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# Physiological and Metabolic Responses to Hypoxia in Invertebrates

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## 1 Introduction

On Earth the transition from a mainly anoxic hydrosphere and atmosphere to oxic ones is thought to have occurred at about 2 billion years ago. Indicators of progressive oxygen accumulation are, for example, the disappearance of banded iron formations, no further deposition of the highly oxidizable uraninite, the occurrence of blue–green algae whose chains of tiny cells included thick-walled heterocysts, which may have shielded oxygen-sensitive nitrogenase enzymes in a similar way as in their modern representatives. By then the atmospheric oxygen content had probably reached about 1% of its present level (Cloud 1983; Jenkins 1991). It may have been sufficient for several prokaryotes to employ an oxygen-dependent energy metabolism which increased the energy yield considerably as compared to the original oxygen-independent energy requisition. The build-up in free oxygen and the increase in metabolic efficiency was certainly a principal driving force in evolution, leading to the rapid development of eukaryotes including the animal kingdom already present in a rich diversity during the Cambrian period.

Although present-day oxygen constitutes one fifth of the telluric atmosphere, oxygen may, for some animal species, nevertheless become scarce either owing to changes in the partial pressure of oxygen ($Po_2$) within particular habitats or as a result of an increased oxygen demand of the animal itself. It is therefore conceivable that some animal species still exploit primordial mechanisms to survive adverse hypoxic conditions. Using such specific adaptations, species should be able to continuously gain energy during hypoxia albeit at a reduced rate to maintain a morphological and physiological integrity to such an extent that all vital functions can be performed without any delay when sufficient oxygen is available again.

## 2 Environmental Versus Functional Anaerobiosis

In its original definition, hypoxia represents a state of reduced oxygen availability when the partial pressure of ambient oxygen ($Po_2$) has fallen

below normoxic values. It contrasts with normoxia which refers to a $Po_2$ normally prevailing at sea level, i.e., 150–169 torr.

When the ambient $Po_2$ is decreased below normoxic values most animals continue to gain energy aerobically using the respiratory chain with $O_2$ as electron acceptor and the mitochondrial ATP-synthase for the production of ATP, thus leaving ATP and $O_2$ fluxes constant. The metabolism occurring during normoxia and moderate hypoxia is referred to as aerobiosis. Under severe hypoxia, when the $Po_2$ in the environment becomes increasingly reduced, animals will not be able to transfer oxygen to tissues at a rate sufficient to meet the oxygen requirements of the mitochondria for continuous reoxidation of NADH. As a consequence organic compounds serve as electron acceptors. During severe hypoxia and anoxia many invertebrates gain energy from anaerobiosis, which is also termed "environmental anaerobiosis" because it is due to a limited supply of oxygen within the environment. The transition from moderate to severe hypoxia is certainly a critical situation because the energy gain from anaerobic metabolic pathways is drastically reduced as compared to oxygen-dependent energy provision.

Anaerobiosis in invertebrate animals can also be brought about by physiological activity, for instance, enhanced muscular exercise such as during flight and fight reactions. It requires a higher rate of ATP provision than can be obtained by aerobic ATP-synthesis alone. This mode of anaerobiosis results from a physiological function, thus it is termed "functional anaerobiosis." It is usually short term and restricted to exercising muscles. In contrast to environmental anaerobiosis, the afflicted tissues need not necessarily be in a state of acute hypoxia since the mitochondria may still have sufficient oxygen to generate ATP, but at too low a rate to meet the requirements of the contractile proteins.

Many animal species react with different adaptive mechanisms to both types of anaerobiosis. During functional anaerobiosis the energy demand increases abruptly at the onset of muscular activity and it quickly falls to preexercise levels at the end of enhanced activity. Although oxygen consumption rises at the very beginning of activity, it cannot match the energy requirements of the contractile proteins and, therefore, stored high-energy phosphate compounds and fermentative processes contribute ATP in addition to oxidative phosphorylation. After extensive but usually short-term exercise, recovery can rely on aerobic energy production, but in some species anaerobiosis may also continue until energy-rich phosphates are recharged again (Grieshaber 1978; Ellington 1983a).

When animals are exposed to hypoxia within their habitat, a great variety of reactions and mechanisms are exploited to cope with the

reduced oxygen availability. These are increased respiratory movements to augment oxygen uptake, circulatory changes, modulation of oxygen-carrying capacities of the respiratory pigments, a reduction of oxygen consumption accompanied by a conspicuous decrease of the overall energy expenditure far below the standard metabolic rate, and finally distinct fermentative pathways for substrate level ATP synthesis.

The different responses of invertebrate animals during functional and environmental anaerobiosis have elicited much scientific interest to unravel the systemic and biochemical mechanisms used to adapt and overcome these adverse conditions. A deluge of publications has appeared since this topic gained renewed interest about 25 years ago. Within the past decade, the anaerobic metabolism of free-living invertebrates has been reviewed several times (Gäde 1980a; Schöttler 1980; Ellington 1983a; Gäde 1983a; Livingstone 1982, 1983; Livingstone and de Zwaan 1983; de Zwaan 1983; Storey and Storey 1983; Hochachka 1986; de Zwaan and Putzer 1985; Gäde and Grieshaber 1986; Bryant 1991). Although work on anaerobiosis revealed many species-specific details, it also demonstrated a general theme of the energy metabolism in hypoxically living invertebrates. This review is therefore intended to give a more generalized account of a topic resulting from comparative investigations on "worms, mollusks, and crustaceans."

## 3 Occurrence and Extent of Oxygen Limitations in Different Habitats

Although the present-day telluric oxygen concentration is plentiful for most organisms to remain aerobic, various physicochemical and biological features can lead to fluctuations in oxygen concentrations in different habitats. Depending on the nature of the milieu, whether it is aerial or aquatic, a variety of factors such as temperature, stratification, viscosity, or restricted gas exchange can profoundly influence oxygen availability. In addition, biological factors including oppulent plant growth or the density of animal population can aggravate differences in gas tension, in particular in confined habitats. It is therefore conceivable that an animal species can encounter oxygen concentrations from normoxia to moderate hypoxia, and in some cases severe hypoxia and even anoxia (Boutilier 1990). Also, in some habitats hyperoxic conditions can develop with $Po_2$ values three times as high as during normoxia (Dejours 1975).

Assuming an effective mixing of oxygen between a distinct habitat and the atmosphere, the oxygen concentration ($Co_2$) is a function of the

oxygen capacitance $\beta o_2$ and the $Po_2$

$$Co_2 = \beta o_2 \cdot Po_2 \ (\mu mol \cdot l^{-1}). \quad (1)$$

The capacitance coefficient is defined as the increment of concentration per increment of partial pressure. The value of $\beta o_2$ depends on the nature of the milieu (e.g., chemical binding of a gas), on the temperature, and on the barometric pressure. In Table 1 a few values of $\beta o_2$ are shown, and their application for the calculation of oxygen concentrations may give a first hint of oxygen availability for an animal. The capacitance coefficient of distilled water is by a factor of 30.1 lower than that of air at 20°C. The capacitance of both media declines with increasing temperature. The proportional decrease is more pronounced in water than in air, and a rise in salinity of water lowers the oxygen capacitance as well (Dejours 1975).

In order to distribute oxygen from one region to another, either in the same medium or between different ones, convection currents and diffusion are of paramount importance. If, however, convection currents become low, as can happen in secluded aerial habitats or in small aquatic biotopes, the major driving force is diffusion. Following Fick's law, the amount of oxygen ($Mo_2$) diffusing at a certain time span through a specific wall of given thickness (E) and area (A) depends on the concentration difference of oxygen $\Delta Co_2$ between the two media.

$$Mo_2 = Do_2 \cdot \Delta Co_2 \cdot A \cdot E^{-1} \quad [nmol \cdot sec^{-1}] \quad (2)$$

The coefficient of diffusion $Do_2$ [$cm^2 \cdot sec^{-1}$] is equivalent to the quantity of oxygen diffusing per second through 1 $cm^2$ cross-sectional area under a unit of concentration difference of a gas. If biological membranes are separating the media, $Do_2$ is not sufficient to describe gas exchange. It has to be corrected for the capacitance $\beta o_2$ [$nmol \cdot ml^{-1} \cdot torr^{-1}$] which allows for specific characteristics of the membrane material or the medium. The product of $Do_2 \cdot \beta o_2$ is termed

**Table 1.** Capacitances ($\mu mol \cdot l^{-1} \cdot torr^{-1}$) of $O_2$ and $CO_2$ for distilled water, sea water, and air at different temperatures (from Dejours 1975)

| Temperature (°C) | Air ($\beta g$) | Distilled water ($\beta wO_2$) | Distilled water ($\beta wCO_2$) | Sea water ($\beta wO_2$) | Sea water ($\beta wCO_2$) |
|---|---|---|---|---|---|
| 5 | 57.68 | 2.52 | 84.17 | 2.03 | 70.79 |
| 10 | 56.66 | 2.23 | 70.57 | 1.83 | 60.00 |
| 15 | 55.68 | 2.01 | 60.23 | 1.67 | 51.58 |
| 20 | 54.73 | 1.82 | 51.89 | 1.54 | 44.74 |
| 25 | 53.81 | 1.66 | 44.86 | 1.43 | 39.01 |
| 30 | 52.92 | 1.53 | 39.30 | 1.32 | 34.47 |

Krogh's constant of diffusion ($Ko_2$, $nmol \cdot sec^{-1} \cdot cm^{-1} \cdot torr^{-1}$). As can be easily calculated from Table 1, the diffusion coefficients or the capacitances for oxygen are much higher in air than in water. As a consequence, hypoxic conditions occur more frequently in aquatic than in terrestrial habitats (Dejours 1975).

### *3.1 Terrestrial Habitats*

In the free atmosphere the gas composition is kept nearly constant owing to a continuous mixing by convection and diffusion (20.95% $O_2$, 0.03% $CO_2$, 78.09% $N_2$, and 0.93% Ar). The only aerial habitat which is characterized by permanent hypoxia is high altitude. With increasing altitude the atmospheric pressure becomes lower according to the equation

$$\log PB_h = \log PB_{NN} - h \cdot [72 \cdot (256.4 + t)]^{-1} \quad (3)$$

where $PB_{NN}$ is the barometric pressure at sea level (usually assuming 760 torr and 15°C) in torr, $PB_h$ is the barometric pressure at a certain height (h) in torr, h is the height in meters, and t is the mean temperature (°C) of a column of air of height h (Zuntz et al. 1906). This formula differs from the theoretical barometric equation with regard to the constants, but fits the pressures actually measured very well. It seems likely that the constants were chosen to make the formula fit conditions in mountains rather than in free air (Pugh 1957).

At extreme altitudes, like on Mount Everest where the $Po_2$ is approximately one quarter of that at sea level, only a few animal species can survive for brief periods. A specimen of the large vulture, Rueppel's griffon (*Gyps indicus*), collided with an air-plane at 11 280 m (Laybourne 1974) and the bar-headed goose (*Anser indicus*) has been repeatedly observed to fly from near sea level to elevations as high as 9200 m within a few hours (Black and Tenny 1980). Several animal species live permanently at lesser altitudes, but still high enough to experience hypoxia. Since almost all of the species investigated are vertebrates, their well-documented physiological adaptations will not be dealt with in this review, the reader is referred to the works of Bouverot (1985) and Jürgens (1989) instead.

### *3.2 Limnic Habitats*

Compared to air, low levels of oxygen are frequent in aquatic environments due to limiting physical parameters (slow diffusion and weak

convection currents) and to biological factors such as dense animal population and plant growth. The latter will aggravate hypoxia when plant respiration surpasses photosynthesis, e.g., at night. It is therefore not surprising that hypoxic conditions have been repeatedly reported from different limnic ecosystems as well as from marine habitats (Boutilier 1990).

The sources of oxygen in fresh water ecosystems are photosynthesis and gas exchange with the atmosphere at the surface. The latter is predominant in flowing waters when turbulence and cascades cause intensive mixing and keep oxygen concentrations close to saturation. In slow-moving rivers and creeks devoid of tumbling water, as well as in ditches crowded with aquatic plants, daily cycles of variations in $Po_2$ can occur. During intensive sun exposure photosynthetic $O_2$ production can exceed animal and plant respiration leading to $Po_2$ values up to approximately 500 torr. At night respiratory oxygen consumption can reduce $Po_2$ to almost zero (Jones 1961; Garey and Rahn 1970). These diurnal cycles of oxygen fluctuations can be aggravated by high temperature and introduced organic material, in particular by discharges of unproperly treated sewage.

Short-term measurements by Gameson and Griffith (1959) in the polluted River Hiz (England) showed typical diurnal fluctuations in oxygen concentrations which were smaller during winter than in summer. Repeated measurements during six consecutive months allowed the compilation of the frequency of maximal and minimal oxygen concentrations. The highest values were mainly reached between 12 and 6 P.M.

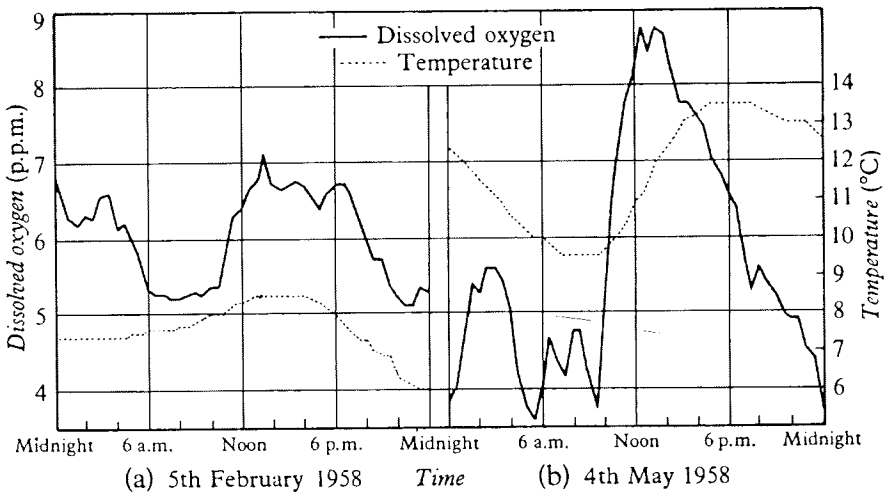



**Fig. 1.** Changes of oxygen concentrations ($ml \cdot l^{-1}$) and temperature in River Hiz on two different days. (From Gameson and Griffith 1959)

whereas the minima occurred from midnight to dawn (Fig. 1). From this example it can be concluded that in some flowing waters acute hypoxic conditions can occur regularly, but they are usually of limited duration. Modern treatment of sewage, however, considerably reduces the danger of hypoxia in rivers.

In standing waters, gas exchange with the atmosphere relies on wind-caused wave action and inflowing tributaries. Photosynthesis is a prominent source of oxygen in those parts of a lake which can be penetrated by light. Shallow lakes with clear water and low productivity are usually completely saturated with oxygen during the year. Oxygen concentration in near-saturating condition is more or less uniformly distributed in relation to depth. Most large lakes in temperate zones, however, are stratified in summer because the surface water becomes warmer and thus less dense than bottom water. It floats on the cooler, deeper water, sealing it off from the exchange with atmospheric oxygen. Decomposition of organic matter in the lower water body consumes most of the oxygen resulting in hypoxia. In stratified eutrophic lakes acute hypoxic conditions can usually be found for short periods above and within the sediment during summer time (Cole 1983; Schwörbel 1993).

Hypoxic conditions can also occur during winter months when a pond or lake becomes covered with ice and snow. Gas exchange with the atmosphere is inhibited, as is photosynthesis, because the snow blanket

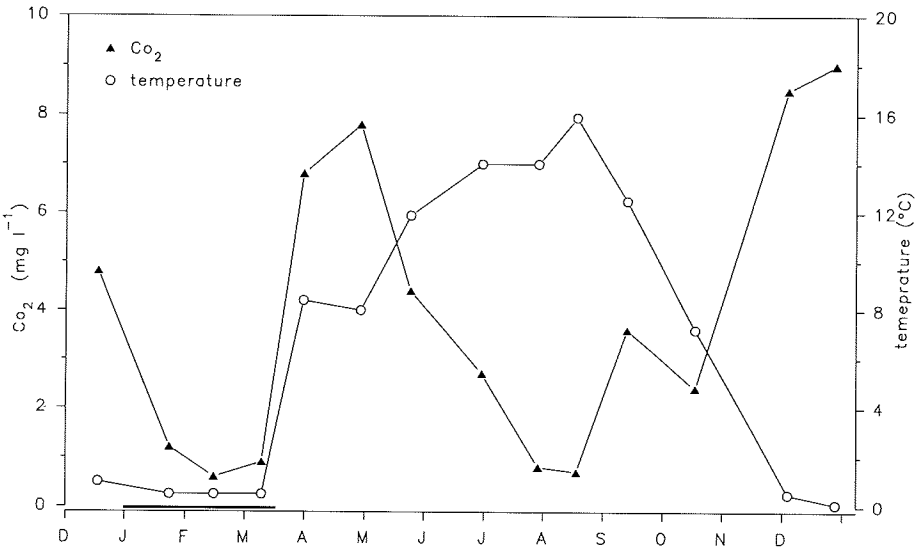



**Fig. 2.** Seasonal variation of oxygen contents $Co_2$ ($mg \cdot l^{-1}$) and temperature (°C) in a shallow pond. *Dark bar* depicts period of ice coverage of the pond (Seuss et al. 1984)

prevents light penetration. In such a sealed-off lake the water can become severely hypoxic both spatially and temporarily. Seuss et al. (1984) measured the oxygen concentration and temperature in a mesophilic pond throughout the year. The authors selected this habitat because it housed a dense population of *Tubifex*, a particularly hypoxia-resistant oligochaete. Two hypoxic periods were found: the first one developed under the ice cover and lasted for 10–11 weeks at a temperature of around 1°C, whereas the second hypoxic period occurred for approximately 2 weeks during summer stagnation at a temperature of around 16°C (Fig. 2). Thus, during the latter period, *Tubifex* has to cope with severe hypoxia at an elevated temperature.

In most lakes the different forms of stratification are abolished during autumn and spring storms which can overturn the water body now having a similar temperature and density in relation to depth. The water becomes aerated and normoxic conditions usually prevail for longer time periods than hypoxia (Schwörbel 1993).

### *3.3 Marine Habitats*

Although oxygen tensions in the ocean are usually in equilibrium with the atmosphere, naturally occurring hypoxic conditions can be found in certain areas. Hypoxic regions have been reported from upwelling systems as on the Peruvian shelf, where intensive biomass production and subsequent aerobic decomposition of the decaying and sinking material result in an oxygen consumption higher than the flushing rate within this area (Walsh 1975). In recent years conspicuous hypoxic events, which may have been caused at least in part by anthropogenic introduction of waste material, were reported from the coastal area of New York Bight (Falkowski et al. 1980), off the upper coast of Texas (Harper et al. 1981), in the German Bight of the North Sea (Dethlefsen and von Westernhagen, 1983), and in certain areas of the Baltic Sea (Ehrhardt and Wenk 1984; Gerlach 1984).

Particularly alarming oxygen deficiencies were observed in the bottom water of the German Bight and the waters off Jutland between 1981 and 1983 (Hickel et al. 1989). Although no detailed oxygen measurements had been performed before 1981, it can be assumed from the hydrographical structure of the German Bight that the bottom water was hypoxic for short periods during many summer periods. In this area stratification of the water body is common because the less saline coastal waters overlay the North Sea water leading to density stratification with a distinct halocline. In addition, thermal stratification can develop

during calm weather. Both factors may seal off bottom water from oxygen-rich surface water thus eliminating oxygen exchange. Benthic animals could be affected by these conditions.

Normally, many bottom-dwelling species must have resisted these natural conditions, since no mass mortalities had been reported by fishermen and other observers before 1981. However, the increasing discharge of nutrient rich water from the River Elbe added an additional load on the German Bight due to the fertilizing effect of organic material. Consequently, large plankton blooms developed from time to time. These organisms can drift to calmer areas and settle in deeper, less turbulent water ("marine snow"). The decomposing algae decrease the oxygen content which is already low in the deeper water body. The additive effects of thermal and saline stratification together with anthropogenic organic discharge may cause severely hypoxic conditions for prolonged periods, putting too much stress on many species of the macrobenthos. As a result, mass mortalities such as those reported from the German Bight between 1981 and 1983 can occur (Frey 1990; von Westernhagen et al. 1986).

