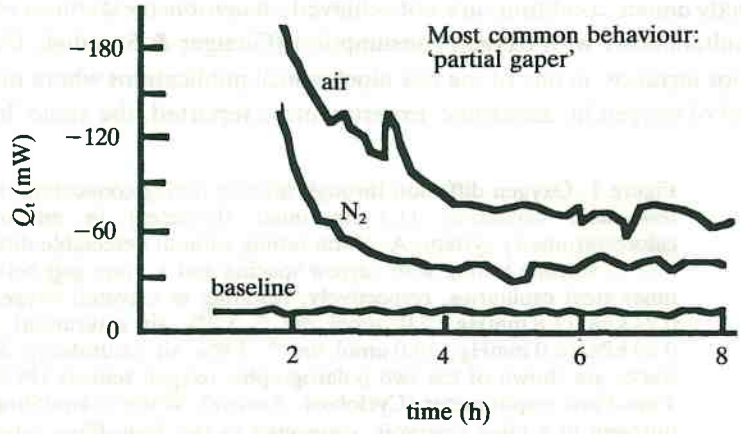


Figure 3. Heat flux of the intertidal mussel *Mytilus edulis* during exposure to air and nitrogen gas. From the difference between the traces at steady state it is concluded that 40% of total heat flux in air is aerobic. Heat flux was measured in a 25 ml stainless steel chamber of a Thermal Activity Monitor (ThermoMetric, Sweden) at 15 °C (from Shick *et al.*, 1986).

0.5 ml O_2' per litre is specified for anaerobic energy metabolism in an isopod (De Zwaan & Skjoldal, 1979). At experimental conditions (13 °C; seawater) the oxygen concentration at 100% air saturation is 264 μmol dm^{-3}; therefore the oxygen concentration of 0.5 ml l^{-1} (22.3 μmol dm^{-3}) corresponds to 8.4% air saturation (1.7 kPa). Although the actual oxygen content may have been less and this particular isopod species is probably not an oxygen regulator, it is important to note that the oxygen consumption of some Crustacea is virtually unaffected down to oxygen pressures of 1.7 kPa (Figure 2).

Another example illustrates the relative meaning of 'anaerobic'. During exposure to air, the intertidal mussel *Mytilus edulis* maintains an aerobic metabolism responsible for up to 40% of the total (anaerobic and aerobic) heat flux (Figure 3). Thus, the simultaneous operation of aerobic and anaerobic pathways of *M. edulis* exposed to air is important, and the ratio depends on season and nutritional status (Widdows & Shick, 1985; Shick *et al.*, 1988). Obviously, total metabolism of mussels exposed to air cannot be referred to as anaerobic without testing for aerobic contributions to total ATP turnover. Too little attention has been paid to the quantitative significance of residual oxygen consumption relative to anaerobic ATP generation under 'anaerobic' conditions.

Methods at low oxygen

Most studies on the oxygen dependence of respiration have been carried out in closed respirometric chambers under conditions of continuously declining oxygen pressure. In this approach limited time is available for observation of hypoxic respiration. Transient conditions may elicit a different response compared with steady-state conditions, which can be maintained for long periods of time in open- or intermittent-flow respirometers (Bayne & Livingstone, 1977). Oxygen consumption of *Cyclops abyssorum* (Figure 2) and of the aquatic oligochaete *Lumbriculus variegatus* was measured in a Cyclobios Twin-Flow respirometer at various constant P_{O_2} levels of the inflow (Gnaiger, 1983*b*). The inflow–outflow P_{O_2} difference varies according to the experimental oxygen uptake at a constant perfusion flow maintained through the system (Figure 4, arrows).

If the inflow water is immediately mixed with the bulk water in a well-stirred animal chamber, then the outflow P_{O_2} is the relevant partial pressure experienced by the animal. In an unstirred chamber the turbulence generated by the animals is irregular and undefined. Then the average P_{O_2} in the gradient between inflow and outflow is a more accurate choice than

Table 1. *Amperometric and redox titration in chemical Winkler analysis of dissolved oxygen in air-equilibrated and N_2-equilibrated water samples*
Values are means±standard deviations; n=4.

	Amperometric (μmol O_2 dm^{-3})	% Air	Redox (μmol O_2 dm^{-3})	% Air
Air	261.6±1.06	98.56	263.8±0. 75	99.36
Air	264.7±0.31	99.06	256.3±0.28	99.34
N_2 techn.	1.8±0.09	0.67	1.9±0.06	0.71
N_2 pure	1.5±0.19	0.58	1.5±0.16	0.57

From Gnaiger (1983*c*).

reference to the inflow oxygen pressure (Gnaiger, 1983*b*; Hughes *et al.*, 1983). At low perfusion flow and low oxygen levels, respiration may be limited by the total oxygen delivery into the system rather than by P_{O_2}, particularly when the outflow P_{O_2} remains near zero irrespective of changes in the inflow P_{O_2}. The oxygen delivery is the inflow oxygen concentration multiplied by the perfusion flow. Different problems are associated with either perfusion or closed-chamber respirometry when defining the dependence of oxygen flux on oxygen pressure, and a systematic investigation of these methodological aspects is required.

Calorespirometric studies show that at a P_{O_2} of 0.1 kPa (0.5% air saturation), aerobic metabolism may amount to as much as 33% of total heat flux (see Figure 6). It is worrying that this oxygen pressure is near the limit of detection in some routine applications of polarographic oxygen sensors with small cathodes (Hitchman, 1983). This is overcome in the Twin-Flow respirometer by frequent, automatic calibration of the oxygen sensors with large cathodes (Figure 1). Moreover, 0.5% air saturation is at the limit of detection of the Winkler method in N_2 equilibrated water (Table 1).

The sensitivity of a polarographic oxygen sensor, with a guard cathode for scavenging the oxygen that diffuses from the electrolyte and sensor body (Orbisphere, Switzerland) (Hitchman, 1983), is extended to oxygen concentrations of 0.003 μmol dm^{-3} (*ca.* 0.001% air saturation, 0.2 Pa or 0.002 mmHg). This sensor was elegantly used to study the respiration of isolated myocytes (Wittenberg & Wittenberg, 1985). Optical methods, particularly for studies of tissue P_{O_2}, have been reviewed by Tamura *et al.* (1989).

Critical and limiting oxygen pressure

Oxygen regulators are characterized by a critical P_{O_2} (*Pc*), above which the resting, routine or standard metabolic energy flux is relatively independent of oxygen pressure. Below the *Pc* the regulation of oxygen flux becomes less efficient (Bayne & Livingstone, 1977). The *Pc* of regulators is significantly below normoxia. Metabolic hypoxia is indicated as a reduced oxygen flux below the critical oxygen pressure (Figure 2) and is either fully or partly anaerobic. The metabolism of the planktonic copepod *Cyclops abyssorum* is completely aerobic at 2 kPa (15 mmHg) as measured by simultaneous direct and indirect calorimetry (calorespirometry; Gnaiger, 1983*b*, and unpublished results). In the lugworm *Arenicola marina*, the *Pc* is near air saturation at 16 kPa (120 mmHg) (Toulmond, 1975) but anaerobic processes are not switched on until partial oxygen pressure reaches 6 kPa (45 mmHg) (Schöttler *et al.*, 1983). Above this 'second critical' (Toulmond, 1975) or 'limiting' oxygen pressure (Schöttler *et al.*, 1983) there is an extended phase of fully aerobic hypoxia. Anaerobiosis compensates progressively for the declining oxidative energy supply below the limiting oxygen pressure.

Oxygen flux of *L. variegatus* is nearly independent of oxygen pressure between 50 and 100% air saturation (94% of the normoxic respiration observed at 8 kPa) and decreases non-linearly under hypoxic conditions (Figure 4; Figure 5*a*, heavy line). A hyperbolic fit (Figures 2 and 4) does not imply that the theory of Michaelis–Menten kinetics is applicable to a complex animal system. Interpretation of a calculated K_M as the whole organism's oxygen affinity is not meaningful (50% of normoxic respiration is reached at 0.6 and 1.2 kPa; Figures 2 and 4). Furthermore, a 50% drop in oxygen flux does not reflect the functional aspect of hypoxia. Neither can an unambiguous definition of the *Pc* be applied when a sharp discontinuity does not occur in the oxygen flux-pressure relation (Rosenthal *et al.*, 1976). If the critical P_{O_2} is arbitrarily defined as the P_{O_2} at which respiration declines below 75% of the normoxic value, then *C. abyssorum* has a *Pc* of *ca.* 1.5 kPa (Figure 2), and in *L. variegatus* the *Pc* is at 3 kPa (Figure 4). As an oxygen regulator, *L. variegatus* shows a steep decline of oxygen consumption in the microxic region towards anoxia. A significant oxygen uptake is observed at the lowest P_{O_2} of 0.08 kPa (0.6 mmHg) (see also Figure 6).

The distinction between the critical and limiting P_{O_2} is also important at the cellular level. Resting myocytes isolated from heart muscle regulate oxygen flux down to 0.05 kPa (0.4 mmHg). The limiting P_{O_2} of these cells,