Investigations on the species composition in the oxygen-deficient areas of the North Sea and the Kiel Bight of the Baltic Sea where extremely severe hypoxia also persisted in 1981 and 1983 demonstrated that bottom-dwelling animals react differently to a long-lasting lack of oxygen. Crustaceans and echinoderms which are usually sensitive to prolonged periods of hypoxia were reduced in numbers. In particular, the echinoderm *Echinocardium cordatum* nearly disappeared from these North Sea areas in 1983. The polychaete species *Lanice conchilega*, *Chaetozone setosa*, and *Spiophanes bombyx* were also less abundant than in previous years. Several mollusk and polychaete species, however, survived with very limited changes in population size (Hickel et al. 1989). In severely hypoxic area, Kröncke (1985) found a dominating abundance of the mollusks *Tellina fabula* and *Venus striata*, the polychaete *Magelona*, as well as the genera *Phoronis* and *Edwardsia*. In some parts of the Kiel Bight, Weigelt and Rumohr (1986) reported that all benthic species except three clams (*Arctica islandica*, *Astarte spp.*, *Corbula gibba*) and the priapulid *Halicryptus spinulosus* were extinguished in 1981. The surviving animals are of interest since they must possess systemic and cellular defense mechanisms allowing them to resist severe hypoxia within their environment for prolonged periods.

Rock pools of the intertidal zone represent another habitat where low oxygen concentrations or low $Po_2$ values are frequently found. On rocky shores the receding tide leaves pools which are usually densely populated with algae and animals. When such a rock pool becomes exposed during

a sunny day, plant photosynthesis produces partial pressures of oxygen within the water which can be three times as high as normoxic values. Exposure at night, on the other hand, leads to severe hypoxia since animal and plant respiration consumes the oxygen of the water now isolated from the main water body (Fig. 3). Thus, animals which live in these ponds quite often experience hyperoxic and hypoxic conditions (Truchot and Duhamel-Jouve 1980; Morris and Taylor 1983).

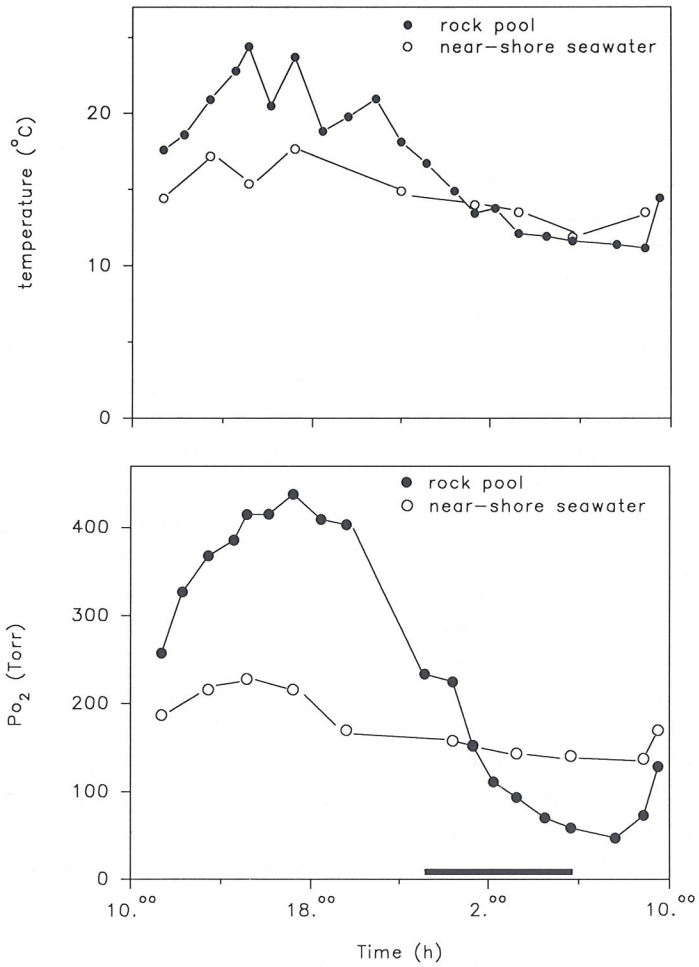



**Fig. 3.** Recordings of oxygen partial pressure ($Po_2$ in torr; *lower tracing*) and temperature (°C; *upper tracing*) in a rock pool (*filled circles*) and in near-shore seawater (*open circles*) during 24-h period in July 1982. The *dark bar* depicts the emersion of the rock pool at night. (Modified after Morris and Taylor 1983)

The availability of oxygen in coastal sediments, in particular in those of the intertidal zone, can vary considerably. There is hardly an overabundance of oxygen since the diffusion coefficient of oxygen in water is small and the increased diffusion distance in interstitial water as well as a high biological and chemical oxygen demand contribute to keep $Po_2$ low. In intertidal habitats a two-layered sediment structure can often be recognized. An upper brightly-colored surface stratum is followed by a dark, usually sulfidic smelling sediment (Fig. 13). For many years it was thought that the upper part of the sediment contained an oxygen gradient declining steeply to zero towards the beginning of the black layer. This presumable reduction in oxygen content seemed to be accompanied by a transition of the redox potential from positive to negative values, and was therefore called the redox potential discontinuity (RPD) (for review see Watling 1991).

The recent development of microelectrodes for oxygen (Revsbech and Jørgensen 1986), however, changed the view of the physico-chemical situation of fine sandy and muddy sediments. Most electrode measurements reveal an oxygen-containing layer only in the upper few millimeters of the sediment (Jørgensen 1988). The redox potential of around +300 mV coincides with the normoxic oxygen content of the interstitium. It decreases abruptly when the sediment becomes anoxic, but remains constant at albeit still positive values for several centimeters of the sediment. The redox potential becomes negative only in much deeper layers, conspicuously separated by an anoxic transition zone from the oxygen-containing sediment (Fig. 13). Thus redox potential measurements do not reflect the actual oxygen availability (Watling 1991). With the exception of coarse sandy habitats, most sediments are anoxic a few millimeters below the surface.

Early investigations by Brafield (1964), who measured the oxygen content of interstitial water in sandy shores, showed a correlation between the oxygen level and the amount of fine sand (0.25 mm diameter). If the percentage of fine sand is more than 15%, a $Po_2$ of less than 10 torr will be found during low tide. Predominantly coarse sand allows for drainage of water as the tide recedes and the interstices will become air filled. Interstitial air saturation is even more pronounced when the beach has a greater slope and consequently better draining. During low tide, oxygen from the air can penetrate the substratum at least down to 20 cm, and normoxia can persist as long as the tide is out. If the beach is immersed again, interstitial oxygen will be used and, since it cannot be replenished from air, hypoxic conditions can occur within less than 1 h (Gordon 1960). Oxygen carried by the water will quickly be extracted by highly abundant microorganisms.

In the sublittoral and continental slope at 3000–4000 m water depth, or in deep sea sediments of the tropical Atlantic and the North Pacific at more than 4000 m depth, oxygen can penetrate several centimeters into the sediment because in those sediments the content of organic material and the biological oxygen demand are extremely low (Reimers et al. 1986).

In the field, the sediment layers are not static, but become disturbed by biotic and abiotic activities. Many invertebrate species such as the peanut worm *Sipunculus nudus*, the lugworm *Arenicola marina*, the fat innkeeper *Urechis caupo* or the clams *Astarte borealis*, *Scrobicularia plana*, *Mya arenaria*, as well as the crustaceans *Callianassa californiensis*, *Upogebia pugettensis* burrow in intertidal sediments. When immersed, most of the species ventilate normoxic sea water and remain aerobic. At low tide, some species, particularly those which inhabit sandy mud areas such as the lugworm, cease to ventilate, become inert and completely asphyxic (Toulmond 1973). Measurements of concentrations of dissolved oxygen in water samples from burrows of lugworms exposed for 5 h showed 0.5 $ml \cdot l^{-1}$ $O_2$ corresponding to a $Po_2$ of 13.4 torr (Jones 1955) and in the exposed burrow of the fat innkeeper Arp et al. (1992) measured a partial pressure of oxygen between 25 and 91 torr (or 1.64 to 5.41 $ml \cdot l^{-1}$), i.e., moderately hypoxic values.

Not only mud-burrowing species can experience severe hypoxia during low tide, but several invertebrates such as the sea mussel *Mytilus edulis* or the crustacean *Balanus balanoides*, which live on the substratum of the intertidal zone, can also become anaerobic, because they close their shells tightly when emersed to avoid desiccation.

## 4 The Transition from Normoxia to Hypoxia

In contrast to the rapid changes in the sediments of the intertidal zone, the $Po_2$ of most habitats prone to hypoxia is reduced gradually and it may take days or even weeks before anoxic conditions occur. In these habitats animals do not experience an instantaneous and complete lack of oxygen, they will rather be exposed to gradually declining partial pressures of oxygen instead. It is, therefore, appropriate to consider first the physiological mechanisms which allow animals to stay aerobic in the face of a slowly declining $Po_2$.

Several mobile animals will move from hypoxic regions to sites of higher partial pressures of oxygen. Given the choice, *Corophium arenarium* selects well-aerated water (Gamble 1971), the freshwater snails

*Planorbis corneus* and *Lymnea stagnalis* float on the surface of hypoxic ponds and increase their reliance on aerial respiration (Jones 1961), whereas the shore crab *Carcinus maenas* (Taylor et al. 1973, 1977) or the crayfish *Austropotamobius pallipes* (Taylor and Wheatly 1980, 1981) will try to leave the hypoxic water and breathe air. Many species, however, cannot escape these adverse conditions and must adapt to hypoxia.

### *4.1 Ventilatory Responses*

During moderate hypoxia many invertebrates first use systemic mechanisms to supply enough oxygen to the tissues ensuring a constant rate of oxygen consumption. Almost all invertebrates that are water breathers regulate their ventilatory activity in response to changes of the oxygen partial pressure of the inspired medium. Among tube- or sediment-dwelling sipunculids and annelids the ventilation of respiratory surfaces, either containing gills or not, is achieved by intermittent peristaltic contractions of the circular musculature running along the worm-like piston or by undulatory movements of the body. As early as in 1938 van Dam observed an eightfold increase in the volume of water pumped when *Arenicola marina* was exposed to low oxygen. Studies by Toulmond and Tchernigovtzeff (1984) demonstrated the complexity of the mean ventilatory water flow in relation to ambient $Po_2$. Ventilatory activity is low during hyperoxia and increases linearly towards normoxic values. Between ambient $Po_2$ of 150 and 100 torr, the rate of water ventilation rose steeply from 7 to 12 $ml \cdot h^{-1} \cdot g^{-1}$ fresh weight and dropped to zero at approximately 20 torr.

Ventilatory responses to reduced oxygen levels in mollusks demonstrate that some species such as *Arctica islandica* or *Mytilus perna* rely on a continuous increase in their ventilatory rate over a wide range of hypoxic oxygen concentrations. *Mytilus edulis*, *Mya arenaria*, or *Rangia cuneata* enhance their ventilation slightly at the beginning of moderate hypoxia and then gradually reduce it as the $Po_2$ declines towards a critical oxygen tension, the so-called $P_c$ (Bayne 1971; Mangum and Burnett 1975; Taylor and Brand 1975). At this $P_c$ all physiological parameters, including the rate of oxygen consumption, decline over-proportionally in both types of animals. Conversely, anaerobic energy provision commences (Pörtner et al. 1985; Pörtner and Grieshaber 1993).

Air- and water-breathing crustaceans are widely studied with respect to physiological adaptations. Their respiratory responses to varying oxygen tensions of inspired water have been reviewed by McMahon and

Wilkens (1975), Taylor (1982), and McMahon (1988). Animals kept undisturbed in normoxic water ventilate the gills at moderate rates and often show ventilatory pauses during which one or both scaphognathites stop beating. This pausing behavior may save energy by drawing up oxygen from stores built up during ventilation periods (McMahon and Wilkens 1975; Butler et al. 1978; Burnett and Bridges 1981). During the beginning of moderate hypoxia ventilation remains the same as before, but without apnoea. As the level of moderate hypoxia deepens ($Po_2 \leq 120$ torr) respiratory rates increase steadily with falling oxygen tensions. Crayfish, shore crabs, or shrimps, for example, are able to increase ventilation by as much as 2.5 to three or even five times the resting level at normoxia (McMahon et al. 1974; A.C. Taylor 1976; E.W. Taylor et al. 1977; Wheatly and Taylor 1981; Morris and Taylor 1985), whereas McMahon and Wilkens (1975) and Butler et al. (1978) recorded an increase of 1.2–1.4 times in lobsters. Enhanced water currents flowing over the gills can be achieved by increases of the frequency of the scaphognathite beat, its stroke volume and force (Dejours and Beekenkamp 1977). Chronic exposure to moderate hypoxia of the crayfish *Orconectes virilis* also resulted in an increased efficiency of the branchial pumping mechanism. After 7–8 days, branchial water flow was still twice as high as in normoxic controls, but the rate of scaphognathite beating was only 20% above that of controls (McMahon et al. 1974). From these data it can be concluded that an increase in ventilatory activity is a common respiratory response in many species of crustaceans under moderately hypoxic conditions.

At oxygen pressures usually below 30–60 torr, ventilatory compensation cannot keep up with oxygen requirements and it has been reported for several crustaceans that they reduce the level of branchial ventilation (McMahon and Wilkens 1975; McMahon et al. 1974; Wheatly and Taylor 1981; Morris and Taylor 1985). This limit is probably due to a progressive increase in the aerobic energy demand of the respiratory pump. At a certain level of acute hypoxia the amount of respired oxygen will not even be sufficient to meet the energy requirements of the enhanced muscular work necessary for the increased stroke volume and frequency of the scaphognathites. The critical $Po_2$ is reached and anaerobiosis sets in.

### *4.2 Circulatory Adaptations*

The amount of oxygen made available during enhanced ventilatory activity may also depend on circulation. An increase in cardiac output by

chronotropic (frequency) or inotropic effects (stroke volume) could result in an enhanced blood flow and gill perfusion. The rate of $O_2$ uptake at the gills would be enhanced and more oxygen could be extracted from the inspired water. Thereby a faster oxygen transport to the tissues could augment oxygen consumption. In many invertebrate species, the circulatory system, however, has not evolved to a level elaborate enough to allow for an inotropic regulation of blood flow.

The gills of *Arenicola marina* shrink and swell rhythmically with the peristaltic waves of the body wall, injecting oxygenated blood into its efferent vessel, then sucking venous blood from the afferent one (Milne-Edwards 1838). Toulmond (1975) computed the blood flow through the gills in the confined lugworm. Although the ventilatory rate is enhanced between an ambient $Po_2$ of 150 and 120 torr, blood flow remained relatively constant at 14 $ml \cdot h^{-1}$ in a 10-g animal. When the partial pressure of oxygen fell from 120 to 90 torr, blood flow was even found to decrease to 3 $ml \cdot h^{-1}$. Below 80 torr blood flow decreased further but more slowly until it reached 0.45 $ml \cdot h^{-1}$ at an ambient $Po_2$ of 20 torr. In *Nereis virens* and *Nereis succinea* the blood pulse rates in the dorsal vessel were visually observed. From incubations at two hypoxic oxygen tensions the authors conclude that no regulatory compensation occurs during hypoxia (DeFur and Mangum 1979). Thus, at least in the few polychaete species investigated, there is no increased blood flow to augment oxygen supply to the tissues. They mainly rely on an enhanced ventilation to extract as much oxygen as possible from the surrounding medium.

Although there has been increasing interest in the "simple" circulatory systems of mollusks during the last 10 years, direct measurements of blood flow or cardiac output in gastropods and lamellibranchs during hypoxia are scarce (Jones 1983; Smith 1990). The comparison of available studies shows a certain regime of adaptations with constant or slightly increased heart rates at moderate hypoxia and a bradycardiac response at low oxygen tensions. Exposure of *Aplysia californica* to stepwise decreases in $Po_2$ resulted in an initial increase in the heart rate of 5% to 20%, but a severe bradycardia occurred at $Po_2$ values below 60 torr (Dieringer et al. 1978). A constant heart rate was also found in the gastropod *Busycon canaliculatum* at oxygen tensions close to normoxia changing to a progressive bradycardia below a $Po_2$ of 100 torr. The surf clam *Spisula solidissima* seems to be exceptional, since a constant heart rate was measured over the whole range of hypoxia (DeFur and Mangum 1979). A gradual increase in heart rates was monitored in *Arctica islandica* beginning as soon as the oxygen tension of the water was reduced below 90 torr. At a critical oxygen tension of

15–30 torr a distinct bradycardia, but no cardiac arrest was observed (Taylor and Brand 1975). Bayne (1971) subjected the sea mussel *Mytilus edulis* to stepwise decreases in $Po_2$ levels and found a constant heart rate but no tachycardia at the beginning of moderate hypoxia. As the $Po_2$ of the water was further lowered to 60 to 40 torr, there was a slight rate increase followed by a steep decline and finally by cardiac arrest at 20 torr. When *Mytilus edulis* was exposed to air, which stimulates the natural conditions of emersion during low tide better than immersion in hypoxic water, because the animal closes its valves, the heart rate dropped from approximately 25 $min^{-1}$ to 10 $min^{-1}$. In the ribbed mussel *Modiolus demissus* the heart rate remained constant between normoxia and 80 torr, then increased slightly before a severe bradycardia developed at lower hypoxic levels (Booth and Mangum 1978). The heart rate of *Mytilus edulis* exposed within its habitat fell rapidly to below 10 $min^{-1}$ and remained constant throughout exposure during low tide. As is common in field studies, wider fluctuations were measured in some animals, but prolonged periods of air exposure led to an even more extensive suppression of the heart beat and no tachycardia was reported (Helm and Trueman 1967; Coleman 1973). Field studies of the cockle *Cardium edule* also showed a slight bradycardia becoming more pronounced at the end of exposure (Trueman 1967).

From these studies, it may be concluded that in some mollusks the heart rate remains constant, while in others a slight tachycardia compensates for reduced $O_2$ content of the hemolymph during moderate hypoxia. At lower levels of moderate hypoxia or below the $P_c$, bradycardia is pronounced. It is, however, entirely possible that during moderate hypoxia some gastropods and lamellibranchs react similarly to cephalopods (Smith 1990; DeFur and Mangum 1979) and adjust their cardiac output mainly by stroke volume and not by rate changes. Unfortunately detailed studies on stroke volume in snails and mussels are not yet available.

During exposure to moderate hypoxia, crustaceans adjust their hemolymph flow according to the $Po_2$ of the inspired water and try to maintain $O_2$ delivery to the tissues (McMahon and Burnett 1990). In contrast to the ventilatory response of the scaphognathite beat, an increase in heart rate as demonstrated in the spider crab *Libinia emarginata* (DeFur and Mangum 1979) is rarely observed during hypoxic exposure. The more general pattern shows a stable heart rate at $Po_2$ levels above the $P_c$. The cardiac response rate of *Carcinus maenas*, which was examined by several investigators (E. W. Taylor et al. 1973; A.C. Taylor 1976; E. W. Taylor et al. 1977), remained constant at moderate hypoxia and it only became significantly lower at a $Po_2$ range of

40–50 torr. A similar pattern of changes in heart rate was found in lobsters (McMahon and Wilkens 1975; Butler et al. 1978). In the crayfish *Austropotamobius pallipes* (Fig. 4) a heart rate of $63 \pm 3$ beats·min$^{-1}$ was recorded during normoxia, followed by a slight decrease at the beginning of moderate hypoxia. At a $Po_2$ of 70 torr a progressive bradycardia

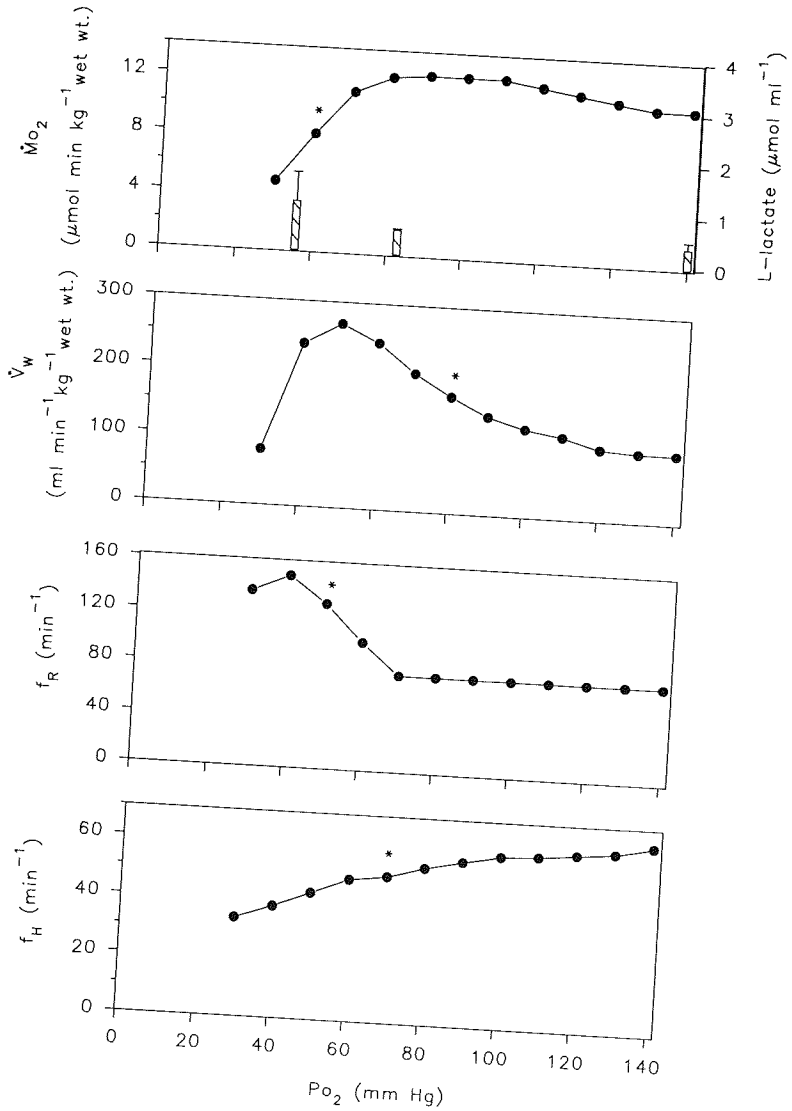



**Fig. 4.** Changes in the rate of oxygen consumption ($\dot{M}o_2$, μmol·min$^{-1}$·kg$^{-1}$ wet weight), water flow over the gills ($V_W$, ml·min$^{-1}$·kg$^{-1}$ wet weight), scaphognathite movement ($f_R$, min$^{-1}$), heart rate ($f_H$, min$^{-1}$), and lactate concentrations (*hatched bars* in the *top panel*; μmol·ml$^{-1}$) in the blood of the crayfish *Austropotamobius pallipes* during progressively decreasing ambient oxygen partial pressure ($Po_2$, torr). (Modified from Wheatly and Taylor 1981)

developed and at 30 torr the heart rate was reduced to half of its initial value (Wheatly and Taylor 1981). When exposed to air for 24 h, this crayfish showed transient increases in heart rate and scaphognathite frequency which recovered within 1 h and remained at levels characteristic of animals in normoxic water. Despite these respiratory responses the animals became hypoxic during the early phase of emersion but recovered later on (Taylor and Wheatly 1981).