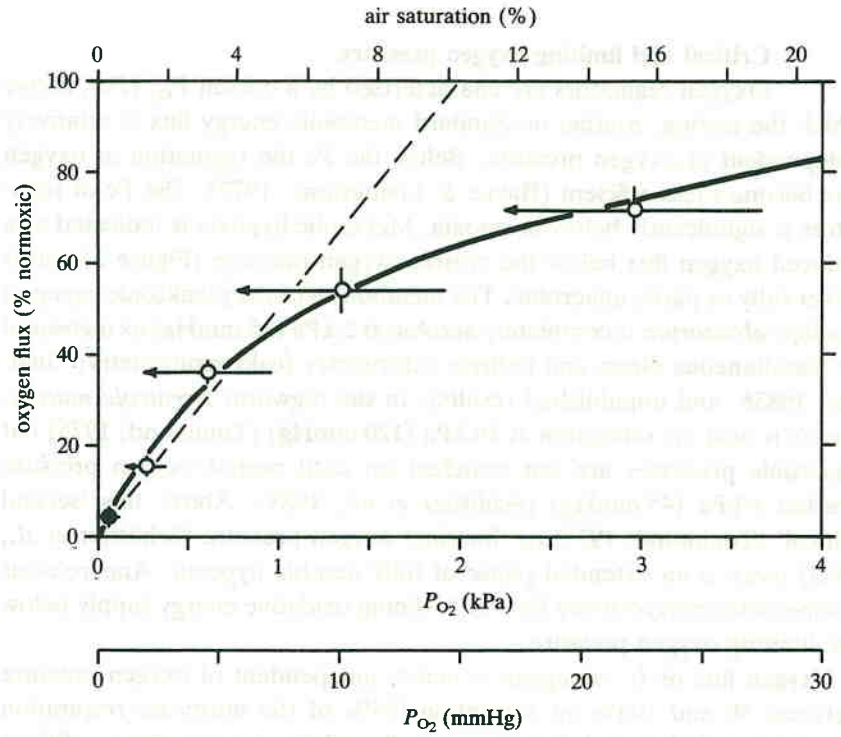


Figure 4. Oxygen flux–pressure relation in the aquatic oligochaete *Lumbriculus variegatus*. Oxygen flux was measured at 20 °C in a 0.5 cm^3 glass chamber of a prototype Twin-Flow respirometer at 7 steady-state P_{O_2} levels. The P_{O_2} of the inflow and outflow water is shown by horizontal arrows. The vertical bars show the ranges of oxygen flux of 2 (open circles) or 4 experiments (full circle) normalized relative to the oxygen consumption at normoxia (11.8±4.6 nmol O_2 s^{-1} g^{-1} dry mass, n=6). At the lowest P_{O_2} (full circle) respiration was calculated as the difference of the oxygen diffusion into the system in the absence and presence of animals (Gnaiger & Staudigl, 1987; see also Figure 6). Steady-state respiration was observed for at least two hours at each P_{O_2}. (The broken line and heavy line are those of Figure 5*a*.)

observed as a steep increase in lactate production, is about 5 times lower (Wittenberg & Wittenberg, 1985) (Figure 7).

Microxic regulators versus oxygen conformers

In contrast to oxygen regulators, oxygen conformers show a linear decline in oxygen flux as P_{O_2} decreases from the normoxic level. Conformity and regulation are a matter of degree, depending on various environmental and physiological variables (Bayne, 1971; Mangum & van

Winkle, 1973). The oxygen flux–pressure relation reflects the operation of complex regulatory feedback mechanisms of oxygen supply and ATP demand, described as a dynamic balance of drive-coupled and load-coupled metabolism (Gnaiger, 1987).

Various types of conforming pattern are distinguished depending on the intercept of the linear slope between oxygen flux and P_{O_2} (Herreid, 1980). In a perfect conformer the relation between oxygen flux and pressure is not only linear but proportional (for a discussion of generalized flux–pressure relations, see Gnaiger (1989)). A steeper slope with a negative intercept results in zero or very low oxygen flux before completely anoxic conditions are reached. Thus, uptake of oxygen can be diminished owing to cessation of blood circulation, to retraction behaviour or to valve closure.

A linear slope is commonly observed between oxygen flux and partial pressure over an extended hypoxic range, with a positive intercept when extrapolated to zero oxygen. Examples are found in polychaetes (Petersen & Johansen, 1967), nematodes (Atkinson, 1973), sipunculids (Pörtner *et al.*, 1985), crustaceans (reviewed by Herreid, 1980), fish (Hughes *et al.*, 1983; Pullin *et al.*, 1980; Ultsch *et al.*, 1981) and snakes (Abe & Mendes, 1980). The extrapolated intercept can be as high as 40% of the oxygen flux at normoxia, with a linear slope from a P_{O_2} of 20 kPa down to 4–6 kPa (150 to 30–45 mmHg). Although classically described as conformers with emphasis on the hypoxic region, these animals behave just like regulators from the perspective of the microxic region around 1% air saturation (0.2 kPa or 1.5 mmHg) (Figure 5*a*). Microxic regulation, therefore, effectively increases the slope of the flux–pressure relation in the microxic region. The slope of an ideal conformer is equivalent to a 5% increase of oxygen flux per kPa (Figure 5*a*).

Irrespective of the position of the *P*c below or above normoxia in a regulator or conformer, respectively, the onset of anaerobic processes at the limiting P_{O_2} can be defined experimentally by specific biochemical indices (Livingstone & Bayne, 1977; Burton & Heath, 1980; Pörtner *et al.*, 1985; Schöttler *et al.*, 1983), by more general acid–base balance studies (Toulmond, 1975), and most generally by calorespirometry (Gnaiger *et al.*, 1989) (see below).

The importance of 'microxic regulation' (Figure 5*a*) for total energy metabolism at very low oxygen is illustrated by a simplified model of physiological energetics at anoxic and various microxic steady states. Based on a stoichiometric and thermodynamic analysis (Gnaiger, 1983*a*), the energy saving on account of residual oxygen consumption under 'anaerobic' conditions can be calculated. It is assumed (Figure 5*b–d*) that

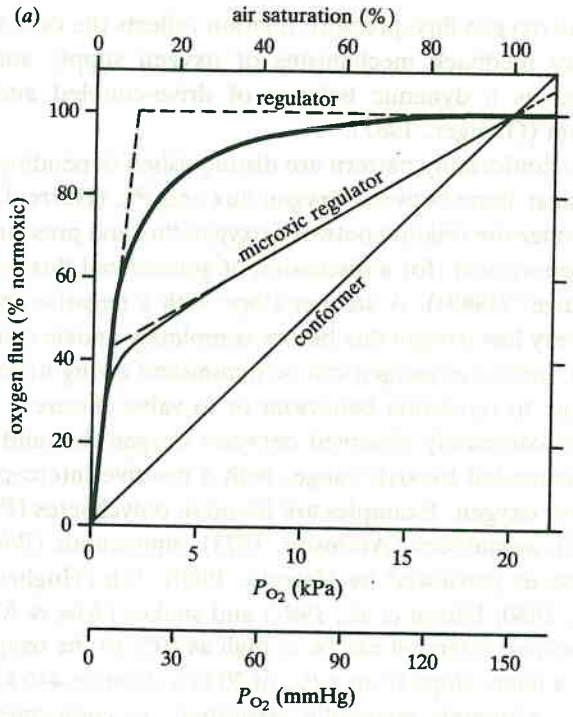
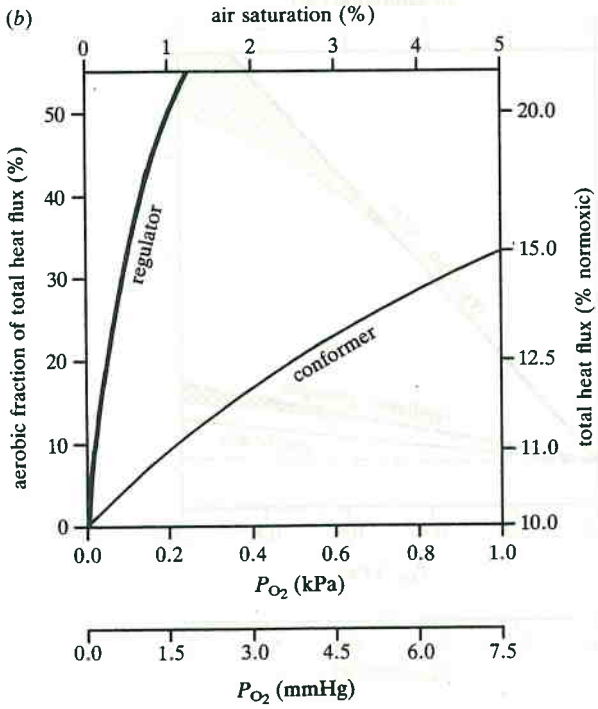


Figure 5. Energetics at low oxygen: Comparison of an ideal conformer with an ideal regulator and a microxic regulator. (*a*) Oxygen flux–pressure relations, normalized relative to the flux under normoxia. The thick hyperbolic line for the regulator is the experimental observation in *Lumbriculus variegatus* (Figure 4). (*b*) Contribution of aerobic metabolism to total heat flux under microxic conditions, assuming that anoxic heat flux is constant in the microxic region at 10% of heat flux under normoxia (see

the animal utilizes glycogen exclusively; the propionate–acetate pathway is the only anoxic process, with excretion of the acids; anoxic heat flux is 10% of normoxic heat flux, and the corresponding propionate and acetate production is maintained at a constant level at P_{O_2} from 0 to 0.5 kPa. In this region, the metabolism of the regulator and microxic regulator is identical, with a slope 10 times that of the perfect conformer (Figure 8.5*a*).

At 1% air saturation (0.2 kPa or 1.5 mmHg), 50% of the total heat flux is aerobic in the regulators. Even the conformer is 9% aerobic (Figure 5*b*). Few respiratory measurements have been made with animals at microxic conditions.

The calculated ATP turnover increases steeply in the regulators, reaching nearly 150% of the anoxic ATP turnover at 0.2 kPa (1% air saturation),



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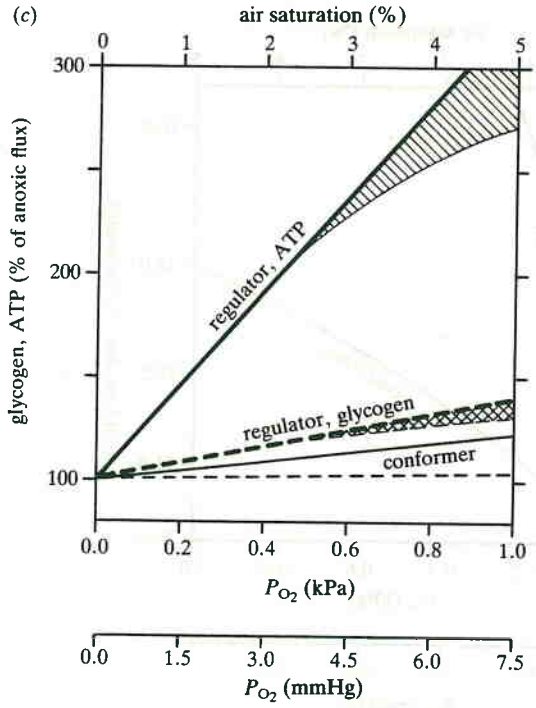
text). (*c*) Glycogen utilization (solid lines) and ATP turnover (broken lines) in the microxic range, normalized relative to the fluxes under anoxia. The hatched area indicates the range of deviation between the regulator (upper boundary line) and the microxic regulator (lower boundary line). Heat flux (*b*) is converted into the flux of glycogen utilization by division by the catabolic enthalpy per mol glycosyl-unit, $\Delta_k H_{Glyc}$,