Data on cardiac output which were obtained in crustaceans using either Fick estimates together with heart rate (DeFur and Mangum 1979; Burnett 1979), or the elegant thermodilution technique (Burnett et al. 1981) demonstrate that in *Libinia emarginata* both heart rate and stroke volume contribute to enhanced blood flow. Jørgensen et al. (1982, cited in detail by McMahon 1988) measured a twofold increase of cardiac stroke volume in the Dungeness crab *Cancer magister* when the ambient $Po_2$ was lowered to 100 torr. A marked reduction of cardiac output but no cardiac arrest occurred below a $Po_2$ of 60 torr. Measurements of cardiac output in *Homarus americanus* also indicated that a decrease in ambient oxygen may be associated with a five- to tenfold increase of the stroke volume (Bourne and McMahon 1989). A.C. Taylor (1976) estimated a cardiac output of 0.07–0.09 $ml \cdot g^{-1}$ body weight in normoxic *Carcinus maenas* again using the Fick principle. As the $Po_2$ was gradually lowered, cardiac output was continuously reduced. At 30 torr it amounted to about 25% of the level of normoxic control animals. In contrast to other studies, the cardiac output never increased during moderate hypoxia. Although the amount of data is still somewhat limited, it is safe to conclude that cardiac stroke volume is as important as heart rate for an increased blood flow in many crustaceans.

### *4.3 Oxyregulator Versus Oxyconformer*

In an attempt to compile systematically the numerous data on the different physiological responses of animals to declining oxygen tensions, Prosser and Brown (1961) distinguished two types of organisms with regard to their rate of oxygen uptake: (a) animals that keep their oxygen consumption independent within a certain range of oxygen tensions are referred to as "oxygen regulators"; and (b) animals that reduce their oxygen uptake in a nearly linear function when the ambient $Po_2$ is lowered are categorized as "oxygen conformers." Below a certain ambient $Po_2$, oxygen consumption rates start to fall rapidly in both types of animals. This $Po_2$ is traditionally called the critical oxygen tension ($P_c$; Fig. 5).

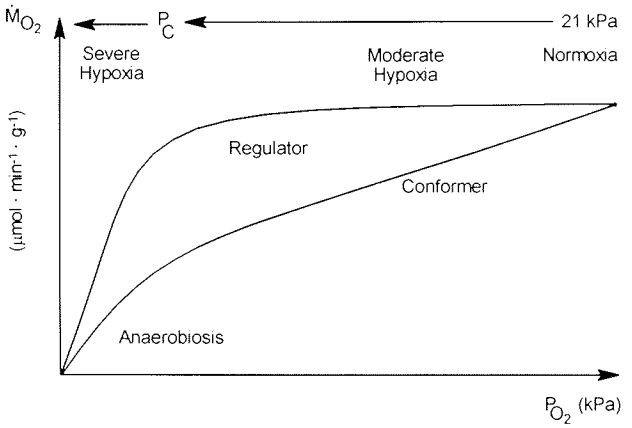



**Fig. 5.** General scheme of oxygen consumption ($\dot{M}o_2$, $\mu$mol·min$^{-1}$·g$^{-1}$) at different ambient partial pressure of oxygen ($Po_2$, kPa). Moderate hypoxia is compensated for by physiological mechanisms, whereas anaerobiosis commences during severe hypoxia and anoxia below the critical $Po_2$ ($P_c$) (Grieshaber et al. 1992).

The clear distinction between the two modes of oxygen consumption at declining $Po_2$ has been questioned. The mode of oxygen consumption may vary, depending upon the physiological state of the individual, the prevailing temperature or salinity, upon activity, or even the experimental conditions. Mangum and van Winkle (1973) tried to explain different responses on a mathematical basis using a polynomial model to predict a species oxygen consumption at various $Po_2$ (van Winkle and Mangum 1975). Later, Herreid (1980) proposed that the responses of both oxyregulators and oxyconformers should be incorporated into a general model which assumed that all animals do exhibit a $P_c$ (even though this may be highly variable) below which the resting $\dot{M}o_2$ starts to decline indicating the transition to oxyconformity.

Available studies reviewed by Pörtner and Grieshaber (1993) suggest that the transition to oxyconformity in oxyregulators is mainly linked to the onset of anaerobic processes. In aerobic oxyconformers like in *Arenicola marina or Sipunculus nudus* a critical $Po_2$ can be elaborated, too. It is characterized by the onset of an anaerobic metabolism and the lowest possible aerobic metabolic rate, the standard metabolic rate.

As a corollary the ambient $Po_2$ or $P_c$ at which the pronounced decrease in $\dot{M}o_2$ and the transition to anaerobiosis occur reflects an important set point of respiratory and metabolic regulation in many species. It might therefore be useful to relate the abiotic environmental factor "oxygen tension" to the $P_c$. The term "moderate hypoxia" should be assigned to $Po_2$ values between normoxia and the $P_c$, and oxygen

tensions below the $P_c$ should be referred to as "severe hypoxia." Complete lack of oxygen is termed anoxia and oxygen tensions above normoxia reflect hyperoxia. Only normoxia (160 to 150 torr) refers to the oxygen tension close to sea level (Fig. 5).

## 5 Energy Metabolism During Environmental Anaerobiosis

### *5.1 Historical Background of Anaerobiosis*

A striking feature of many invertebrates, in particular those which can survive prolonged periods of severe hypoxia or even anoxia, is the exploitation of different anaerobic energy yielding biochemical pathways. As early as at the turn of the century the classical investigations of Bunge (1890) and Weinland (1901) revealed the formation of short chain fatty acids in some parasites which are obligatory anaerobes. From then on the anaerobic energy metabolism of various parasites has been elaborated in much detail and has been summarized in several reviews (Fairbairn 1970; Saz 1981; Ward 1982; Barrett 1984; Köhler 1988, 1991).

Half a century later, von Brand et al. (1950) studied the anaerobic metabolism of aquatic snails (Planorbidea, Lymnaeidae) known to be intermediate hosts of various parasites. In some anaerobic species these authors found a marked accumulation of lactic acid, the well-known end product of anaerobic glycolysis. In others, however, the formation of lactate could account for only a small fraction of the consumed glycogen. Therefore, it was proposed that anaerobic end products other than lactate must occur in these species. This was consequently confirmed by Mehlmann and von Brand (1951) who demonstrated the anaerobic synthesis of acetate and propionate in the fresh water snail *Australorbis glabratus*.

Several investigators had failed to demonstrate the accumulation of lactate in some other invertebrates. Glaister and Kerly (1936) tried unsuccessfully to stimulate lactate formation in the foot retractor of the sea mussel *Mytilus edulis*. Although they found a high glycogen content, anaerobic lactate formation was small when compared to other animals. Dugal and Fortier (1941) found no anaerobic lactate production in oysters, nor did Wernstedt (1944) in *Dreissenia*. In the lugworm *Arenicola marina*, a lack of lactate synthesis has been reported by several authors (Dales 1958; Zebe 1975; Surholt 1980), but one of these authors had already assumed "that in *Arenicola marina* metabolism of glycogen under anaerobic conditions leads to products other than lactic acid" (Dales

1958). Obviously other anaerobic pathways besides the lactate-yielding anaerobic glycolysis must operate in some free-living invertebrates.

The suggestion by von Brand in 1946 that in some free-living aquatic mollusks anaerobic pathways similar to those of helminthic parasites could be operative gained little interest during the following years. It was not until 1968 when Stokes and Awapara reported succinate and not lactate as a major end product of the anaerobic glucose breakdown in the mantle of the clam *Rangia cuneata*, and until 1969, when Hammen described the rapid incorporation of $^{14}C$-labeled bicarbonate into succinate of mantle tissue of the American oyster (*Crassostrea virginica*) incubated under hypoxic conditions, that the anaerobic energy metabolism in marine invertebrates was reinvestigated. The latter author also concluded that succinate originated from the anaerobic reduction of fumarate to succinate in a very similar way as had already been demonstrated for *Ascaris* in 1962 by Bueding. Only 1 year later, Coles (1970) reported the formation of acetate and propionate in hypoxic *Alma emini*, an oligochaete from the tropical swamps of central Africa. From that time, the investigation of the anaerobic energy metabolism in many invertebrates was taken up by several groups, and their work very much confirmed the early speculations by von Brand (1946). Meanwhile, details of the anaerobic energy metabolism have been elucidated in all major phyla of invertebrates and even in some insect species (Zebe 1991) which are usually considered as completely oxygen dependent.

### *5.2 Anaerobic Metabolism During Severe Hypoxia*

Since many invertebrates possess several respiratory and circulatory mechanisms to remain aerobic during moderate hypoxia, early investigations employed extreme experimental conditions to elicit a full anaerobic metabolism. Animals were usually incubated under a nitrogen atmosphere or in nitrogen-saturated water in order to induce environmental anaerobiosis. As a result, exposure to anoxia which lasted for hours or days started abruptly. In more recent studies, graded levels of hypoxia were imposed on the experimental animals in order to determine the ambient oxygen tension ($P_c$) below which anaerobiosis commences (Pörtner et al. 1985; Pörtner and Grieshaber 1993), and the results obtained in the laboratory were compared with field observations on animals naturally exposed to hypoxia within their habitat. These comparative investigations on many different species finally led to a general scheme of the anaerobic metabolism, even though several interesting modifications between species have been found. Animal diversity is indeed reflected on the level of such a basic aspect as anaerobic energy metabolism.

The anaerobic mode of living is now particularly well understood in the sea mussel *Mytilus edulis* mainly investigated by Albertus de Zwaan (Utrecht), and in *Arenicola marina* as well as in *Sipunculus nudus*, the two species of interest of Ernst Zebe (Münster) and his former group. Based on their data and those from comparative studies, a general account of the biochemical adaptations to environmental and functional anaerobiosis can now be given.

### *5.2.1 Substrates for Energy Provision During Severe Hypoxia*

#### 5.2.1.1 The Role of Phosphagens

Phosphagens are phosphorylated guanidinium compounds which serve as rapidly available stores of phosphate-bond energy. Whereas phosphocreatine is the only phosphagen in all vertebrates, phospho-L-arginine is widespread among invertebrates. In addition to phospho-L-arginine, other phosphorylated guanidinium compounds such as phosphoglycocyamine, phosphotaurocyamine, and phospholombricine are important. In many of the invertebrates studied two or three types of phosphagens are found simultaneously, but usually in unequal amounts, within the same species (Fig. 6; Robin 1964; van Thoai and Roche 1964; van Thoai et al. 1964; van Thoai and Robin 1969).

Quantitative measurements of tissue phosphagen levels are known, for example, for phosphocreatine, phospho-L-arginine (e.g., Beis and Newsholme 1975), phosphotaurocyamine (Pörtner et al. 1979), phosphoglycocyamine (Schöttler et al. 1990), and phospholombricine (Hoffmann 1981). As can be seen in Table 2, phosphagen levels in muscle tissues vary considerably in different species, and it seems that mobile animals have higher phosphagen contents than do sedentary species. Owing to the single reaction step necessary to transphosphorylate a phosphagen to ATP, phosphagens can instantaneously provide energy during the beginning of severe hypoxia.

The transfer of the phosphoryl group from a phosphagen to ADP is catalyzed by phosphotransferases, also called phosphokinases. A distinct enzyme is responsible for the transphosphorylation of a specific guanidinium compound, and thus arginine phosphokinase or glycocyamine phosphokinase, among others, is found together with the corresponding phosphagen. Presumably all phosphokinases originated from one ancestral gene which coded for arginine phosphokinase. Arginine phosphokinase is therefore considered the most primitive enzyme of this group (van Thoai 1968; Watts 1968, 1971, 1975).

There has been much debate about the biological significance of this multitude of phosphagens and phosphokinases among invertebrates.

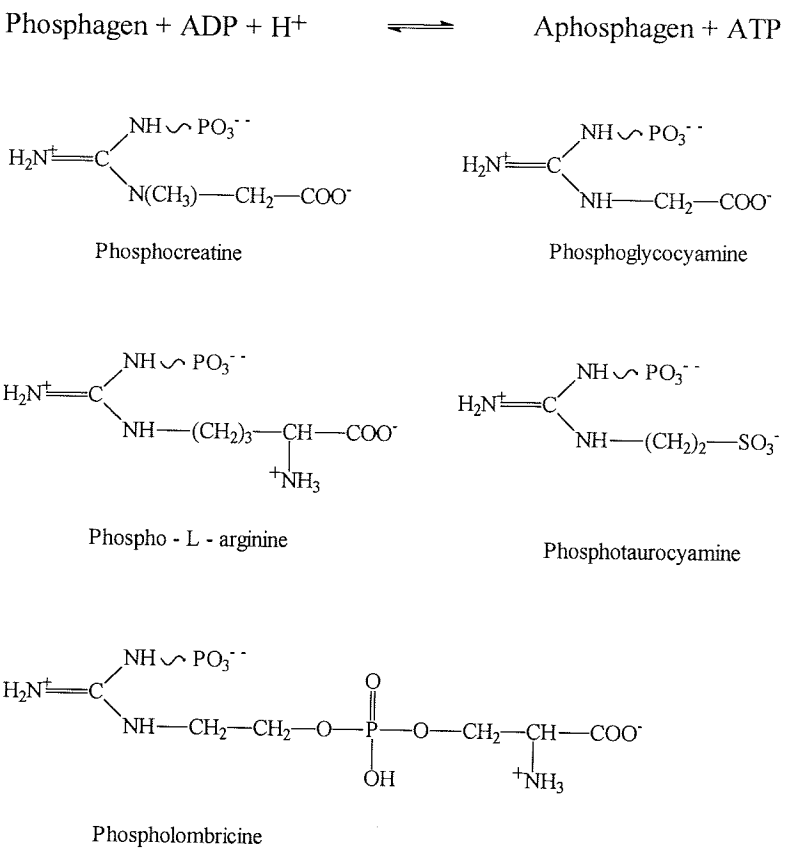



**Fig. 6.** General chemical reaction equation of the phosphagen kinase reaction and chemical structures of some phosphagens

Watts (1975) argued that creatine phosphokinase developed because creatine is a dead-end compound which does not interfere with the intermediary metabolism, in particular with the amino acid metabolism. This rationale may also hold true for the other phosphagens but certainly not for phospho-L-arginine.

An interesting explanation on the evolution of phosphokinases has been forwarded by Ellington (1989) who considered the apparent equilibrium constants for the different phosphokinases

$$K' = [\text{aphosphagen}] \cdot [\text{ATP}] / [\text{phosphagen}] \cdot [\text{ADP}] \cdot [\text{H}^+]$$

as selective factors in the evolution of these enzymes. Whereas the equilibrium constant for the creatine phosphokinase reaction ranges from 100 to 160 under physiological conditions (Lawson and Veech 1979), the $K'$ values for arginine phosphokinase and others are smaller

**Table 2.** Resting steady-state contents ($\mu mol \cdot g^{-1}$ wet weight) of phospho-L-arginine (PLA), phosphotaurocyamine (PTC), phospholombricine (PLC), phosphocreatine (PC) and phosphoglycocyamine (PGC) from some invertebrates

| Species | Tissue | PC | PLA | PTC | PLC | PGC | $MP_f$ | $MP_e$ | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Annelida | | | | | | | | | |
| *Arenicola marina* | Body wall muscle | – | – | 8.9 | – | – | 0.42 | 0.014 | Pörtner et al. (1979) |
| *Nereis diversicolor* | Total animal | 9.3 | – | – | – | 8.1 | – | – | Schöttler et al. (1990) |
| *Nephtys hombergi* | Total animal | – | – | – | – | 23.6 | – | – | Schiedek (1991) |
| *Marenzellaria viridis* | Total animal | – | 3.9 | 0.9 | – | – | – | – | Schiedek (1992) |
| *Tubifex spec.* | Total animal | – | 0.6 | – | 1.4 | – | – | – | Hoffman (1981) |
| Sipunculida | | | | | | | | | |
| *Sipunculus nudus* | Body wall muscle | – | 30.6 | – | – | – | 0.3 | 0.01 | Pörtner et al. (1984a) |
| | Retractor muscle | – | 24.9 | – | – | – | 0.7 | 0.03 | Kreutzer et al. (1985) |
| Mollusca | | | | | | | | | |
| Gastropoda | | | | | | | | | |
| *Buccinum undatum* | Columella muscle | – | 6.2 | – | – | – | 0.7 | – | Koormann and Grieshaber (1980) |
| *Nassa mutabilis* | Foot | – | 9.3 | – | – | – | 5.4 | 0.02 | Gäde et al. (1984) |
| *Busycon contrarium* | Radula retractor | – | 12.5 | – | – | – | 0.07 | 0.04 | Ellington (1982) |
| Bivalvia | | | | | | | | | |
| *Mytilus edulis* | Adductor post | – | 8.8 | – | – | – | – | 0.5 | Ebberink et al. (1979) |
| *Mytilus edulis* | ABRM | – | 8.5 | – | – | – | 1.5 | 0.5 | Zange et al. (1989) |
| *Cardium tuberculatum* | Foot | – | 22.0 | – | – | – | 8.1 | 0.02 | Gäde (1980b) |
| *Chlamys opercularis* | Adductor | – | 20.4 | – | – | – | 3.7 | – | Grieshaber (1978) |
| *Argopecten irradians concentricus* | Adductor | – | 18.5 | – | – | – | 4.8 | 0.04 | Chih and Ellington (1983) |
| *Placopecten magellanicus* | Adductor phasic | – | 22.3 | – | – | – | 3.5 | 0.004 | de Zwaan et al. (1980) |
| | Adductor tonic | | | | | | 0.6 | 0.03 | |
| Cephalopoda | | | | | | | | | |
| *Loligo vulgaris* | Mantle muscle | – | 35.6 | – | – | – | – | – | Grieshaber and Gäde (1976a) |
| Arthropoda | | | | | | | | | |
| Crustacea | | | | | | | | | |
| *Crangon crangon* | Abdominal flexor | – | 20.7 | – | – | – | 61.2 | – | Onnen and Zebe (1983) |
| *Cherax destructor* | Pleon muscle | – | 25.0 | – | – | – | – | – | England and Baldwin (1983) |
| *Orconectes limosus* | Pleon muscle | – | 20.8 | – | – | – | 23.0 | 0.4 | Gäde et al. (1984) |
| Chelicerata | | | | | | | | | |
| *Filista hibernalis* | Prosoma and legs | – | 9.5 | – | – | – | 25.5 | – | Prestwich (1988) |
| *Lycosa lenta* | Prosoma and legs | – | 8.3 | – | – | – | 24.9 | – | Prestwich (1988) |
| Insecta | | | | | | | | | |
| *Locusta migratoria* | Tibia muscle | – | 19.6 | – | – | – | – | – | Schneider et al. (1989) |

$MP_f$ and $MP_e$, rates of phosphagen depletion during functional and environmental anaerobiosis ($\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight). – not detected; open space = not determined yet.

ranging between 13% and 32% of that of the creatine phosphokinase reaction. These results and the lower changes in the free energy of hydrolysis of phosphagens as compared to phosphocreatine (Ellington 1989) indicate that the invertebrate phosphagens are thermodynamically more stable than phosphocreatine under the same physiological conditions. Invertebrate phosphokinases can buffer ATP at lower [ATP]/[ADP] ratios. These characteristics are well suited to sustain a prolonged anaerobic energy provision which is accompanied by low ATP turnover rates, low [ATP]/[ADP] ratios and an intracellular acidosis. Under these circumstances phosphocreatine would be rapidly dissipated, but other phosphagens decline gradually and act as an effective energy buffer during environmental hypoxia (Fig. 7).