while glycogen consumption is only increased to 108% of the anoxic demand (Figure 5*c*). This difference is due to the high ATP coupling stoichiometry of aerobic metabolism, 37 mol ATP per mol glycosyl-unit compared with 6.4 mol ATP per mol glycosyl-unit in the propionate–acetate pathway (Gnaiger, 1983*a*). Thus, microxic regulation is an effective mechanism to increase simultaneously the flux of ATP turnover and the ATP stoichiometry per unit of substrate (Figure 5*d*).

Various quantitative aspects of microxic regulation require modification for analysis of specific cases. (i) Glycogen is rarely the exclusive or even the predominant substrate of aerobic catabolism under normoxia (see, for example, Hawkins *et al.*, 1985). Information on the substrate of aerobic catabolism under hypoxic and microxic conditions is rarely available.



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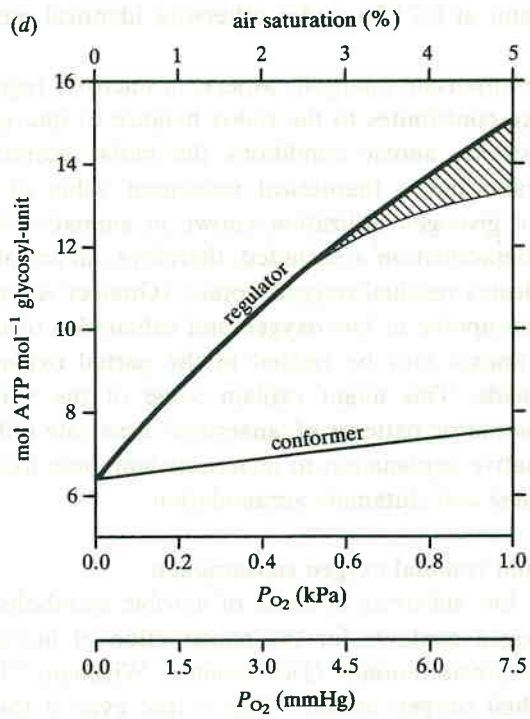
which is −2868 and −226 kJ mol^{-1} in aerobic oxidation and anaerobic propionate–acetate excretion, respectively (Gnaiger, 1983*a*). At an anoxic heat flux of 10% of the normoxic level, therefore, anoxic glycogen utilization (100% in *c*) is 10×−2868/−226=127% of the normoxic glycogen utilization. Heat flux is converted into the catabolic flux expressed as ATP-turnover by division by the catabolic enthalpy per mol ATP

Therefore, the small increase of glycogen utilization (Figure 5*c*) merely illustrates the demand for organic carbon. (ii) Accumulation and excretion of different anaerobic end-products occurs, depending on the P_{O_2}, temperature and time of exposure (Livingstone, 1978). Accordingly, the appropriate thermochemical constants have to be used, ranging from −160 to −260 kJ mol^{-1} glycosyl-unit, or −35 to −72 kJ mol^{-1} ATP turnover with glycogen as the substrate (Gnaiger, 1983*a*). (iii) Anaerobic metabolism is constant in the microxic range up to 2 kPa (15 mmHg) in *Arenicola marina* (Schöttler *et al.*, 1983). Even if aerobic ATP turnover is simply added to a constant anaerobic flux, microxic regulation is a highly significant strategy (Figure 5). Microxic regulation, however, is energetically even more



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turnover, $\Delta_k H_{\infty ATP}$, which is -77.5 and -35.3 kJ mol^{-1}, respectively. At an anoxic heat flux of 10% of the normoxic level, therefore, anoxic ATP turnover (100% in *c*) is $10\times-77.5/-35.3=22\%$ of the normoxic ATP turnover. (*d*) Stoichiometric coefficient of ATP production per unit glycogen utilization, increasing with an increasing aerobic contribution to total heat flux.

important if anaerobic catabolism is progressively compensated by increasing aerobic ATP turnover in the microxic P_{O_2} range.

Aerobic compensation is actually observed in the microxic regulator *Sipunculus nudus*, where anaerobic metabolism is reduced from a P_{O_2} of *ca.* 0 to 0.4 kPa (3 mmHg) (Pörtner *et al.*, 1985), and in the regulator *Lumbriculus variegatus*, where anaerobic ATP turnover at 0.2 kPa is *ca.* 60% of ATP turnover under anoxia (Putzer, 1985). The stoichiometric coefficient of ATP production increases from 6.4 to 9.6 mol ATP mol^{-1} glycosyl-unit with an increase in the P_{O_2} from 0 to 0.2 kPa in the 'additive' microxic regulator (Figure 5*d*), whereas aerobic compensation at the extent observed in *L. variegatus* increases the stoichiometry to 10.0 mol

ATP mol^{-1} glycosyl-unit at 0.2 kPa under otherwise identical metabolic conditions.