As soon as severely hypoxic conditions prevail, *Arenicola marina* uses phosphotaurocyamine to provide ATP. During the first 3 h the phosphagen store is depleted at a rate of 1.5 $\mu$mol·h$^{-1}$·g$^{-1}$ wet weight, whereas during the following 21 h it decreases only at a rate of 0.03 $\mu$mol·h$^{-1}$·g$^{-1}$ wet weight (Schöttler et al. 1984a). In anaerobic *Sipunculus nudus*, which has a much higher phosphagen store, the degradation of phospho-L-arginine is similar as in the lugworm during

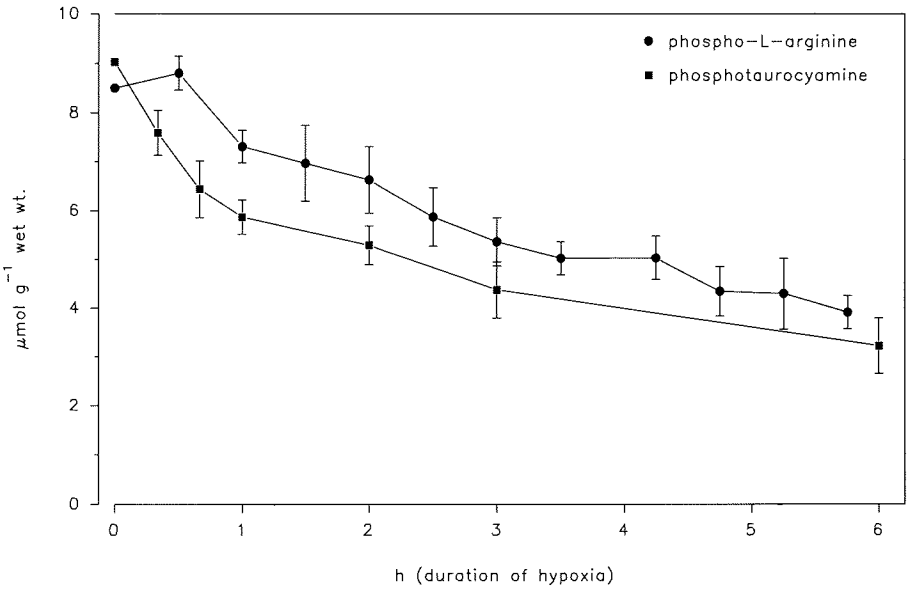



**Fig. 7.** Transphosphorylation of phospho-L-arginine in the isolated and anoxically perfused ventricle of the whelk *Busycon contrarium* and of phosphotaurocyamine in the body wall musculature of the anoxically incubated lugworm *Arenicola marina*. (Modified after Ellington 1983b; Graham and Ellington 1985; Schöttler et al. 1984a)

the first 6 h (1.2 $\mu$mol·h$^{-1}$·g$^{-1}$ wet weight) and decreases to a low rate of 0.3 $\mu$mol·h$^{-1}$·g$^{-1}$ wet weight during the remaining time (Pörtner et al. 1984a). Whereas these results were obtained with classical enzymatic assays, levels of high-energy phosphates in the anaerobic ventricle of the gastropod *Busycon contrarium* were monitored by enzymatic assays and by $^{31}$P-NMR (Ellington 1983b; Graham and Ellington 1985). Continuous measurements by the $^{31}$P-NMR technique revealed an almost linear reduction of the phospho-L-arginine pool and transphosphorylation of approximately half of the phosphagen during 6 h of severe hypoxia. Additional quantitative enzymatic estimations of phospho-L-arginine showed steady-state levels of $8.5 \pm 1.5$ $\mu$mol·g$^{-1}$ wet weight in the same tissue (Fig. 7). A transphosphorylation rate of 0.7 $\mu$mol·h$^{-1}$·g$^{-1}$ wet weight can be calculated. These studies therefore confirm the theoretical considerations of Ellington (1989) that the phosphagen pool of invertebrates is preferentially exploited during environmentally induced severe hypoxia.

#### 5.2.1.2 The Coupling of Aspartate Transamination to Glycogen Fermentation

In many marine invertebrates the pool of free amino acids is large, but only a few contribute substantially to the pool size. Glycine, D, L-alanine, taurine, L-aspartate, L-glutamate, and L-glutamine account for the majority of the free amino acids (Schoffeniels and Gilles 1972). As early as in 1973, Hochachka et al. proposed that L-aspartate and L-glutamate may serve as substrates during hypoxia. Both amino acids were thought to be metabolized along with glucose via the following reactions:

$$\text{L-aspartate} + \text{2-oxoglutarate} \rightleftarrows \text{L-glutamate} + \text{oxaloacetate} \quad (1)$$

$$\text{L-glutamate} + \text{pyruvate} \rightleftarrows \text{L-alanine} + \text{2-oxoglutarate} \quad (2)$$

$$\text{oxaloacetate} + \text{NADH} \rightleftarrows \text{malate} + \text{NAD}^+ \quad (3)$$

The three enzymes, glutamate oxaloacetate transaminase (Eq. 1), glutamate pyruvate transaminase (Eq. 2), and malate dehydrogenase (Eq. 3), which exhibit high activities in most marine invertebrates (Felbeck and Grieshaber 1980), integrate L-aspartate and glucose fermentation according to the equation:

$$\text{L-aspartate} + \text{pyruvate} + \text{NADH} \rightleftarrows \text{malate} + \text{L-alanine} + \text{NAD}^+ \quad (4)$$

The equilibrium constants of the two transaminating reactions are close to unity (Barman 1969), and the reduction of oxaloacetate is in favor of

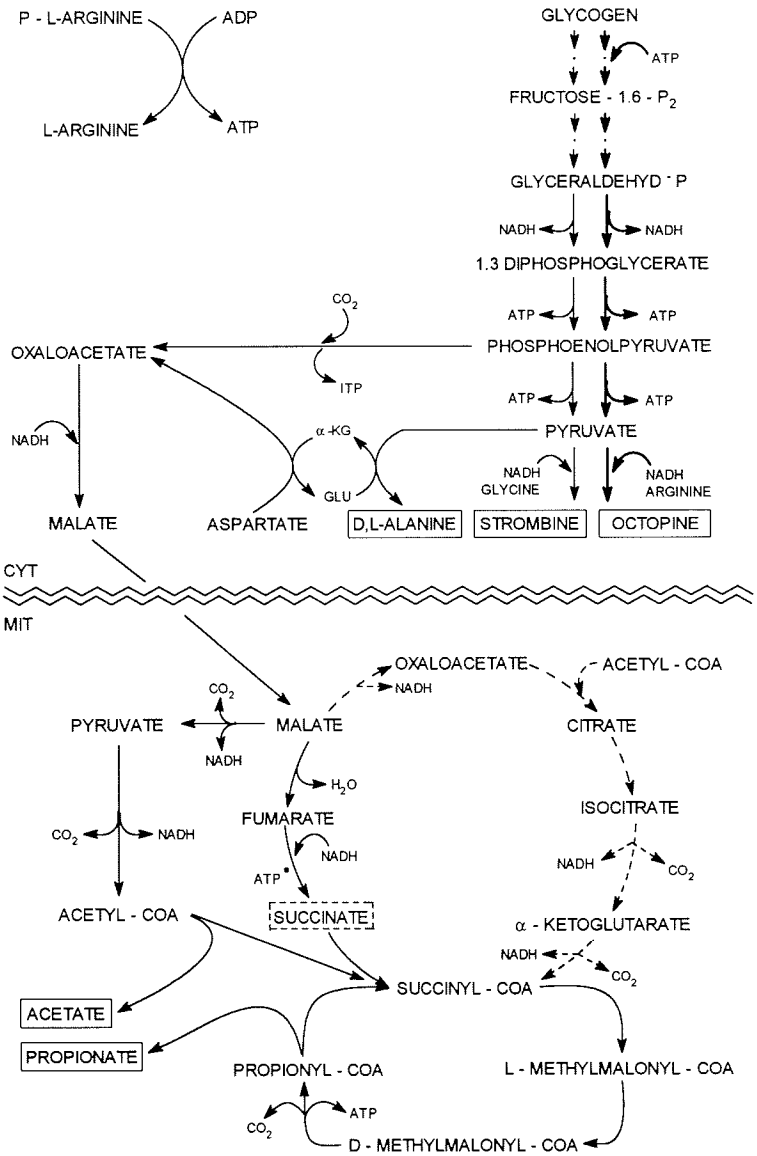
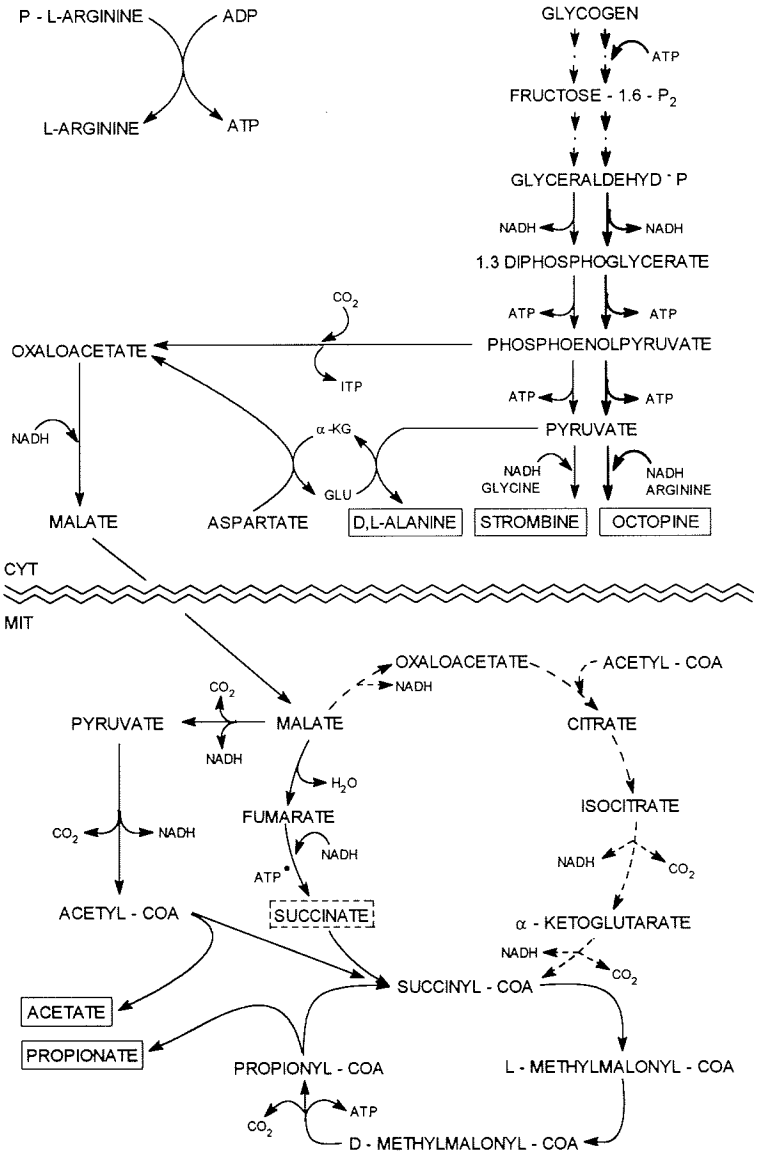



**Fig. 8.** Metabolic scheme of energy provision during functional (*thick arrows*) and environmental anaerobiosis (*thin arrows*) in the cytoplasmic (*CYT*) and mitochondrial (*MIT*) compartments of the peanut worm *Sipunculus nudus*. Species differences occur mainly in the phosphagens and the glycolytic end products accumulated. In *Arenicola marina*, for example, the opines strombine and alanopine accumulate. Note, mitochondrial ATP formation results from the electron transport chain and not from substrate level phosphorylation. Anaerobic end products are *framed*; succinate is a transient end product and therefore *framed* by a *dashed line*. (Data from Kreutzer et al. 1989; Pörtner et al. 1984a; Schöttler 1983, 1986)

malate synthesis. From mass action ratios of these reactions in skeletal muscle it has been suggested that glutamate transaminase operates near equilibrium and that changes in substrate concentrations influence the direction of this metabolic sequence (Garber et al. 1976). Thus, an increased supply of pyruvate from glycolysis and an elevated level of NADH due to scarce oxygen availability would spark the transamination of aspartate. The anaerobic breakdown of aspartate is only possible, however, if malate dehydrogenase restores $NAD^+$ for the continuous oxidation of glyceraldehyde-3-phosphate. As a corollary, pyruvate can act as an acceptor for the amino group instead of serving as a sink for glycolytic hydrogen. Alanine must, therefore, be considered as an end product of anaerobic glycolysis in particular in many marine invertebrates during environmental hypoxia. This conclusion is supported by the work of Stokes and Awapara (1968) who incubated hypoxic mantle tissue of the bivalve *Rangia cuneata* with $^{14}C$-glucose and found a large percentage of the label incorporated into alanine.

Felbeck and Grieshaber (1980) incubated *Arenicola marina* in nitrogen-saturated sea water and measured the levels of metabolites involved in the transamination reactions in the body wall musculature. In control animals the contents of asparatate and L-alanine were found to be 10 and 41 $\mu mol \cdot g^{-1}$ wet weight, respectively. Malate was estimated to 1.0 $\mu mol \cdot g^{-1}$ wet weight, L-glutaminate to 15 and pyruvate to 0.02 $\mu mol \cdot g^{-1}$ wet weight. After 6 h of anaerobiosis, the concentrations of malate and glutamate did not differ significantly from the aerobic controls, but the pyruvate content increased to 0.06 and that of L-alanine to 50 $\mu mol \cdot g^{-1}$ wet weight. During the same time, the aspartate level decreased to 2 $\mu mol \cdot g^{-1}$ wet weight. Similar data were obtained with isolated pieces of body wall tissue incubated under hypoxia, but the degradation of aspartate was more pronounced during the first 2 h of anaerobiosis than during the remaining 4 h of incubation. Unfortunately no NADH levels were measured in *Arenicola marina*, but recent investigations in isolated introvert retractor muscles of *Sipunculus nudus* demonstrated an increase of the NADH content from 1.4 to 2.5 $nmol \cdot g^{-1}$ wet weight, (Kreutzer et al. 1989). Thus it can also be assumed for *Arenicola marina* that steady-state levels of metabolites involved in the transamination reaction change to such an extent that the formation of alanine is favored under hypoxic conditions.

During early anaerobiosis the degradation of L-aspartate and the concomitant synthesis of L-alanine has been demonstrated in many species, such as the cockle *Cardium edule* (Meinardus and Gäde 1981), the sea mussel *Mytilus edulis* (Zurburg and de Zwaan 1981), *Sipunculus nudus* (Pörtner et al. 1984a), or *Scoloplos armiger* (Schöttler and Griesha-

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ber 1988). It is a characteristic feature of many anoxia-tolerant marine invertebrates which usually have a higher pool of free amino acids than closely related limnic species (Schoffeniels and Gilles 1972). However, there is usually a discrepancy between the amount of metabolized L-aspartate and the amount of alanine accumulated (Collicutt and Hochacka 1977; Zurburg and de Zwaan 1981). This discrepancy becomes even more pronounced when both stereoisomers of alanine are present.

Felbeck (1980) surprisingly reported that D-alanine arises in quantities similar to L-alanine in hypoxic *Arenicola marina*. L- and D-alanine were also found in equal amounts in *Sipunculus nudus* (Pörtner et al. 1986a), the brackish or intertidal bivalves *Corbicula japonica*, *Tapes philippinarum*, and *Meretrix lamarckii*, whereas *Mytilus edulis* and *Crassostrea gigas* possess only L,-alanine. The formation of the racemic mixture is dependent on an alanine racemase which is present in those species which accumulate D-alanine (Matsushima et al. 1984).

Several speculations have been proposed for additional sources of amino groups required to explain the formation of D,L-alanine. Protein degradation as it occurs in *Modiolus demissus* (Bishop et al. 1981) may contribute to D,L-alanine accumulation. Another possibility could be the formation of glutamate from free ammonia via the glutamate dehydrogenase reaction followed by the transamination to alanine with the arising 2-oxoglutarate being oxidized to succinyl-CoA, thus regenerating $NAD^+$ according to the following scheme:

$$NH_3 + \text{2-oxoglutarate} + NADH \rightleftarrows \text{L-glutamate} + H_2O + NAD^+ \quad (5)$$

$$\text{L-glutamate} + \text{pyruvate} \rightleftarrows \text{2-oxoglutarate} + \text{L-alanine} \quad (6)$$

$$\text{2-oxoglutarate} + NAD^+ \rightleftarrows \text{succinyl-CoA} + NADH \quad (7)$$

This scheme, which has recently been proposed by de Zwaan (1991) on the basis of experiments with isolated mitochondria from *Mytilus edulis*, requires a continuous supply of 2-oxoglutarate from the citric acid cycle. Since Schroff and Schöttler (1977) and Schöttler (1977) have demonstrated that in anaerobic mitochondria of *Arenicola marina* and *Tubifex spec.* the metabolites of the citric acid cycle continue to flow in the same direction as in aerobic mitochondria, albeit at a reduced flux rate, a continuous supply of 2-oxoglutarate can be anticipated.

The fate of the carbon skeleton of L-aspartate has long been known during anaerobiosis. Using $^{14}C$-labeled aspartate several authors have demonstrated that most of the aspartate appears in succinate (de Zwaan and van Marrewijk 1973; Zebe 1975; Collicutt and Hochachka 1977). Malate which arises from the transamination reactions enters the mito-

a Pyruvate oxidoreductase

b

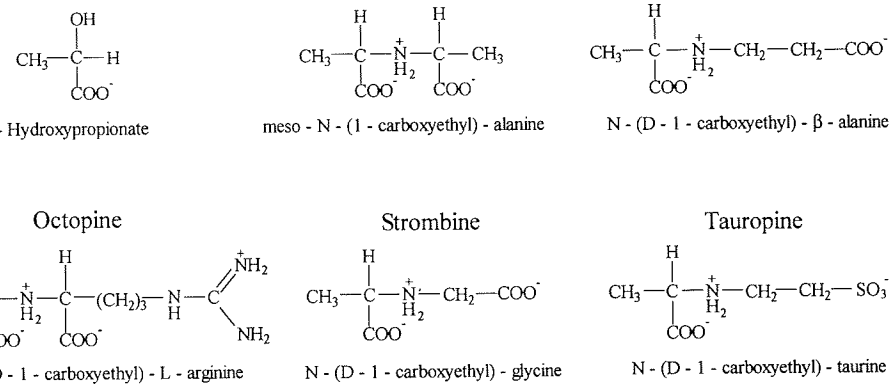


**Fig. 9. a** General chemical reaction equation of pyruvate oxidoreductase (opine dehydrogenase) catalyzed reactions, and **b** chemical structures of the different opines so far known to occur in different invertebrate species

chondria where it is dehydrated to fumarate which in turn is reduced to succinate (Fig. 8). The last reaction which is catalyzed by the fumarate reductase complex is also coupled to the formation of 1 mol ATP per 1 mol aspartate metabolized (see below).

### *5.2.2 Glycogen Fermentation and the Formation of Lactate and Opines*

Whereas phosphagens and L-aspartate are primary substrates for anaerobic energy provision, glycogen is the major energy source during anaerobiosis. Glycogen can be stored in higher amounts than compounds of low molecular weight since it does not increase the osmotic pressure within the cell due to its polymeric nature. The carbohydrate is dispersed as glycogen bodies throughout the cytoplasm, in particular in muscle cells. Glycogen content in marine invertebrates can account for approximately 10%–35% of the dry weight (de Zwaan and Zandee 1972). In *Mytilus edulis* the average glycogen content is 110–390 $\mu$mol glycosyl units$\cdot g^{-1}$ wet weight, whereas in *Arenicola marina* values

between 58 and 93 μmol glycosyl units·g$^{-1}$ wet weight have been reported (Dales 1958; Schöttler et al. 1984a). In the body wall of *Sipunculus nudus* a value of 70 μmol glycosyl units·g$^{-1}$ wet weight has been determined (M.K. Grieshaber and H.-O. Pörtner, unpublished results).

During environmental anaerobiosis glycogen is fermented via the Embden–Meyerhof–Parnas pathway which is located in the cytoplasm. Glycolysis generates 3 mol ATP per 1 mol glycosyl unit thus providing 2 or 0.5 mol ATP more than the transphosphorylation of a phosphagen or the fermentation of L-aspartate to succinate (1 mol ATP from fumarate reduction and 1.5 mol ATP from alanine formation via glycolysis), respectively (Fig. 8). ATP synthesis by substrate phosphorylation results, first, from the formation of an acylphosphate during the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and, secondly, from an enolphosphate (phosphoenolpyruvate) formed during the dehydration of 2-phosphoglycerate. The oxidation reaction also generates NADH which must be reoxidized if anaerobic glycolysis is to proceed.

In the glycolytic reaction sequence only pyruvate can act as a hydrogen acceptor. Consequently, the transamination of aspartate to alanine via pyruvate and the reoxidation of NADH with the concomitant reduction of pyruvate compete. Therefore, anaerobic glycolysis is terminated not only by D,L-alanine, but also by the products of pyruvate reduction which are the stereoisomers of lactate and the opines (Fig. 9).

The formation of the anaerobic glycolytic end products is catalyzed by at least six different pyruvate reductases, also called pyruvate oxidoreductases, and more will be probably discovered in the future. They include the classic lactate dehydrogenase (D-, L-lactate: NAD oxidoreductase) which catalyzes the reaction

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \rightleftarrows \text{lactate} + \text{NAD}^+ . \quad (8)$$

The other five enzymes are usually called opine dehydrogenases and they catalyze the reductive condensation of pyruvate with an amino acid resulting in the synthesis of an imino acid [called opine by Jaques Tempé in analogy to the first isolated compound, octopine (Morizawa 1928); see also Schell et al. (1979)] and water. Specifically, octopine dehydrogenase [*N*-α-D-1-carboxyethyl)-L-arginine: NAD$^+$ oxidoreductase] (Eq. 9), strombine dehydrogenase [*N*-(carboxymethyl)-D-alanine: NAD$^+$ oxidoreductase] (Eq. 10), alanopine dehydrogenase [meso-*N*-(1-carboxyethyl)-alanine: NAD$^+$ oxidoreductase] (Eq. 11), β-alanopine dehydrogenase [*N*-(D-1-carboxyethyl)-β-alanine: NAD$^+$ oxidoreductase) (Eq. 12) and tauropine dehydrogenase [N-D-1-carboxyethyl)-

taurine: $NAD^+$ oxidoreductase] (Eq. 13) catalyze the following reactions:

$$\text{pyruvate} + \text{L-arginine} + NADH + H^+ \rightleftarrows \text{octopine} + NAD^+ + H_2O \quad (9)$$

$$\text{pyruvate} + \text{glycine} + NADH + H^+ \rightleftarrows \text{strombine} + NAD^+ + H_2O \quad (10)$$

$$\text{pyruvate} + \text{L-alanine} + NADH + H^+ \rightleftarrows \text{alanopine} + NAD^+ + H_2O \quad (11)$$

$$\text{pyruvate} + \beta\text{-alanine} + NADH + H^+ \rightleftarrows \beta\text{-alanopine} + NAD^+ + H_2O \quad (12)$$

$$\text{pyruvate} + \text{taurine} + NADH + H^+ \rightleftarrows \text{tauropine} + NAD^+ + H_2O. \quad (13)$$

All six enzymes, which are located in the cytoplasm, are functionally analogous and potentially important in the maintenance of a low redox status in many invertebrates during functional and environmental anaerobiosis. Their presence and physiological role has been demonstrated in many species during anaerobiosis and several reviews exist which describe the details of these enzymes as well as their products (Gäde 1980a; Livingstone and de Zwaan 1983; de Zwaan and Dando 1984; Kreutzer et al. 1985; Gäde and Grieshaber 1986; Grieshaber and Kreutzer 1986).

For the sake of completeness and to demonstrate the enormous flexibility of animal systems to adapt to their environment, the occurrence of an ethanol dehydrogenase activity in the larvae of the midges *Chironomus tentans* and *Chironomus thummi thummi* must be mentioned (Harms 1972). Since ethanol dehydrogenase activity could also result from bacteria associated with these chironomid larvae, Wilps and Schöttler (1980) took great care to localize the decarboxylation of pyruvate to acetaldehyde and its reduction to ethanol. Cytosolic fractions of a cell homogenate from larvae of *Chironomus tentans* were shown to metabolize fructose-1,6-bisphosphate to pyruvate only. No ethanol could be detected despite a high cytosolic activity of ethanol dehydrogenase. In contrast to yeast cells, pyruvate was found to be decarboxylated only in the mitochondrial compartment where an additional ethanol dehydrogenase is present. NADH required for the mitochondrial reduction of acetaldehyde to ethanol could arise from the oxidative decarboxylation of pyruvate. Whether acetaldehyde is also exported to the cytoplasm remains unknown, but seems likely in the presence of a cytoplasmic ethanol dehydrogenase. This enzyme could serve to maintain a low cytoplasmic redox ratio when pyruvate, instead of serving as an electron acceptor, is transaminated to alanine, another major anaerobic end product in midge larvae.

### 5.2.2.1 The Phylogenetic Distribution of Pyruvate Reductases

For many years lactate dehydrogenase was considered the only enzyme terminating anaerobic glycolysis in aminal tissues. The first opine dehydrogenase activity was discovered by van Thoai and Robin who in 1959 reported the enzymatic formation of octopine using tissue extracts from several marine invertebrates. These authors already speculated that octopine, which was reported in muscle extracts from *Octopus octopodia* (hence the name) by Morizawa (1928), could function as hydrogen acceptor in mollusks during vigorous activity. Later, van Thoai et al. (1969) purified octopine dehydrogenase from the adductor muscle of *Pecten maximus*. The discovery of strombine/alanopine dehydrogenase in oyster adductor muscle by Fields (1976) and its subsequent characterization (Fields and Hochachka 1981), the partial purification of for example, strombine dehydrogenase (Nicchita and Ellington 1984), tauropine dehydrogenase (first called rhodoic acid dehydrogenase) by Sato and Gäde (1986) as well as by Doumen and Ellington (1987), and β-alanopine dehydrogenase by Sato et al. (1987) indicated a wide variety of possibilities for the termination of anaerobic glycolysis in the animal kingdom.

Several comprehensive investigations on the distribution of pyruvate reductases have been published (Livingstone 1983; Livingstone et al. 1983, 1990; de Zwaan and Dando 1984). These results reveal that members of the phyla Crustacea, Insecta, and Echinodermata and all Vertebrata possess only one pyruvate reductase activity, i.e., lactate dehydrogenase. This enzyme was also found in some species of Annelida and Mollusca.

A few exceptional species amongst Crustacea and Echinodermata are reported to possess opine dehydrogenases besides lactate dehydrogenase. Strombine dehydrogenase activity was measured in the marine goose barnacle *Lepas anatifera* and the freshwater red swamp crayfish *Procambarus clarkii*, or in the two echinoderm species *Astropecten irregularis and Evasterias troschelli* (Livingstone et al. 1983, 1990).

Octopine dehydrogenase is present in Cnidaria, Nemertina and in particular high activities in the Mollusca. No octopine dehydrogenase has been found in the Crustacea. Amongst the Annelida, traces of this enzymatic activity are present only in a few species, alanopine and strombine dehydrogenase being the main enzymes terminating anaerobic glycolysis in this phylum (Livingstone et al. 1983, 1990) (Table 3).

A striking feature of several species of Mollusca and Annelida is the presence of several pyruvate oxidoreductases within the same tissue (Table 4). All five opine dehydrogenases and lactate dehydrogenase are

**Table 3.** Activities ($U \cdot g^{-1}$ wet weight) of pyruvate oxidoreductases from various tissues of some invertebrates

| Species | Tissue | LDH | StrDH | AloDH | β-AloDH | ODH | TDH | D-LDH | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Porifera | | | | | | | | | |
| *Halichondria panicea* | total animal | – | 0.1 | – | – | – | – | | Livingstone et al. (1983) |
| Nemertina | | | | | | | | | |
| *Cerebratulus lacteus* | anterior part | | – | 3.0 | | 50.0 | | 0.5 | Gäde (1983b) |
| Annelida | | | | | | | | | |
| *Arenicola marina* | body wall muscle | 0.2 | 10.5 | 99.0 | | – | | – | Siegmund et al. (1985) |
| *Scoloplos armiger* | whole animal | 0.8 | 96.7 | 73.8 | | – | – | – | Schöttler and Grieshaber (1988) |
| *Nereis diversicolor* | body wall muscle | – | – | – | | – | | 48.8 | Schöttler pers. comm. |
| *Glycera convoluta* | total animal | – | 51.9 | 171.4 | – | – | | – | Siegmund and Grieshaber unpublished results) |
| *Nephtys hombergi* | body wall | – | 44.2 | 50.3 | – | – | | – | Schöttler (1982) |
| Sipunculida | | | | | | | | | |
| *Sipunculus nudus* | body wall muscle | – | 9.2 | 49.3 | | 445.4 | | – | Pörtner et al. (1984a) |
| Mollusca | | | | | | | | | |
| Gastropoda | | | | | | | | | |
| *Littorina littorea* | foot | | – | 2.4 | | – | | 14.2 | Livingstone et al. (1983) |

**Table 3.** (*Cont.*)

| Species | Tissue | LDH | StrDH | AloDH | β-AloDH | ODH | TDH | D-LDH | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *Buccinum undatum* | foot & columelar muscle | | – | 9.3 | | 68.8 | | 3.1 | Livingstone et al. (1983) |
| *Nassa mutabilis* | foot | | 2.4 | 29.3 | | 186.0 | | 10.7 | Gäde et al. (1984) |
| Bivalvia | | | | | | | | | |
| *Mytilus edulis* | adductor | | 4.9 | 2.7 | | 17.5 | | 13.3 | Livingstone et al. (1983) |
| *Crassostrea angulata* | adductor | – | 36.7 | 34.5 | | – | | – | Livingstone et al. (1983) |
| *Solen marginatus* | foot | – | 6.1 | 39.1 | | 131.7 | | – | Grieshaber (unpublished results) |
| *Cardium tuberculatum* | adductor | – | 33.4 | 40.9 | | 153.0 | | 3.2 | Meinhardus Hager and Gäde (1986) |
| *Pecten maximus* | foot | – | 18.1 | 21.5 | | 117.2 | | 4.0 | Gäde et al. (1978) |
| | adductor | | | | | 1.7 | | | |
| *Anodonta cygnea* | adductor | 6.0 | | | | 10.3 | | | Gäde and Zebe (1973) |
| *Dreissena polymorpha* | adductor | 4.3 | | | | 7.7 | | | Gäde and Zebe (1973) |
| Cephalopoda | | | | | | | | | |
| *Loligo vulgaris* | mantle | – | | | | 120.0 | | – | Grieshaber (unpublished results) |
| *Nautilus pompilius* | spadix retractor | 5.8 | | | | 83.6 | | | Hochacka et al. (1977) |

LDH, lactate dehydrogenase; StrDH, strombine dehydrogenase; AlODH, alanopine dehydrogenase; β-AloDH, β-alanopine dehydrogenase; ODH, octopine dehydrogenase; TDH, tauropine dehydrogenase; –, not detected, open space, not yet determined.

**Table 4.** Activities (act) of pyruvate oxidoreductases ($U \cdot g^{-1}$ wet weight) and contents of glycolytic end products accumulated after bursts of muscular activity in some marine invertebrates (modified from Grieshaber and Kreutzer 1986)

| Species | act<br>ΔEP | LDH<br>Lactate | StrDH<br>Strombine | AloDH<br>Alanopine | ODH<br>Octopine |
|---|---|---|---|---|---|
| *Arenicola* | act | 0.2 | 4.0 | 45.0 | bl |
| *marina* | ΔEP | 0.1 | 1.6 | 8.0 | bl |
| *Glycera* | act | ND | 51.9 | 171.4 | ND |
| *convoluta* | ΔEP | bl | – | 0.5 | bl |
| *Sipunculus* | act | bl | 9.2 | 49.3 | 445.4 |
| *nudus* | ΔEP | bl | 3.4 | – | 8.1 |
| *Nassa* | act | 10.7 | 2.4 | 29.3 | 186.0 |
| *mutabilis* | ΔEP | 0.7 | – | 1.5 | 7.9 |
| *Solen* | act | bl | 6.1 | 39.1 | 131.7 |
| *marginatus* | ΔEP | bl | 0.8 | 0.3 | 4.1 |
| *Cardium* | act | 4.0 | 18.1 | 21.4 | 117.1 |
| *tuberculatum* | ΔEP | – | – | – | 2.3 |
| *Ensis* | act | 0.9 | 6.1 | 13.6 | 166.9 |
| *siliqua* | ΔEP | – | – | – | 4.0 |

ΔEP, difference of control and experimental animal in $\mu mol \cdot g^{-1}$ wet weight; bl, below limit of detection; ND, not determined; –, end products not accumulated.

present in the bivalve *Scapharca broughtonii* (Sato et al. 1987). The lugworm *Arenicola marina*, the peanut worm *Sipunculus nudus*, the periwinkle *Littorina littoralis*, the sea mussel *Mytilus edulis* or the cockle *Cardium tuberculatum* possess three or four pyruvate reductase activities, whereas the nemertean *Cerebratulus lacteus*, the annelid *Nephtys hombergii*, or the gastropod *Littorina littorea* contains two. At first glance, these multiple pyruvate oxidoreductase activities have to be considered with care, in particular if lactate dehydrogenase is consistently present in different species of a phylum. Lactate dehydrogenase could mimic strombine or alanopine dehydrogenase using glycine or alanine as additional substrates. Physiological relevance can, therefore, only be specifically attributed to a pyruvate oxidoreductase if the protein has been purified and characterized and the formation of the corresponding opine has been proven under a given physiological situation *in vivo*.

A prerequisite for the study of how invertebrates regulate their cytosolic redox balance during environmental and functional anaerobiosis are sensitive assays to measure the different glycolytic end products. In the case of lactate, octopine, and ethanol, the determinations can be performed according to the optical assay using lactate, octopine, and ethanol dehydrogenase (see below) as analytical tools (Bernt and Gutmann 1974; Grieshaber 1976; Grieshaber et al. 1978; Gäde and Head

1979). Other opines cannot be estimated using enzymatic assays because the corresponding opine dehydrogenases are unspecific or show a very low activity (Storey et al. 1982). Therefore, several different analytical procedures such as high-voltage paper electrophoresis (Sato et al. 1982), isotachophoresis (de Zwaan and Zurburg 1981), gas-liquid chromatography (Storey et al. 1982), and high-performance liquid chromatography (HPLC) without (Siegmund and Grieshaber 1983) and with sample derivatization (Fiore et al. 1984; Sato et al. 1988) have been used. Each assay method has unique features, but in general the HPLC procedures provide high sensitivity and fast determination.

### 5.2.2.2 The Formation of Lactate and Opines and a Short Note on Ethanol Production During Environmental Hypoxia

During lack of ambient oxygen, lactate has been repeatedly reported to accumulate in invertebrates. As early as 1928 Davis and Slater (1928b) observed the formation of this metabolite in the hypoxic earthworm *Lumbricus terrestris*. Gruner and Zebe (1978) confirmed these results and found in addition that *Lumbricus terrestris* synthesizes L-lactate only in the beginning of anaerobiosis, whereas in *Lumbricus rubellus* L-lactate is produced during prolonged severe hypoxia. In three species of *Nereis*, the formation of D-lactate instead of L-lactate contributes considerably to anaerobic energy provision. *Nereis pelagica*, which can only survive for a limited period at a low $Po_2$, mainly derives energy from the Embden-Meyerhof-Parnas pathway, whereas *Nereis virens* exhibits moderate hypoxia tolerance by relying on both anaerobic glycolysis and the succinate–propionate pathway. *Nereis diversicolor*, which survives long-lasting severe hypoxia, accumulates D-lactate predominantly during the first 24 h of low oxygen availability and then switches to the succinate–propionate pathway (Schöttler 1979).

In the crustacean *Cirolana borealis*, which scavenges in the flesh of dead fish, L-lactate was the main end product increasing about 15-fold to 100–150 $\mu mol \cdot g^{-1}$ dry weight during 18 h of anoxia. Similar amounts of lactate were also found in the incubation water (de Zwaan and Skjoldal 1979). *Uca pugnax*, which lives in burrows dug into the often highly reduced mud of salt marshes, can survive 24 h under nitrogen at 21°C while accumulating 45–60 $\mu mol \cdot g^{-1}$ fresh weight of lactate. Specimens taken from their burrows, which showed a $Po_2$ of 4–5 torr in the water puddle at the bottom, contained 6.7 $\mu mol \cdot g^{-1}$ fresh weight lactate as compared to an average of 2.2 $\mu mol \cdot g^{-1}$ fresh weight in well-aerated animals (Teal and Carey 1967). *Upogebia pugettensis* and *Callianassa californiensis*, members of the endofauna of intertidal mud flats, may be

exposed to hypoxic conditions during low tide. They can survive experimental lack of oxygen for 3 days. Pritchard and Eddy (1979) observed lactate concentrations in the blood of *Upogebia pugettensis* to increase by 41.3 $\mu mol \cdot ml^{-1}$ but only by 5.6 $\mu mol \cdot ml^{-1}$ in *Callianassa californiensis* after 18–20 h of nitrogen exposure. These different concentrations of blood lactate between the two animals led the authors to suggest that the ghost shrimp *Callianassa californiensis* might also use another anaerobic metabolic pathway such as, for example, succinate–propionate formation (see below). Zebe (1982) reinvestigated the anaerobic metabolism of both thalassenid shrimps and confirmed L-lactate as the predominant end product. Alanine and succinate were minor end products which did not differ significantly between the two species. Bridges and Brand (1980) monitored concentrations of blood lactate in the burrowing *Corystes cassivelaunus*, *Atelecyclus rotundatus*, as well as in *Nephrops norvegicus* during 5 h of anoxia and compared the concentrations of lactate to those of the nonburrowing species *Homarus gammarus*, *Galathea strigosa*, and *Carcinus maenas* which are less likely to be exposed to hypoxia. Lactate rose continuously to a net gain of 8.8 $\mu mol \cdot ml^{-1}$ in the blood of *Corystes cassivelaunus* and *Nephrops norvegicus*. The other species had somewhat lower blood lactate concentrations, but nevertheless *Homarus vulgaris* and *Carcinus maenas* can survive short periods of severe hypoxia by means of anaerobic glycolysis. Reduced concentrations of oxygen may also be encountered by *Orconectes limosus* which lives in eutrophic lakes and slowly flowing rivers. As expected, the crayfish shows a nearly linear buildup of 15.1 $\mu mol \cdot g^{-1}$ wet weight L-lactate in the tail muscle and 57.9 $\mu mol \cdot ml^{-1}$ in the blood during 16 h of anoxia. Only a small rise of D-alanine and succinate occurred (Gäde 1984). From these data, and more could be added, one can conclude that anaerobic glycolysis terminating with the formation of lactate is a major if not the only source of energy during environmental hypoxia in Crustacea (Albert and Ellington 1985).

L-lactate has been demonstrated to accumulate in some insects during ambient lack of oxygen. Adult cockroach *Periplaneta orientalis* increase L-lactate within their body from 2 to 10.9 $\mu mol \cdot g^{-1}$ fresh weight during 2 h (Davis and Slater 1928a) and larvae of *Tenebrio molitor* from 2.7 to 7.3 $\mu mol \cdot g^{-1}$ wet weight during 4.5 h of anoxia (Gilmour 1941). Particular interest focused on the larvae of the midge *Chaoborus cristallinus*, living in the benthic mud of freshwater lakes during daylight but migrating into the water column during night. In anoxic larvae of *Chaoborus cristallinus* L-lactate increased by 74.3 $\mu mol \cdot g^{-1}$ dry weight and alanine by 183 $\mu mol \cdot g^{-1}$ dry weight. Again, both compounds are derived from pyruvate probably originating from glycogen breakdown

(Englisch et al. 1982). Larvae of the midge *Chironomus thummi thummi* which also inhabit the often hypoxic sediments of shallow ponds and lakes have already been mentioned because of their peculiar possession of lactate and ethanol dehydrogenase activities (Harms 1972). Wilps and Zebe (1976) found an average increase of 20 $\mu$mol·g$^{-1}$ fresh weight lactate during 24 h of anoxia with most of the lactate accumulation occurring during the first 6 h of incubation (Redecker and Zebe 1988), but the degradation of glycogen was much higher than would have been necessary for the formation of the lactate levels found. Even additional end products present in anaerobic larvae, such as alanine and succinate, could not explain the enormous amounts of glycogen metabolized (approximately 600–700 $\mu$mol glycosyl units per 1 g dry weight during 48 h of severe hypoxia). As already expected from the presence of ethanol dehydrogenase, ethanol was found within the animals (42 mmol·g$^{-1}$ dry weight) and in particular large amounts in the incubation water (990 $\mu$mol·g$^{-1}$ dry weight of midges). The predominant synthesis of ethanol started after a lag period and proceeded at a constant rate during the entire period of incubation (Wilps and Zebe 1976).

Besides this peculiar formation of ethanol in the larvae of midges, the main anaerobic end product in insects is lactate. Thus crustacea, insects, and vertebrates predominantly terminate anaerobic glycolysis with a single end product, i.e., D- or L-lactate.