In addition to these important energetic aspects of microxic regulation, residual oxygen uptake contributes to the redox balance of intermediary metabolism. Under strictly anoxic conditions the molar acetate:(succinate+propionate) ratio has a theoretical maximum value of 0.5 in metabolic pathways of glycogen utilization known in animals (Gnaiger, 1983*a*). If microbial contamination is excluded, therefore, an acetate ratio of more than 0.5 indicates residual oxygen uptake (Gnaiger & Staudigl, 1987). Residual oxygen uptake at low oxygen and exhaustion of internal oxygen stores under anoxia may be related to the partial oxidation of protein and amino acids. This might explain some of the variability observed in the stoichiometric patterns of 'anaerobic' aspartate utilization and provide an alternative explanation to anaerobic ammonia fixation in the case of excess alanine and glutamate accumulation.

Calorimetry and residual oxygen consumption

Owing to the low substrate demand of aerobic metabolism, the measurement of glycogen contents for the construction of biochemical carbon balances is not precise enough (De Zwaan & Wijsman, 1976) for the detection of residual oxygen uptake. This is true even if the other potential aerobic substrates, protein and lipid, are not utilized. The problem arises owing to the interindividual variability of organic biomass and composition, which affects the estimation of biochemical change by the destructive whole-body analysis. Since calorimetry is a non-invasive method which monitors the enthalpy change of metabolic processes directly, a higher accuracy is obtained.

The caloric quotient of heat production to glycogen consumption is -2868 kJ mol^{-1} glycosyl-unit in aerobic dissipative catabolism, but only -226 kJ mol^{-1} when propionate and acetate are excreted (Gnaiger, 1983*a*). If only 1% of the total anaerobic glycogen consumption is due to residual oxygen uptake, then the calorimetric signal of heat flux increases by 12% relative to completely anoxic catabolism. Therefore, calorimetry is a particularly sensitive method for the detection of such residual oxygen uptake (Figure 6). The corresponding P_{O_2} may be as low as 0.02 kPa (0.15 mmHg or 0.1% air saturation) in a microxic regulator (Figure 5*b*); this P_{O_2} is below the limit of detection of conventional analytical methods (Figure 7).

Open-flow calorespirometry was applied by Widdows *et al.* (1989) to study the energetics of the oyster *Crassostrea virginica* at low oxygen and