The formation of other glycolytic end products such as alanopine, $\beta$-alanopine, octopine, strombine, and tauropine is also well established in many anaerobic invertebrates. The highly mobile bloodworm *Glycera dibranchiata*, which has only a limited capacity to survive anoxic conditions, accumulated linearly up to 20 $\mu$mol·g$^{-1}$ fresh weight alanopine but no strombine during 24 h of nitrogen exposure (Fig. 10) (M.K. Grieshaber and L.E. Burnett, unpublished results). $\beta$-Alanopine was the major anaerobic end product in the adductor muscle of the blood shell *Scapharca broughtonii* where it increased from 2.5 to 21 $\mu$mol·g$^{-1}$ fresh weight during 24 h of aerial exposure. In addition, alanopine, strombine, and tauropine were accumulated although to a lesser extent (Sato et al. 1988). The simultaneous accumulation of strombine from 0.76 to 9.62 $\mu$mol·g$^{-1}$ wet weight and of D-lactate from 0.06 to 3.0 $\mu$mol·g$^{-1}$ wet weight was demonstrated in the adductor muscle of the cockle *Clinocardium nuttali* during 7 h of nitrogen exposure (Fig. 10) (J.H.A. Fields and M.K. Grieshaber, unpublished results). In *Mytilus edulis* the formation of strombine was extensively studied. During 24 h of aerial exposure strombine was synthesized to only about 3 $\mu$mol·g$^{-1}$ wet weight, but approximately 9 $\mu$mol·g$^{-1}$ fresh weight were accumulated in the adductor muscle after 4 h of reimmersion in well-aerated sea water. Only traces of

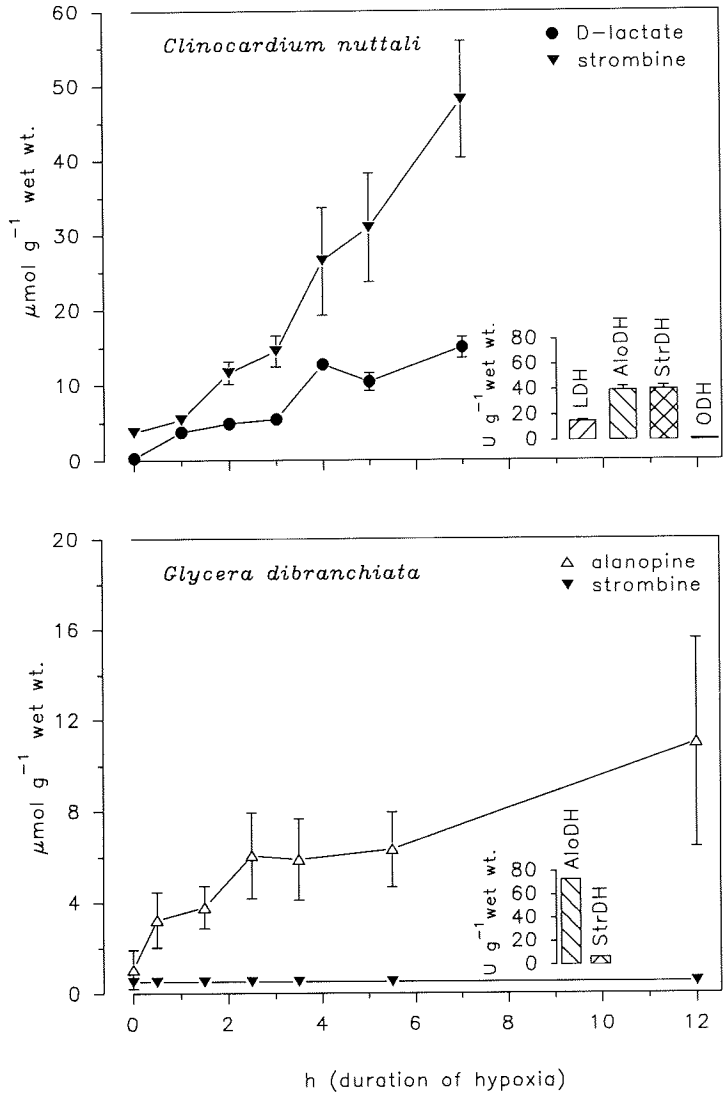


**Fig. 10.** Contents ($\mu$mol·g$^{-1}$ wet weight) of glycolytic anaerobic end products and activities ($\mu$mol·min$^{-1}$·g$^{-1}$ wet weight) of pyruvate oxidoreductases in the shell adductor muscle of the cockle *Clinocardium nuttali* (J. H. A. Fields and M. K. Grieshaber, unpublished results), and in the body wall musculature of the bloodworm, *Glycera dibranchiata* (M. K. Grieshaber and L. E. Burnett, unpublished results). Note, there is no formation of alanopine or octopine in the shell adductor muscle of the cockle, despite the presence of the corresponding enzymatic activities

octopine and D-lactate were found (de Zwaan et al. 1983a). In the body wall musculature and introvert retractor muscle of *Sipunculus nudus* approximately 5 and 2.5 $\mu mol \cdot g^{-1}$ wet weight strombine, respectively, accumulated during early anaerobiosis (Pörtner et al. 1984a). Strombine was also synthesized in the body wall musculature of *Arenicola marina* where it amounted to 2.3 $\mu mol \cdot g^{-1}$ wet weight (Siegmund et al. 1985). The formation of tauropine was investigated in the shell adductor muscle and in the foot of the ormer *Haliotis lamellosa* (Gäde 1988). A substantial accumulation of tauropine was measured in the adductor muscle after 6 h of nitrogen exposure, whereas only a small, but significant, formation occurred in the foot muscle. In the latter tissue, the main anaerobic end product was D-lactate, which also accumulated in the adductor muscle, but to a much lesser extent. Besides these experiments which demonstrate the pronounced formation of opines in various anaerobic tissues, several reports on minor opine accumulation exist. Small amounts of alanopine and strombine were found in the clam *Mercenaria mercenaria* (Korycan and Storey 1983) and in the oyster *Crassostrea virginica* (Eberlee et al. 1983) during hypoxic incubation. In the latter species, opine accumulation continued to some degree during the following aerobic recovery. A minor but significant accumulation of octopine was found in the resting and anoxic anterior byssus retractor muscle of *Mytilus edulis* (Zange et al. 1989). There are also some early reports in which the separation as well as the quantification of individual opines was not achieved and where only the sum of opine accumulation was measured (Baldwin and England 1982a; Ellington 1982; Nicchitta and Ellington 1983; Chih and Ellington 1983). Nevertheless, the majority of studies clearly demonstrated that the anaerobic reduction of pyruvate leads to the formation of opines in many annelids and mollusks.

These investigations on the distribution of ethanol dehydrogenase, lactate dehydrogenase, and opine dehydrogenases, and on the formation of the corresponding end products throughout the animal kingdom demonstrated that anaerobic glycolysis is indeed present in all species investigated. Even those species like the sea mussel *Mytilus edulis* (Glaister and Kerly 1936) or the lugworm *Arenicola marina* (Dales 1958), which were once considered to possess only a limited capacity of the Embden–Meyerhof–Parnas pathway, are no longer exceptional. During the beginning of severe environmental hypoxia, in particular when only short periods of restricted oxygen availability must be overcome, the Embden–Meyerhof–Parnas pathway is used for energy provision. Different end products terminate anaerobic glycolysis and the enzymes catalyzing the formation of opines are functionally analogous to the lactate dehydrogenase in keeping the cytoplasmic redox ratio $NADH/NAD^+$ low.

This mode of anaerobic energy metabolism is, however, only used for a limited time period which generally does not last longer than the first 3–6 h of a severe hypoxic exposure. During sustained anaerobiosis the anaerobic degradation of glycogen is not restricted to the Embden–Meyerhof–Parnas pathway, instead the mitochondrial compartment becomes involved in the anaerobic energy provision.

### *5.2.3 Formation of Succinate, Acetate, and Propionate During Sustained Severe Hypoxia*

#### 5.2.3.1 Phosphoenolpyruvate Branchpoint

During sustained severe hypoxia, anaerobic glycogenolysis also follows the initial reactions of the Embden–Meyerhof–Parnas pathway until the formation of phosphoenolpyruvate is achieved. At this level, however, the metabolite flux deviates from its normal aerobic route, which leads to the pyruvate kinase catalyzed formation of pyruvate. Instead, phosphoenolpyruvate carboxykinase preferentially catalyzes the carboxylation of phosphoenolpyruvate to oxaloacetate (Fig. 8). Energetically both reactions are of equal value and deliver ATP. At this stage, oxaloacetate, which in the beginning of anaerobiosis originated from aspartate, is now exclusively derived from the degradation of glycogen (Schöttler et al. 1984a).

The diversion of the metabolite flux at the phsophoenolpyruvate branchpoint, leading to oxaloacetate instead of pyruvate, can be demonstrated by monitoring the concentrations of different end products after 6 h of severe hypoxia. In the body wall musculature of normoxically incubated *Arenicola marina* there is a steady-state concentration of 0.2 and 1.2 $\mu mol \cdot g^{-1}$ wet weight alanopine and strombine, respectively, and 0.03 $\mu mol \cdot g^{-1}$ wet weight succinate. During severe hypoxia the contents of these metabolites increase as anticipated (Fig. 11) (B. Siegmund and M.K. Grieshaber, unpublished results). If, however, phosphoenolpyruvate carboxykinase is inhibited by 3-mercaptopicolinic acid (DiTullio et al. 1974), more pyruvate is delivered via pyruvate kinase. The increased supply of pyruvate results in an enhanced accumulation of alanopine and strombine, whereas succinate formation is less, due to a reduced supply of oxaloacetate. Reduced succinate formation in 3-mercaptopicolinic acid-treated, anaerobic lugworms was also demonstrated by Schöttler and Wienhausen (1981), as well as by Schöttler et al. (1984a). Similar treatment of the adductor muscle with mercaptopicolinate had no effect on succinate formation in *Mytilus edulis* collected during winter, but in animals collected in summer a marked reduction of succinate

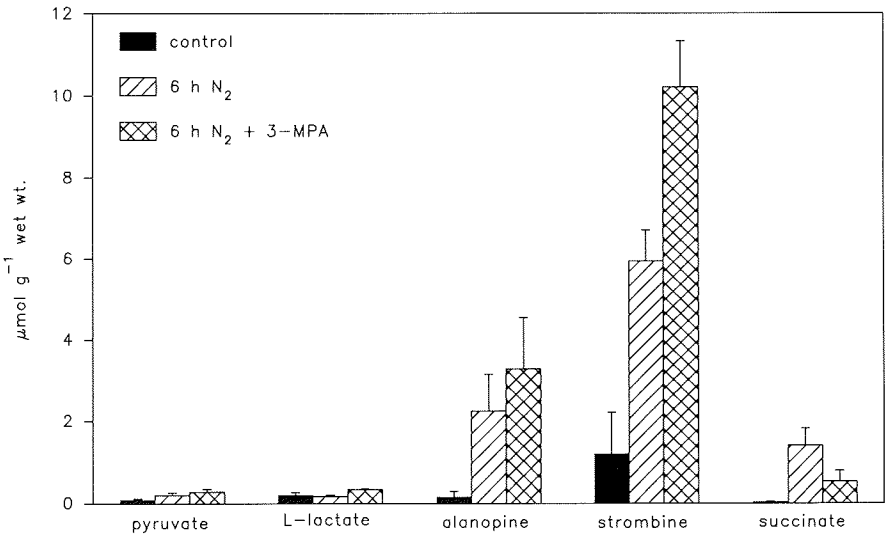


**Fig. 11.** Contents ($\mu$mol·g$^{-1}$ wet weight) of pyruvate, L-lactate, alanopine, strombine, and succinate in the body wall musculature of the lugworm, *Arenicola marina* L. during environmental anaerobiosis (6 h $N_2$) and in specimens additionally treated with 3-mercaptopicolinic acid (6 h $N_2$ + 3-MPA) to inhibit phosphoenolpyruvate carboxykinase (B. Siegmund and M. K. Grieshaber, unpublished results)

accumulation was found (de Zwaan et al. 1983b). Thus, the diversion of the metabolite flux at the phosphoenolpyruvate branchpoint to oxaloacetate enables these two species, and probably others with a pronounced capability to survive sustained anoxia, to use the mitochondrial compartment for additional anaerobic energy provision.

Switching at the phosphoenolpyruvate branchpoint depends on the regulation of the two enzymes competing for this metabolite, pyruvate kinase and phosphoenolpyruvate carboxykinase. The diversion of the metabolite flux is effected by the inhibition of pyruvate kinase and the activation of phosphoenolpyruvate carboxykinase. Different regulatory parameters have been proposed. Hochachka and Mustafa (1972) found different pH optima for both enzymes, the latter one being activated at more acidic pH. Therefore, the decrease in $pH_i$ was assumed to initiate phosphoenolpyruvate carboxylation because the catalyzing enzyme shows a more acidic pH optimum. Conversely, in *Sipunculus nudus* this shift at the phosphoenolpyruvate branchpoint already occurred during the initial period of intracellular alkalosis. At least in this species and probably also in some others, a drop in $pH_i$ cannot cause the activation or inhibition of the two competing enzymes (Pörtner et al. 1984 b,c).

From the work of Siebenaller (1979), Holwerda et al. (1981, 1983, 1984), Plaxton and Storey (1984a,b), Storey (1988), as well as Englisch (1989) it seems certain that the covalent phosphorylation of pyruvate kinase reduces the activity of this enzyme. Therefore, phosphoenolpyruvate carboxykinase outcompetes pyruvate kinase for phosphoenolpyruvate.

Oxaloacetate resulting from this reaction is reduced to malate by the cytoplasmic malate dehydrogenase, thus replacing the pyruvate oxidoreductases in keeping the redox balance $NADH/NAD^+$ low. Malate in turn is transported to the mitochondria where the ensuing anaerobic metabolism takes place.

The further metabolic fate of malate follows two routes (de Zwaan et al. 1981): it can either be oxidized to oxaloacetate by the mitochondrial malate dehydrogenase or dehydrated to fumarate catalyzed by fumarase. The latter metabolite is of utmost importance in the anaerobic reaction sequence because it assumes the role of an electron acceptor in the absence of oxygen giving rise to succinate.

#### 5.2.3.2 Fumarate Reductase Activity

The establishment of an enzymatic activity catalyzing the reduction of fumarate to succinate using NADH as a cosubstrate in facultative anaerobic animals was indeed an important step in providing an explanation of succinate accumulation in the absence of oxygen. A "fumarate reductase activity" was demonstrated for the first time in free-living invertebrates in the marine bivalve *Crassostrea virginica* and in the brachiopod *Glottidia pyramidata* by Hammen and Lum (1966). Later Schroff and Schöttler (1977) and Schöttler (1977) demonstrated a pronounced fumarate reductase activity in the lugworm *Arenicola marina* and in a mixture of riverworm species such as *Tubifex*. Fumarate reduction was also reported in *Mytilus edulis* (Holwerda and de Zwaan 1979, 1980) and in the terrestrial gastropod *Achatina achatina* (Umezurike and Chilaka 1982). It is now established that many eukaryotic organisms with a high potency for anaerobiosis possess this enzymatic activity.

Electron transfer from NADH to fumarate has long been known in the intestinal parasite *Ascaris suum* (Kmetec and Bueding 1961) or in the liver fluke *Fasciola hepatica* (Barrett 1978). In contrast to the investigations on facultatively anaerobic animals, reports on the structure of fumarate reductase from parasites are numerous and provide a detailed picture of this enzymatic activity. Recent comprehensive reviews on the fumarate reductase activity have been published by Oya and Kita (1989) and Behm (1991).

Fumarate reductase activity has been purified from the intestinal nematode *Ascaris suum* (Takamiya et al. 1984, 1986; Oya and Kita 1989) and the lung fluke *Paragonimus westermani* (Ma et al. 1987). The complex II from mitochondria of muscle tissue of the adult stage of *Ascaris suum* possesses a succinate–ubiquinone reductase activity and most of the *b*-type cytochrome is cytochrome $b_{558}$ (Kita et al. 1988 a,b). In addition, complex II contains an iron sulfur subunit and a flavoprotein. The cytochrome $b_{558}$ in complex II can be reduced with NADH, α-glycerophosphate, and succinate and it is rapidly reoxidized with fumarate. In the presence of methyl viologen as an electron donor, complex II shows high fumarate reductase activity. In addition, rhodoquinone seems to replace ubiquinone. Physiologically, complex II acts as a fumarate reductase by its reverse reaction (Takamiya et al. 1986) which is corroborated by a low succinate dehydrogenase activity. Fumarate reductase must therefore be considered as an enzyme complex consisting of NADH–rhodoquinone–oxidoreductase (complex I) and succinate –quinone–oxidoreductase (complex II). If oxygen is lacking, electrons are transported from complex I to complex II via rhodoquinone, thus reversing the normal aerobic electron flow from complex II to rhodoquinone. The reduction of fumarate appears to be accompanied by the establishment of a proton motive force which is obtained via complex I where the proton translocation occurs. Consequently fumarate not only serves as a terminal electron acceptor but also permits the synthesis of an additional mole of ATP (Fig. 8).

The fumarate reductase reaction is also common in facultative anaerobes. Although the fumarate reductase has not yet been purified in any of the free-living invertebrates, a similar reaction mechanism can be deduced from submitochondrial particles isolated from *Arenicola marina*, *Tubifex*, and *Mytilus edulis*. Experiments using inhibitors which act on different sites of the respiratory chain and on succinate dehydrogenase indicate that in facultative anaerobes the route of hydrogen transfer from NADH to fumarate is similar to that in *Ascaris suum*. Obviously, under severe hypoxic conditions the electron flow in complex II is reversed. Instead of oxidizing succinate to fumarate, the succinate dehydrogenase complex reduces fumarate to succinate. From the data available, it can be tentatively assumed that the ubiquinone–fumarate oxidoreductase is identical with complex II. In addition 1 mol ATP is synthesized per mole fumarate reduced (Fig. 8), thus increasing the energy yield by 2 mol of ATP per glycosyl unit fermented (Schroff and Schöttler 1977; Holwerda and de Zwaan 1979).

Since *Arenicola marina* has, in contrast to *Ascaris suum*, a complete and functional system of aerobic energy provision, it was important to

exclude the possibility that electron transport from cytochrom *b* to cytochrom *c* would occur. This was proven by Schroff and Schöttler (1977) who found a pronounced succinate accumulation in the presence of antimycin A which inhibits electron flow between cytochromes *b* and $c_1$. In addition, malonate reduced the rate of fumarate reduction, thus indicating that succinate dehydrogenase catalyzes the transfer of hydrogen from ubiquinone to fumarate. Recently, Schöttler and Bennet (1991) argued that a quinone other than ubiquinone must be present in the mitochondria of *Arenicola marina* because the redox potential of ubiquinone ($E' = +0.10$ V) does not permit the transfer of electrons to fumarate ($E' = -0.03$ V). Which quinone, however, participates in this reaction is presently still unknown.

The reduction of fumarate to succinate using NADH as cosubstrate is another example for a substrate-dependent hydrogen transfer catalyzed by mitochondria. Klingenberg and von Häfner (1963) and Klingenberg (1964) reported the mitochondrial reduction of acetoacetate to 3-hydroxy-butyrate using succinate as hydrogen donor. Although both reactions demonstrate the reversibility of mitochondrial metabolic pathways, they are quite different concerning the energy budget. In the first reaction, the electron flow follows the redox potential from fumarate to succinate with the concomitant synthesis of ATP, which results from the redox potential difference of complex I. Conversely, the latter reaction pumps hydrogen from one substrate to the other against the redox potential consuming energy. Thus, succinate formation, i.e., the reversion of the normal "clockwise" metabolite flux of the Krebs cycle, transforms energy into phosphate-bond energy, whereas the intersubstrate hydrogen transfer from succinate to acetoacetate is made possible by the reversibility of oxidative phosphorylation.

A reversal of the electron flux in complex II from fumarate to succinate is only possible if the oxygen concentration within the mitochondria is sufficiently low to exclude any electron flow to oxygen. An aerobic electron flow will only be prevented, if the intramitochondrial $Po_2$ is below 0.2 torr, the $P_{50}$ of the cytochrome oxidase (Oshino et al. 1974). At these low partial pressures of oxygen, the respiratory chain will become oxygen limited and succinate will accumulate. Thus, succinate is the ideal indicator of the onset of an anaerobic metabolism within the mitochondria. It is a much better signal of anaerobiosis than lactate or opines which are only indicative of anaerobic metabolism within the cytoplasmic compartment.