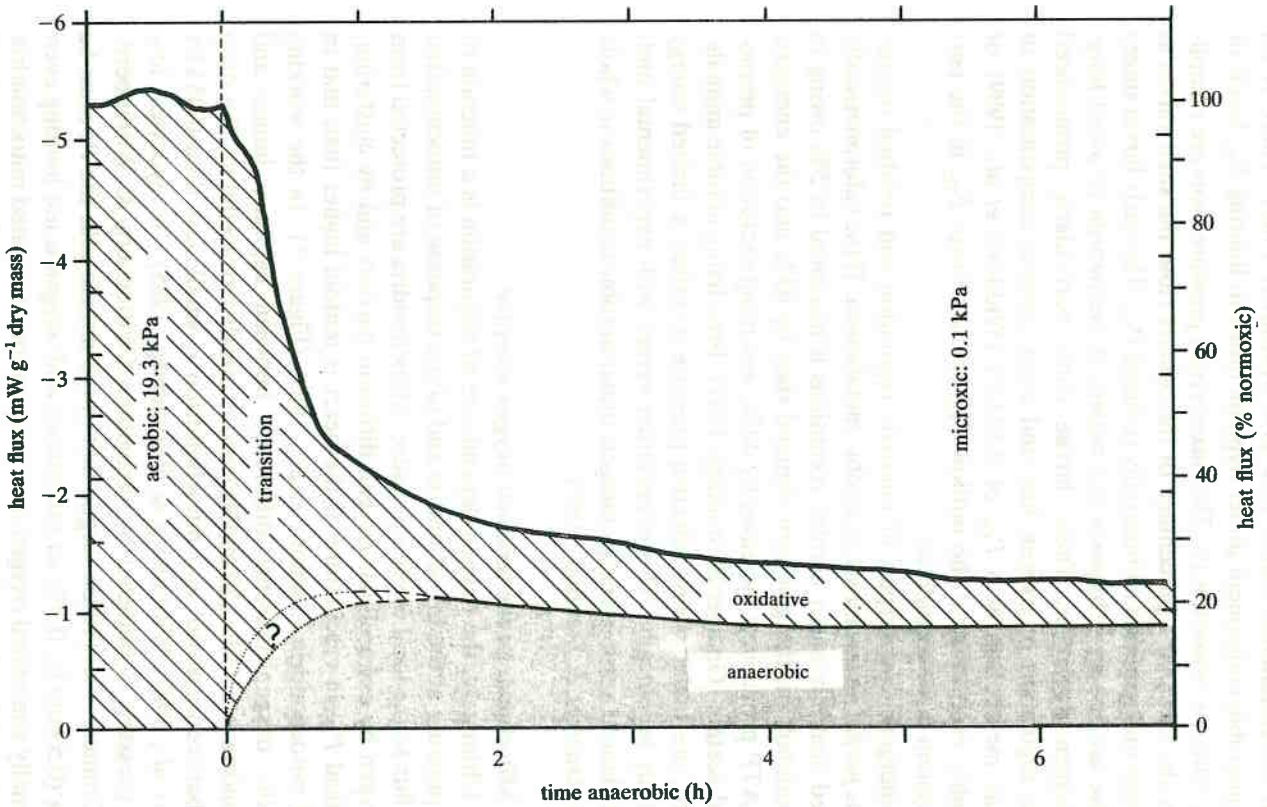

Figure 6. Heat flux of *Lumbriculus variegatus* at 20 °C under normoxic conditions (P_{O_2}=19.3 kPa, fully aerobic) and microxic conditions ('anaerobic', P_{O_2}=0.1 kPa, 0.75 mmHg, 0.5% air saturation), measured in a calorespirometer system. The low time resolution of the Twin-Flow respirometer during the aerobic–anaerobic transition does not allow for an accurate separation of aerobic and anoxic sources of heat during the transition period (? on graph). At 0.1 kPa a significant contribution to total heat flux is aerobic (oxidative, 30%) owing to residual oxygen consumption (modified after Gnaiger & Staudigl, 1987).

different developmental stages (see also Widdows, 1989). There is no major anaerobic component under hypoxia down to limiting P_{O_2} levels of 1.3 kPa, which is below the *P*c. The flux–oxygen pressure plots are complicated by the fact that a percentage of the oysters close the shell valves at low oxygen and generate an internally reduced P_{O_2}. The early larval stages are active under severe hypoxia and anoxia, a mechanism to avoid long-term oxygen depletion. These larvae show particularly pronounced microxic regulation of oxygen flux and some aerobic compensation of anaerobic metabolism at a P_{O_2} of 0.67 kPa (Widdows *et al.*, 1989) or presumably even lower if the outflow P_{O_2} or an average P_{O_2} in the perfusion system were considered.

The energetic counterpart of microxic regulation and residual oxygen uptake is *partial* anaerobiosis in aerobic metabolism. If the calorimetrically measured heat flux under aerobic conditions is increased by 5% owing to anoxic catabolism, the glycogen demand rises by 63% and the energetic cost of ATP production increases by 44%, assuming excretion of propionate and acetate (Gnaiger & Staudigl, 1987). Free-living aerobic animals, therefore, are under strong selective pressure to utilize a limited energy supply fully aerobically. This expectation agrees with experimental findings of balanced aerobic energy budgets under aerobic conditions in whole animals (Gnaiger & Staudigl, 1987).

Mitochondria: the terminal oxygen acceptor

Ultimately, the oxygen-dependence of respiration is a function of oxygen pressure at the mitochondria and of the response of mitochondrial oxygen flux to the local oxygen pressure. Mitochondria are protected from high oxygen by extracellular oxygen diffusion barriers and by clustering. The critical P_{O_2} in vasculature of the heart is tenfold higher than that in isolated mitochondria (Tamura *et al.*, 1989) (Figure 7). In the working heart, the oxygen pressure difference between capillary lumen and mitochondria is 2.7 kPa (20 mmHg) and is mostly extracellular. The drop in P_{O_2} between cystosol and mitochondrion is small, less than 0.03 kPa (Clark *et al.*, 1987; Wittenberg & Wittenberg, 1985). In myocytes, low oxygen pressure is buffered by myoglobin (Wittenberg & Wittenberg, 1985). Connett *et al.* (1985*a*) argue that in red muscle cells the *Pc* must be 0.07 kPa (0.5 mmHg; 0.3% air saturation) and oxygen is not limiting even at maximally stimulated oxygen flux. Respiration of isolated mitochondria is not affected by decreasing oxygen pressure until below 1 μmol dm^{-3} (0.01 to 0.1 kPa, depending on metabolic state) (Oshino *et al.*, 1974; Sugano *et al.*, 1974; Cole *et al.*, 1982).