The formation of succinate from fumarate requires a constant supply of NADH. Since the redox state of the cytoplasm is balanced, NADH must originate within the mitochondria. In a detailed analysis, Schöttler

(1977) presented evidence that malate is metabolized in a clockwise and counterclockwise direction within the citric acid cycle. Since the formation of oxaloacetate and acetyl-CoA is required to start the citric acid cycle in the clockwise direction, oxidative decarboxylation of malate to acetyl-CoA via malic enzyme- and pyruvate dehydrogenase-mediated reactions is necessary. Further metabolization of citrate via isocitrate and 2-oxoglutarate to succinyl-CoA has been proven with 1,4-$^{14}$C-malate which gave rise to an almost stoichiometric formation of $^{14}CO_2$. When 2,3-$^{14}$C-malate was offered as a substrate almost no radioactively labeled $CO_2$ was recovered. The clockwise route of malate metabolization results in the formation of 5 mol NADH per 2 mol malate and this NADH is made available for the reduction of 5 mol fumarate originating from the counterclockwise dehydration of 5 mol malate. Thus for *Tubifex* and *Arenicola marina* the following stoichiometric equations apply:

$$2 \text{ malate} + 5 \text{ NAD}^+ \rightleftarrows 1 \text{ succinate} + 5 \text{ NADH} + 4 \text{ CO}_2$$

$$5 \text{ malate} + 5 \text{ NADH} \rightleftarrows 5 \text{ succinate} + 5 \text{ NAD}^+$$

$$7 \text{ malate} \rightleftarrows 6 \text{ succinate} + 4 \text{ CO}_2$$

Similar, but more detailed investigations were performed by de Zwaan et al. (1981) using mitochondria isolated from the mantle of the sea mussel, *Mytilus edulis*. These authors reported that malate was not only metabolized to succinate, but also to pyruvate, alanine, acetate, and propionate. Therefore, only 0.61 mol succinate was obtained from 1 mol malate. The remaining part of malate was distributed to the other metabolites, with acetate and propionate accounting for 0.11 and 0.08 mol respectively, of the offered malate.

The anaerobic formation of succinate from glucose was reported for terrestrial, fresh water, and marine invertebrates. Examples are the edible snail *Helix pomatia* (Wieser 1981), the earth worms *Lumbricus terrestris* and *Lumbricus rubellus* (Gruner and Zebe 1978), the fresh water bivalve *Anodonta cygnea* (Gäde et al. 1975), the aquatic oligochaetes *Tubifex spp.* (Schöttler and Schroff 1976; Hoffmann et al. 1983), the medical leech *Hirudo medicinalis* (Zebe et al. 1981; Hoeger et al. 1989; Hildebrandt 1992), the sea mussel *Mytilus edulis* (Kluytmans et al. 1975), the lugworm *Arenicola marina* (Schöttler et al. 1984a) and *Sipunculus nudus* (Pörtner et al. 1984a). In all species investigated the succinate content of aerobic animals is low, usually at or below 0.1 $\mu mol \cdot g^{-1}$ wet weight. During the initial phase of severe hypoxia the succinate content increases about five- to tenfold and sooner or later reaches a plateau which remains at the same level as long as a severely hypoxic $Po_2$ remains (Fig. 12). From these results and those on the mechanisms of anaerobic succinate

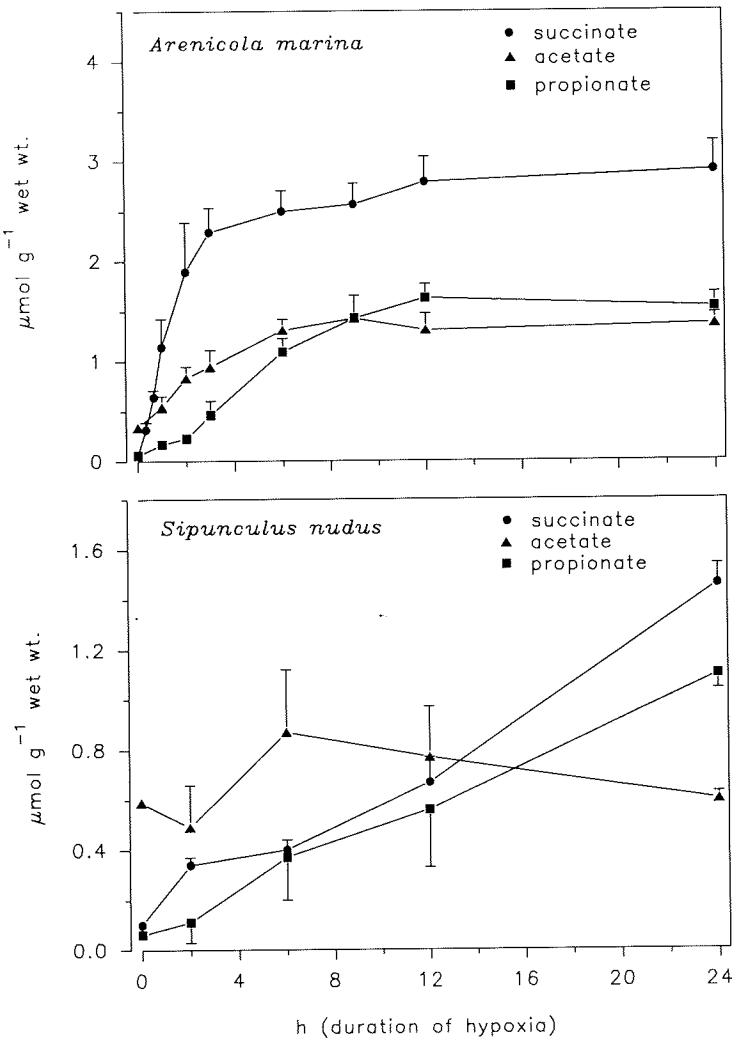


**Fig. 12.** Contents ($\mu$mol·g$^{-1}$ wet weight) of succinate and volatile fatty-acids in the body wall musculature of anoxically incubated *Arenicola marina* and *Sipunculus nudus* (After Schöttler et al. 1984a; Pörtner et al. 1984a)

formation, it is evident that this anaerobic end product can be used as a reliable indicator for the onset of anaerobic energy metabolism in hypoxia-exposed invertebrates.

Brinkhoff et al. (1983) used succinate to monitor whether some mollusks from the intertidal zone rely on anaerobiosis within their habitat. Four species were selected (Fig. 13): the limpet *Patella vulgata* and the mussel *Mytilus edulis* which are both attached to rocks and solid

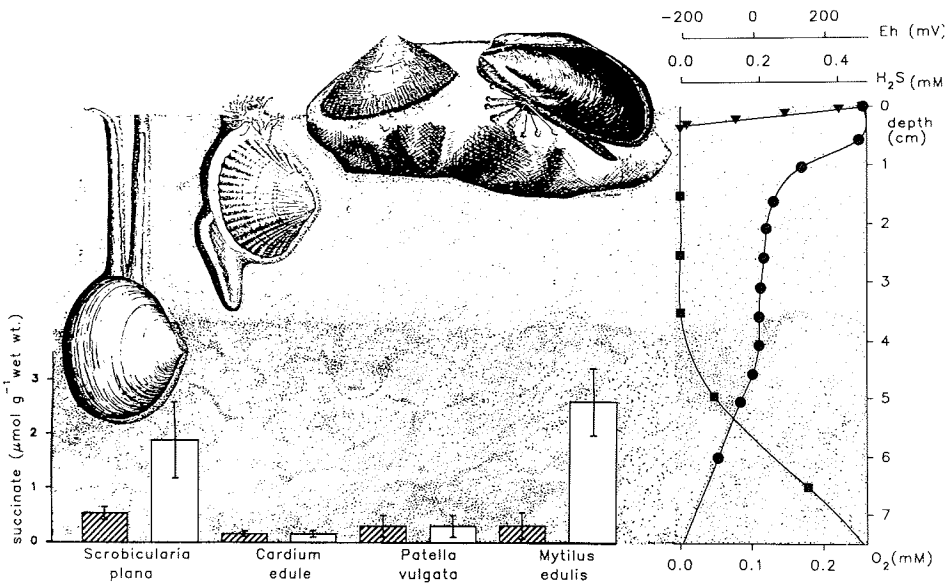


**Fig. 13.** Distribution pattern of normoxic, anoxic and sulfidic sediments as well as redox potential (*circles*), oxygen concentration (*triangles*) and hydrogen sulfide (*squares*) from intertidal flats (*right side of the panel*) (based on figures from Revsbech and Jørgensen 1986 as well as Giere 1992) and succinate contents in four different mollusks in the beginning (*hatched bars*) and at the end of low tide (*open bars*) (drawn from data of Brinkhoff et al. 1983)

surfaces of the intertidal zone, thus representing the epifauna, the cockle *Cardium edule* and the razor blade clam *Scrobicularia plana* which both belong to the endofauna. The cockle lives just below the surface of mixed sand and mud between mid to low shore levels. The razor blade clam occurs between the tide marks, burrowed about 10–15 cm deep in mixed sand, mud, or finely graded mud.

From Fig. 13 it is obvious that the four species respond differently to emersion within their habitat. The succinate contents of *Mytilus edulis* and *Scrobincularia plana* increase during 6 h of emersion to levels similar to those found during experimental anoxia. Therefore, it can be concluded that both species do resort to anaerobiosis within their habitat when emersed during the tidal cycle. In contrast, *Patella vulgata* and *Cardium edule* never showed an increase of succinate contents, although both species do so during experimentally induced anoxia (Brinkhoff et al. 1983). Obviously these species remain aerobic during emersion. The different response can be understood from the biology of these species. *Mytilus edulis* closes the valves during air exposure (Coleman 1973) and *Scrobicularia plana* withdraws its siphons from the surface of the sedi-

ment into the mantle cavity. Therefore, both clams can hardly respire air. *Cardium edule*, although it is buried, albeit just below the sediment's surface, gapes air or traps air bubbles within the mantle cavity and thus consumes oxygen from air at a rate of between 28% and 78% of that from water (Boyden 1972; Widdows et al. 1979). *Patella vulgata*, which leaves a chink between its shell and the rock it is attached to, obtains oxygen via diffusion from air into the water held between the body and the shell, thus bathing the gills. Using this mechanism, the limpet takes up as much oxygen in air as in water (Branch 1979). Therefore, the latter two species can avoid anaerobiosis when emersed during low tide and consequently do not accumulate succinate.

Besides mollusks, the annelids *Arenicola marina* and *Nereis diversicolor* were studied in their natural habitat to investigate whether or not an anaerobic energy production occurs in these worms during low tide (Schöttler et al. 1984b). In both species succinate contents increased in the same range as those found under experimental anoxia, i.e., 14-fold in *Arenicola marina* and twofold in *Nereis diversicolor*. In the latter species, there was also a marked increase in the D-lactate content, whereas levels of opines in the lugworm have not been measured. When the burrows of both worms had been dry for 8 h, a significant decrease of the phosphagen pool could also be substantiated. These results and those from the study on mollusks clearly demonstrate that, first, succinate can be used as a valuable marker of the in situ occurrence of an environmentally induced anaerobiosis, and, secondly, that some species to resort do anaerobic energy production within their habitat.

#### 5.2.3.3 Formation of Volatile Fatty Acids

A comparison of the amount of glycogen metabolized and the amounts of alanine, opines, and succinate produced during severe hypoxia revealed that these end products can only account for about 50% of the glycogen loss in *Mytilus edulis* (Kluytmans et al. 1975). From the available literature at that time, it could already be assumed that volatile fatty acids are major end products formed during anaerobiosis in free-living invertebrates.

Apart from the results of Mehlmann and von Brand (1951) which indicated that volatile fatty acids could be formed by a few species of fresh water snails, synthesis and accumulation of acetate and in particular propionate were reported on a qualitative basis from the marine tube worm *Eudistylia vancouveri* (Idler and Fagerlund 1953), the swamp worm *Alma emini* (Coles 1970) and *Arenicola marina* (Zebe 1975). In a very detailed quantitative analysis Kluytmans et al. (1975) and Gäde et

al. (1975) reported the formation of acetate and propionate in *Mytilus edulis* and *Anodonta cygnea*. Both volatile fatty acids, which account for at least half of the glycogen broken down during sustained anaerobiosis, are mainly excreted into the surrounding medium.

The anaerobic formation of acetate and propionate has been confirmed in many invertebrate species. It is particularly common among mollusks as shown for the cockle *Cardium edule* (Gäde 1975), the freshwater snails *Biomphalaria glabrata* as well as *Lymnea stagnalis* (Patience et al. 1983; de Zwaan et al. 1976), and several other species as shown in a comparative study by Kluytmans and Zandee (1983). Anaerobic acetate and propionate production is also widespread in many annelids. Following the early reports in *Arenicola marina* (Zebe 1975; Surholt 1977; Pörtner et al. 1979), *Euzonus mucronata* (Ruby and Fox 1976) and *Tubifex spp.* (Schöttler and Schroff 1976; Seuss et al. 1983), the anaerobic synthesis of both volatile fatty acids was shown in the marine polychaetes *Nephtys hombergi* (Schöttler 1982), various *Nereis* species (Schöttler 1979), and *Scoloplos armiger* Schöttler and Grieshaber 1988), the terrestrial oligochaetes *Lumbricus terrestris*, *Lumbricus rubellus* (Gruner and Zebe 1978), and *Eisenia foetida* (Zebe and Heiden 1983), as well as the leech *Hirudo medicinalis* (Zebe et al. 1981, Hildebrandt 1992). From the lesser groups of Priapulida and Sipunculida the formation of volatile fatty acids has been reported in *Halicryptus spinolosus* (Oeschger 1990) and in *Sipunculus nudus* (Pörtner et al. 1984a). It can thus be concluded that the anaerobic glycogen catabolism leading to the formation and excretion of acetate and propionate during prolonged lack of ambient oxygen is widely distributed among facultative anaerobes and can therefore be considered as another major adaptive mechanism for the survival of sustained environmental hypoxia.

#### 5.2.3.4 Metabolic Route of Acetate and Propionate Synthesis

Schroff and Zebe (1980) demonstrated the anaerobic transformation of malate and succinate into propionate in mitochondria isolated from the body wall musculature of *Arenicola marina*. Subsequent investigations of *Arenicola marina* (Schöttler 1983, 1986; Schöttler and Bennet 1991) and mitochondria isolated form the mantle of *Mytilus edulis* (Schulz et al. 1982, 1983, 1984, Schulz and Kluytmans 1983) revealed details of the enzymatic system involved (Fig. 8). For optimal in vitro production of propionate from succinate, isolated mitochondria required malate, ADP, inorganic phosphate, $Mg^{2+}$ at pH 6.7. The initial substrate for propionate formation is succinyl-CoA. It is not synthesized from succinate by the reversal of the succinyl-CoA synthetase, although this reaction is readily

reversible and concentrations of succinate are increased. Succinyl-CoA initially originates form the clockwise reaction sequence of the tricarboxylic acid cycle which spins at a lesser rate during anaerobiosis providing NADH for the reduction of fumarate (Schöttler 1977; de Zwaan et al. 1981).

Once the succinyl-CoA level is high enough within the tissue, the successive steps of propionate formation can proceed (Fig. 8). They are catalyzed by methylmalonyl-CoA isomerase, methylmalonyl-CoA racemase, propionyl-CoA carboxylase, and acyl-CoA transferase. In this reaction sequence, the decarboxylation of D-methylmalonyl-CoA to propionyl-CoA also leads to the formation of an equimolar amount of ATP, thus increasing the energy yield by another 2 mol ATP per mole glycosyl unit (Schroff and Zebe 1980). Propionyl-CoA synthesized in this reaction, however, is not split into propionate and HS-CoA by thiolytic cleavage. Instead, the CoA moiety is transferred to succinate to regenerate succinyl-CoA. Thus, only a low amount of this CoA-ester is necessary to keep the "succinate–propionate cycle" spinning. Propionate synthesis will continue as long as a sufficiently high concentration of succinate is provided by the fumarate reductase (Schöttler and Bennet 1991).

As mentioned above, in addition to propionate there is also an intramitochondrial anaerobic formation of acetate with equimolar synthesis of ATP (Wienhausen 1981). Although acetyl-CoA was reported to be involved in acetate formation by this author, it was impossible to find any enzymatic activity responsible for the thiolytic cleavage of acetyl-CoA. In a subsequent and more detailed investigation, Schöttler (1986) demonstrated that the acyl-CoA transferase is quite unspecific. Therefore, acetyl-CoA resulting from the metabolization of malate could exchange the CoA moiety with succinate, giving rise to acetate and succinyl-CoA. If the pool of succinyl-CoA becomes too high, it is possible that succinyl-CoA is not completely channeled into the succinate–propionate cycle. Instead, part of this metabolite is cleaved by succinyl-CoA thiokinase. This reaction would explain the acetate coupled formation of ATP (GTP).

The volatile fatty acids acetate and propionate do not remain within the tissue, instead they are excreted to the surrounding medium in the case of annelids and sipunculids or they may be released into the extrapallial fluid of clams. This process is of physiological importance because it prevents the disturbance of osmotic balance which would occur if protons are excreted (see below), but the anions of the acids are retained within the cell.

The excretion of acetate and propionate into the ambient water starts a few hours after the onset of lack of oxygen and becomes particularly

important during sustained environmental anaerobiosis. During a detailed investigation on *Arenicola marina*, Holst and Zebe (1986) found propionate to be linearly released into the incubation water after 6 h of anoxic incubation whereas it remained constant in the body wall musculature. After 24 h the steady-state level of propionate in the muscle tissue was 2.1 $\mu mol \cdot g^{-1}$ wet weight. Between the third and 36th h of anoxic incubation a rate of propionate excretion of 0.23 $\mu mol \cdot h^{-1} \cdot g^{-1}$ wet weight can be calculated.

Excretion of acetate was much lower and resulted in a ratio of about 3.1 propionate/acetate as compared to approximately 1.5:1 in the tissue. Excretion of both end products proceeded via the undifferentiated body wall, there was no special involvement of gills or nephridia found. The rate of excretion depended on the concentration gradient between animal and the ambient water, the chain length of the fatty acid, and the pH of the water. Recently, it was found in *Hirudo medicinalis* and *Arenicola marina* that the ratio of acetate and propionate production and its release into the water strongly depends on $Pco_2$ and pH (Hildebrandt 1992; I. Hardewig, B. Giebels, H.-O. Pörtner, and M. K. Grieshaber, unpublished results).

### *5.2.4 Regulation of Metabolic Fluxes During Short-and Long-Time Anaerobiosis*

In the early phase of anaerobiosis, which is initiated as soon as ambient oxygen has fallen below the critical $Po_2$, energy must be provided to an extent almost similar to aerobic conditions. Therefore mechanisms are exploited which allow an instantaneous anaerobic energy production. Phosphagens are transphosphorylated, aspartate is metabolized to succinate resulting in ATP synthesis from the fumarate reductase reaction and the substrate phosphorylations of the Embden–Meyerhof–Parnas pathway are recruited giving rise to lactate and opine formation. These ATP-supplying reactions, however, cannot match aerobic energy provision, since the efficiency of the anaerobic pathways is too low.

Even though energy expenditure may already be decreased during the beginning of severe hypoxia (Pörtner and Grieshaber 1993), an increase in glycolysis is necessary and can be regulated at the glycogen phosphorylase (Ebberink and Salimans 1982) and phosphofructokinase (Ebberink 1982) reaction. Schöttler (1980) found a rate increase of glycogen catabolism in *Arenicola marina* which is probably controlled by glycogen phosphorylase. This enzyme, which is present in its *a* form during normoxia in this species, might be activated by increasing levels of phosphate and AMP, but not by covalent modification (Kamp 1986).

Both metabolites increase with the beginning of severe hypoxia as demonstrated on the lugworm in NMR studies (Kamp and Juretschke 1989). Conversely, an enhanced glycolytic rate during lack of ambient oxygen would rapidly drain the available glycogen store due to the low energy yield of the anaerobic metabolism. Therefore, most invertebrates investigated drastically reduce their metabolic rate during sustained severe environmental hypoxia. This capacity of metabolic rate depression in certain animal species is as important as the specific metabolic adaptations in order to withstand harsh environmental conditions such as cold, dryness, hypoxia, or even anoxia over extended time periods (Hochachka 1982; Hochachka and Guppy 1987).

#### 5.2.4.1 Metabolic Depression During Anaerobiosis

During the past 10 years the noninvasive techniques of simultaneous direct calorimetry and respirometry as well as biochemical estimations of changes in metabolite levels have become important in determining the total amount of heat dissipation and the relative contributions of aerobic and anaerobic energy production to the total energy expenditure during hypoxia. These investigations also lend themselves well to measure the hypoxia related attenuation of the standard metabolic rate (SMR), which can be defined for most invertebrates as the minimum metabolic rate of an aerobic animal excluding locomotory or other muscular activity (Pörtner and Grieshaber 1993).

Under unfavorable conditions metabolic depression, defined as the reduction of metabolism below the SMR (see above), provides two major advantages: metabolic regulators may be more tolerant of changes of suboptimal conditions in the intracellular milieu since metabolic reactions need not proceed at high velocities. On the other hand, metabolic depression allows for the conservation of fuels under conditions which would normally require enhanced substrate consumption. Thereby the accumulation and consequently the perturbation of the intracellular milieu by anaerobic end products would be limited.

Exposure to sustained severe hypoxia may cause an increase of the glycolytic flux to make up for the low energy output of anaerobic fermentation. To obtain the same amount of ATP per unit of time as compared to oxidative metabolism, the glycolytic rate in glycogen-fermenting organisms must be enhanced by a factor of 12, or by a factor of approximately 5 if the succinate–propionate pathway is also exploited. The acceleration of glycolysis to maintain high energy output under anoxia is called the Pasteur effect since it was observed for the first

time by Louis Pasteur (1861) in yeast cells. Subsequent investigations revealed the same phenomenon in different mammalian tissues (Ramaiah 1974).