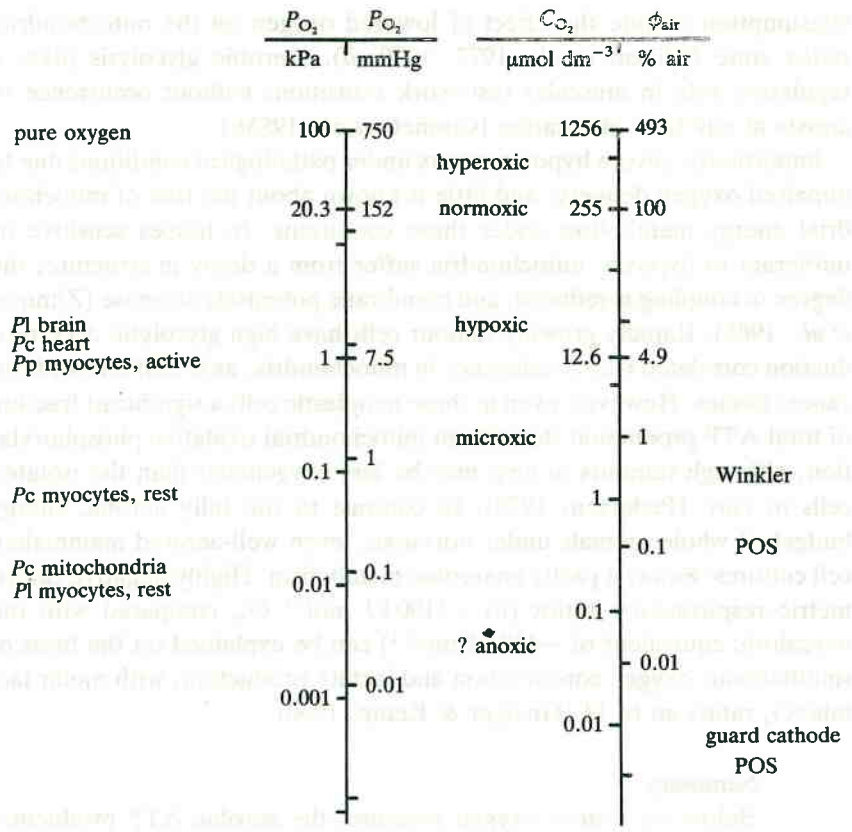

Figure 7. Logarithmic presentation of partial pressure of oxygen, P_{O_2} (1 kPa=0.133322 mmHg). The corresponding oxygen concentration, C_{O_2} (µmol dm^{-3}), is given for 25 °C in pure water. The volume–percentage air saturation values, ϕ_{air}, are for the standard barometric pressure of 100 kPa and corrected for a 3.2 kPa water vapour saturation pressure at 25 °C. Left: some critical and limiting partial oxygen pressure values, *P*c and *P*l, for mammalian systems (see text for references). Right: limit of detection of some routine methods (POS, polarographic oxygen sensor).

Owing to clustering of mitochondria in sites of high ATP demand, the oxygen dependencies of isolated liver mitochondria and mitochondria in intact hepatocytes are different (Jones, 1986). In the liver, glycolysis is known to play an important role in overall energy-yielding metabolism in the activated state of gluconeogenesis (Soboll *et al.*, 1978), possibly owing to local hypoxia (Baumgärtl & Lübbers, 1983). The respiratory flux in intact hypoxic cells is controlled at constant levels by adjustments of the phosphorylation potential, resulting in independence of cellular oxygen

consumption despite the effect of lowered oxygen on the mitochondrial redox state (Wilson *et al.*, 1977, 1979*a*,*b*). Aerobic glycolysis plays a regulatory role in muscular rest–work transitions without occurrence of anoxia at any time or location (Connett *et al.*, 1985*b*).

Importantly, severe hypoxia occurs under pathological conditions due to impaired oxygen delivery, and little is known about the role of mitochondrial energy metabolism under these conditions. In tissues sensitive or intolerant to hypoxia, mitochondria suffer from a decay in structure, the degree of coupling is reduced, and membrane potentials decrease (Zimmer *et al.*, 1985). Rapidly growing tumour cells have high glycolytic ATP production correlated with a deficiency in mitochondria, as is well known from cancer tissues. However, even in these neoplastic cells a significant fraction of total ATP production stems from mitochondrial oxidative phosphorylation, although tumours *in vivo* may be less oxygenated than the isolated cells *in vitro* (Pedersen, 1978). In contrast to the fully aerobic energy budget of whole animals under normoxia, even well-aerated mammalian cell cultures display a partly anaerobic metabolism. Highly negative calorimetric–respirometric ratios (to $-1100\,\text{kJ mol}^{-1}\ O_2$, compared with the oxycaloric equivalent of $-450\,\text{kJ mol}^{-1}$) can be explained on the basis of simultaneous oxygen consumption and lactate production, with molar lactate : O_2 ratios up to 14 (Gnaiger & Kemp, 1990).

Summary

Below the *critical* oxygen pressure, the aerobic ATP production decreases, and below the *limiting* oxygen pressure anaerobic processes compensate increasingly for the diminished aerobic flux. The respiration of cells, tissues, organs and whole organisms is more dependent on external p_{O_2} than that of isolated mitochondria. The capacity of organisms to utilize traces of oxygen at reduced metabolic flux must be studied with great experimental care, and calorespirometry provides a sensitive approach.

Microxic regulation enables a steep increase of oxygen flux with P_{O_2} at very low oxygen levels. This is a metabolic adaptation to environments in the boundary to anoxic conditions, offering an energetic advantage to active organisms.

The difficulties involved in defining an absolute limit between microxic and anoxic conditions are best illustrated by a logarithmic P_{O_2} scale (Figure 7). Ideally the term 'anoxic' (without oxygen) should be restricted to any situation where molecular oxygen is strictly absent. Absence of oxygen, however, has to be practically defined in terms of tested oxygen removal

techniques and relative to the sensitivity limit of analytical methods (Figure 7). Effective anoxia is obtained when a further decrease of P_{O_2} does not elicit any physiological or biochemical response.

The capacity for energy assimilation and growth under anoxia should be considered as the ultimate criterion for 'facultatively anaerobic' animals. Very little direct evidence exists documenting the persistence of animal populations under strict anoxia, except for members of the meiofauna in the sulphide system.

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