Many facultatively anaerobic invertebrates indeed lower their energy requirements under reduced oxygen availability (Shick et al. 1982; Storey 1986). It must, however, be noted that the low ATP yield of anaerobic fermentation, which equals only 6%–20% of the energy output of an aerobic metabolism, does not allow for an equivalent reduction of the glycolytic rate. A depression of the glycolytic rate can only be observed in organisms which are able to decrease their energy metabolism below values of 6%–20% of the normoxic rate (Grieshaber and Hardewig 1993).

In the peanut worm *Sipunculus nudus*, the ATP turnover under anoxia is reduced to 27% of the normoxic control value, but the glycolytic rate is enhanced by a factor of 2 compared to normoxic conditions (Hardewig et al. 1991a). The situation is similar in the lugworm *Arenicola marina*. During anaerobiosis a reduction of ATP turnover by 78% is met by a two fold increase of the glycolytic flux (calculated from data of Schöttler et al. 1983 and Schöttler 1989). Conversely, de Zwaan and Wijsman (1976) found that the energy demand of the sea mussel *Mytilus edulis* drops to 5% of the normoxic value under anoxic incubation. From their data of anaerobic end product accumulation during 48 h of anoxia a decline of the glycolytic rate to 43% of the aerobic value can be calculated. In the cockle *Cardium tuberculatum* a reduction of the ATP consumption to 6% corresponds to a depression of the glycolytic rate by a factor of 2 (Meinardus-Hager et al. 1989).

Besides using the estimations of ATP turnover and substrate depletion, the metabolic rate can be assessed by measuring the dissipated heat per unit of time. Heat dissipation of several species has been determined calorimetrically under both normoxic, hypoxic, and anoxic conditions. Gnaiger and Staudigl (1987) found that heat production was reduced to 23% of the normoxic value in the aquatic oligochaete *Lumbriculus variegatus* when exposed to a nitrogen atmosphere. In *Tubifex tubifex* a reduction to 10%–15% has been observed during anaerobiosis (Famme and Knudsen 1984), and *Mytilus edulis* showed a decline of heat release to 5%–10% during anoxic incubation (Famme et al. 1981). Unfortunately, an estimate of the rates of ATP consumption and glycolysis in these experiments is impossible since there is no constant relationship between any of the three parameters.

The dissipated heat per unit of ATP turnover is approximately $-80\ kJ \cdot mol^{-1}$ ATP for aerobic metabolism, but less than

**Table 5.** Relations between heat dissipation, ATP turnover, and glycolytic rate at different oxygen partial pressures ($Po_2$) in the peanut worm *Sipunculus nudus*. Data from Hardewig et al. 1991a

| $Po_2$ [kPa] | Heat dissipation [$mJ \cdot h^{-1} \cdot g^{-1}$ wet weight] | ATP turnover [$\mu mol \cdot h^{-1} \cdot g^{-1}$ wet weight] | Glycolytic rate [$nmol \cdot h^{-1} \cdot g^{-1}$ wet weight] |
|---|---|---|---|
| 18.0 | 110.1 | 1.60 | 43.3 |
| 2.7 | 35.5 (33%) | 0.60 (37.5%) | 52.9 (122%) |
| 0 | 21.5 (19.5%) | 0.44 (27.5%) | 77.0 (180%) |

Numbers in brackets give the respective percentages of the aerobic control values at 18 kPa.

$-40\ kJ \cdot mol^{-1}$ ATP for propionate fermentation (Gnaiger 1983). This means that the heat release during anoxia may be decreased to 50% of the normoxic value even though the ATP turnover remains constant. The rate of heat dissipation is even less correlated with the glycolytic flux. During an aerobic metabolism $-2968$ kJ are dissipated per mole of oxidized glycosyl unit, whereas the heat production is decreased to $-162\ kJ \cdot mol^{-1}$ glycogen during anaerobic lactate formation (Gnaiger 1983). Table 5 shows that heat dissipation of *Sipunculus nudus* is reduced with decreasing ambient oxygen partial pressure, even though the glycolytic rate increases.

This analysis elucidates that the term "metabolic rate" requires a clear definition, i.e., whether this term refers to ATP turnover, substrate consumption, or heat dissipation. Consequently, metabolic rate should be defined as the rate of ATP turnover, and metabolic depression means a reduction in energy requirements.

### 5.2.4.2 Down-Regulation of Anaerobic Metabolism

The reduction of the metabolic rate under limited oxygen availability is, generally, caused by two mechanisms: first, by the inhibition of ATP-generating pathways, and, secondly, by the depression of ATP-requiring processes.

Since anaerobic glycolysis is the introductory pathway for anaerobic ATP synthesis, strong glycolytic control is essential in facultative anaerobes. Numerous studies have revealed phosphofructokinase (PFK), which catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, as the key regulatory enzyme of glycolysis. Inhibition of PFK by ATP and its activation by AMP and inorganic phosphate is held responsible for the induction of the Pasteur effect (for review see

Ramaiah 1974). Since the PFK of organisms which exhibit no or even a reversed Pasteur effect show the same kinetic properties, additional mechanisms are required to suppress glycolytic activation. Three main regulatory mechanisms to attenuate the glycolytic flux during anaerobiosis are discussed in the literature (for review see Storey 1988; Somero and Hand 1990): (a) allosteric regulation, (b) covalent modification of key enzymes; and (c) enzyme association/dissociation.

#### 5.2.4.3 Allosteric Regulation

The regulation of enzyme activities by allosteric effectors allows for a fast response of flux velocities to changes of intracellular metabolite levels. In recent years, fructose-2,6-bisphosphate (F-2,6-$P_2$) has been revealed as a potent regulator of metabolic processes (Hers and van Schaftingen 1982). Its potential to modulate PFK activity and, thereby, the glycolytic rate, favored this compound to be responsible for the depression of glycolysis under hypoxia. In fact, Storey (1985) observed a strong decrease of the F-2,6-$P_2$ content in soft tissues of different marine invertebrates such as *Ostrea edulis*, *Mytilus edulis*, and *Littorina littorea* after hypoxic incubation. In the muscle tissue of those animals, however, F-2,6-$P_2$ does not seem to hold any regulatory function. Levels of F-2,6-$P_2$ remained constant in the adductor muscle of *Ostrea edulis* and *Mytilus edulis* and even increased in the foot muscle of *Littorina littorea* (Storey 1985). Also in the muscle tissue of *Sipunculus nudus* no changes in F-2,6-$P_2$ concentrations occurred after hypoxic incubation (Hardewig et al. 1991b). In the body wall of the priapulid *Halicryptus spinolosus*, however, a slight transient decrease of fructose-2,6-bisphosphate contents has been observed within 20 days of anoxic incubation (Oeschger and Storey 1990). Not only changes in F-2,6-$P_2$ levels, but also the sensitivity of PFK to these metabolite changes vary between different tissues. Villamarin et al. (1990) showed that PFK from the mantle of *Mytilus galloprovincialis* is far less sensitive towards bisphosphate than the isozyme from the adductor muscle. Obviously the regulatory role of F-2,6-$P_2$ in metabolic rate depression cannot generally be defined, it rather seems to be species as well as tissue specific.

#### 5.2.4.4 Covalent Modification of Key Enzymes

Enzyme modification by covalent incorporation of phosphate allows a profound and rapid change of the active/inactive ratio of enzymes, making it an important mechanism of metabolic control (Cohen 1980). Phosphorylation is responsive to extracellular signals (e.g., hormones) by

stimulation of protein kinases via cAMP and $Ca^{2+}$ as second messengers. In the earthworms *Eisenia foetida* and *Allolobophora calligenosa* anoxic incubation caused significant changes of the kinetic properties of pyruvate kinase (PK). The enzyme variants also eluted in different peaks from a DEAE-cellulose column, indicating an altered charge of the anaerobic enzyme form (Michaelidis and Storey 1990). Similar results were obtained for PFK and PK of the marine whelk *Busycotypus canaliculum* (Storey 1984; Plaxton and Storey 1984a). In general, enzymes isolated from anaerobic tissues showed less sensitivity towards allosteric activators and higher responsiveness towards inhibitors. Treatment of the anaerobic enzyme form with alkaline phosphatase restored the properties of the aerobic enzyme variant (Storey 1984; Plaxton and Storey 1984a). The authors concluded from their data that anoxia induces phosphorylation of PFK and PK inactivates these enzymes and causes a reduced carbon flow through glycolysis. Plaxton and Storey (1984b) found a general increase of $^{32}P$-incorporation into protein in the muscle tissue of the whelk during anoxia. This observation may give some evidence for a broader significance of anoxia-induced inactivation of enzymes by phosphorylation, which could be responsible for an overall metabolic depression (Plaxton and Storey 1984b).

However, this mechanism of metabolic down-regulation at the level of PFK has been questioned by Biethinger et al. (1991). They purified PFK from the mollusks *Helix pomatia* and *Mytilus edulis* and phosphorylated these proteins in vitro by addition of cAMP-dependent protein kinase. The modified enzymes showed a higher sensitivity towards activators such as AMP and F-2,6-$P_2$. Phosphorylation of the PFK from *Mytilus edulis* even caused a significant increase of the maximal activity ($V_{max}$).

These results are inconsistent with the above-mentioned conclusions of Storey (1984), which were based on kinetic studies of PFK in crude extracts from aerobic and anaerobic tissues of *Busycotypus canaliculatum.* Biethinger et al. (1991) therefore doubt that the kinetic differences observed by Storey (1984) are actually due to enzyme phosphorylation and they suggest a reevaluation of the data with purified enzyme preparations where phosphorylation can be unequivocally proven (Biethinger et al. 1991).

#### 5.2.4.5 Association and Dissociation Processes of Enzymes

In recent years a variety of studies have given evidence that metabolic processes are not just operable in the soluble phase of the cytoplasm, but that functionally linked enzymes are organized in metabolons by the

association of enzymes to subcellular structures (Arnold et al. 1968; Arnold and Pette 1970; Clarke and Masters 1974; Srivastava and Bernard 1986). The reversible compartmentation of metabolic pathways provides an effective mechanism of metabolic control. Metabolite channeling, which means the direct transfer of substrates and products from enzyme to enzyme, is much faster than random diffusion of the reactants in the soluble phase. Additionally, kinetic properties of enzymes are partially altered by association to subcellular structures, leading to increased catalytic capacities (Srivastiva and Bernhard 1986).

The reversible association of glycolytic enzymes to actin filaments is well documented for vertebrate muscle tissues (Arnold and Pette 1968; Siegel and Pette 1969; Pette 1975; Masters et al. 1987). Physiological conditions which stimulate the glycolytic flux in vertebrate muscles, such as tetanic stimulation or ischemia, could be shown to cause an enhanced proportion of bound enzyme (Clarke et al. 1984; Weber and Pette 1990). A shift from the soluble to the bound enzyme form provided a markedly increased substrate affinity of aldolase and PFK in mammalian tissues. Conversely, only little information is available on reversible enzyme association in invertebrates. The separation of bound and soluble enzyme fractions by centrifugation of muscle tissue homogenates revealed a significant decrease in the percentage of bound enzyme in *Busycotypus canaliculatum* after 21 h of anoxic incubation (Plaxton and Storey 1986). In the land snail *Otalea lactea*, the dissociation of enzymes from subcellular structures was shown to be a transient effect. After 14.5 h of anoxia the percentage of bound forms decreased significantly and after 45 h control values were reestablished for most of the glycolytic enzymes (Brooks and Storey 1990). The same phenomenon was observed in *Patella caerulea*, where the percentage of bound enzyme already decreased after 4 h of anoxia (Lazou et al. 1989). These results suggest that change of enzyme binding is a fast mechanism to initiate metabolic depression under hypoxic conditions.

#### 5.2.4.6 Coordinated Attenuation of Catabolic and Anabolic Reactions

Besides regulation on the molecular basis of down regulation, metabolic depression also requires the coordinated arrest of both catabolic and anabolic reactions. Since the maintenance of ion gradients over cell membranes, in particular the maintenance of the $Na^+/K^+$ gradient, may require up to 60% of the basal energy turnover, this process is especially sensitive to reduced energy availability. In fact, the breakdown of the membrane potential, followed by increased $Ca^{2+}$ influx into the

cell is the primary reason for cell damage or even cell death in hypoxia-sensitive tissues (Hochachka 1986).

In facultative anaerobes, however, ion gradients are not disturbed, despite a significantly decreased ATP turnover during anaerobiosis (Mangum 1980; Surlykke 1983). Hochachka and Guppy (1987) postulated a reduced membrane permeability under hypoxia in these animals, which prevents equilibration of intra- and extracellular ion concentrations. They proposed the possibility of a hormone-mediated reduction of ion channel densities during anaerobiosis. These ideas are supported by studies on toad bladder, where the density of ion channels is regulated by antidiuretic hormone and aldosterone (Palmer et al. 1982).

Not only passive ion flux, but also active pumping of ions seems to be diminished with reduced metabolic rate. Pörtner et al. (1991) found some evidence that $H^+$ cation countertransport in *Sipunculus nudus* is less during hypoxic incubation than under subsequent aerobic recovery.

Another important component of metabolic maintenance resides in the turnover of structural and catalytic proteins. With the depression of metabolic activity a reduction of protein synthesis is to be expected. An arrest of protein synthesis, however, must be coordinated with a blockage of protein decomposition in order to maintain structural integrity of the organism to make an instantaneous recovery possible when oxygen becomes available again. Even though this is an important aspect of metabolic depression, the problem has achieved only little attention so far. Data on protein biosynthesis during anaerobiosis are available for embryos of *Artemia*. These organisms are able to sustain severe anoxia for months by reaching an almost ametabolic state (Clegg and Jackson 1989). It could be shown that the amplitude of polysome profiles is changed with the entry into anaerobic dormancy. After 15 min of anoxia the number of polysomes is markedly diminished, indicating that the translational activity of the tissue had decreased (Hofmann and Hand 1992). Tracer experiments revealed that synthesis of cytochrome-*c*-oxidase is arrested during anaerobic dormancy (Hofmann and Hand 1990). The half-life of this enzyme is considerably enhanced from 32 h during aerobiosis to periods in the order of weeks under hypoxic incubation (Hand 1993).

It is still unclear which mechanisms mediate between limited oxygen availability and the coordinated depression of both anabolic and catabolic metabolism. A drop in intracellular pH, which is always observed during anaerobiosis (Ellington 1983 b, c; Pörtner et al. 1984b), certainly contributes to the coordination of metabolic events. Juretschke and Kamp (1990) incubated *Arenicola marina* anoxically with and without the addition of an extracellular buffer capacity by the addition of

phenylphosphoric acid. Intracellular pH dropped from 7.3 to 6.7 under hypoxia in control animals, whereas addition of buffer kept the $pH_i$ stable at 7.2. In animals with high intracellular pH the glycolytic rate was enhanced by a factor of 2.3 compared to acidotic animals. In *Artemia* embryos an artificial elevation of $pH_i$ by adding ammonia led to a significant increase of heat dissipation during anaerobiosis (Hand and Gnaiger 1988), whereas aerobic acidosis, induced by artificial hypercapnia, caused a depression of the metabolic rate (Busa and Crowe 1983). The synthesis of cytochrom-*c*-oxidase in *Artemia* embryos can be blocked by aerobic intracellular acidification to the same degree as observed during anoxia (Hofmann and Hand 1990).

These experiments illustrate the importance of intracellular acidosis for metabolic down-regulation. One of the primary effects of increased intracellular proton concentrations is an increased protonation of amino acid residues, mainly histidyl groups. The degree of protonation may have a great impact on the kinetic properties of enzymes. The selective inhibition and activation of regulative enzymes is a widespread mechanism of direct pH control. By changing the charge of peptide chains, shifts of the intracellular pH may also cause an alteration in protein–protein affinities. Thus, intracellular acidification could cause the dissociation of multi-enzyme complexes observed under hypoxia (Somero and Hand 1990).

As mentioned above, the depression or even arrest of protein synthesis, observed during anaerobic dormancy in *Artemia* embryos, can be induced by aerobic acidosis as well. Phosphorylation and dephosphorylation of the translation components, primarily initiation and elongation factors, is a major control mechanism of protein synthesis. Generally, phosphorylation of translation components results in an enhanced rate of biosynthesis (Hershey 1991). In the ciliate *Tetrahymena* a correlation between intracellular acidification and dephosphorylation of the small ribosomal subunit has been shown (Goumard et al. 1990). However, Brooks and Storey (1989) did not find any influence of acidification on the phosphorylation of glycolytic enzymes. They incubated isolated muscle tissue of the whelk *Busycotypus canaliculatum* with and without oxygen and generated tissue pH values of either 7.0 or 7.6 by the addition of buffer substances. Their results demonstrated that phosphorylation-mediated changes of the kinetic properties of pyruvate kinase were correlated with oxygen availability and not with tissue pH. In the same study, the addition of various hormones to isolated tissues could not induce the phosphorylation of PK. Only cAMP and $Ca^{2+}$/calmodulin showed slight effects on the kinetic properties of the enzyme. Brooks and Storey (1989) conclude from their results that

changes of oxygen levels are not detected by a central oxygen sensor and mediated to the tissues by hormonal transmission. Instead, oxygen availability is sensored by each cell individually and the intracellular response is coordinated by second messenger such as cAMP and $Ca^{2+}$/calmodulin.

The depression of the metabolic rate under hypoxic stress provides profound advantages for facultative anaerobes. The reduction of the energy demand to levels which can be met by low rates of a fermentative metabolism prevents a rapid exhaustion of substrate supplies. The glycogen stores of *Mytilus edulis* (250 μmol glycogen per gram wet weight) (de Zwaan and Wijsmann 1976) would only be sufficient to keep up the normoxic rate of ATP provision by anaerobic fermentation for 3 days (calculated with data from de Zwaan and Wijsmann 1976). By strongly reducing their energy requirement, however, these animals are able to sustain anoxic conditions for weeks (Storey 1985).

## 6 Anaerobic Production and Fate of Protons During Environmental Anaerobiosis

During sustained environmental anaerobiosis, metabolism shifts from the initial transphosphorylation of phosphagen, the fermentation of aspartate and glycogen to glycogen depletion only. In the beginning of environmental anaerobiosis, a high rate of glycolytic proton production is counterbalanced by proton removal during phosphagen and aspartate metabolization, thus preventing a pronounced acidification of the intracellular milieu. Later on, the predominant synthesis of the volatile fatty acids acetate and propionate from glycogen provides ATP at a greater yield than glycolysis at low rates of end product and proton formation. In general, facultatively anaerobic invertebrates reduce the extent of proton formation during long-term anaerobic metabolism, by ethanol production or the use of other ("aerobic") mitochondrial mechanisms of proton elimination. Proton elimination results from the depletion of the phosphagen, the accumulation of buffer substances (largely inorganic phosphate), and the use of decarboxylation reactions.

The reduction of $H^+$ formation is linked to metabolic compartmentation and an increase in the ATP output of metabolic pathways (Pörtner 1987a). In addition, protonated end products are released by nonionic diffusion (Pörtner 1993). These mechanisms, together with the reduction of the metabolic rate, result in less proton formation per time unit as compared to lactate or opine accumulation. Therefore, an

important advantage brought about by metabolic depression and the degradation of glycogen to acetate and propionate is a reduction of the metabolically released protons. The net production of protons during anaerobiosis ranges between 0.28 and 1.33 $H^+$ per ATP molecule (Pörtner 1987a,b). It is conceivable that high rates of anaerobic metabolism cannot be maintained over long periods because they quickly lead to a strong acidification of the tissue, which eventually may cause severe cell damage.

Interestingly, in *Sipunculus nudus* an increase in anaerobic metabolic rate is correlated with an increase in anaerobic glycolysis and acetate formation (Pörtner et al. 1986a), which is tantamount to a higher amount of protons in relation to ADP phosphorylation than during propionate synthesis. Kluytmans et al. (1980) also described an increased acetate formation in *Mytilus edulis* during anaerobiosis depending on the season. This comparison reveals that the flexibility of the anaerobic rate in some species depends mainly on the exploitation of the glycolytic pathway making allowance for an enhanced formation of protons or an increase in the overall $H^+$/ATP ratio.

### *6.1 Acid Base Regulation During Anaerobiosis: Quantitative Analysis*

Changes in the acid base status recorded during environmental lack of oxygen in marine invertebrates are small and reflect the metabolic $H^+$ stoichiometries (Pörtner 1987a, b) as well as the depression in metabolic rate. Several studies have been carried out using isolated muscle preparations and $^{31}$P-NMR (Barrow et al. 1980; Ellington 1983b; Graham and Ellington 1985), while other studies have analyzed changes in the acid base status during anaerobiosis in whole animals (Pörtner et al. 1984c; Walsh et al. 1984; Booth et al. 1984; Pörtner et al. 1986b; Pörtner 1987b; Kamp and Juretschke 1989; Juretschke and Kamp 1990; Hardewig et al. 1991). However, only a few studies have addressed the quantitative basis of acid base changes during anaerobiosis.

Acid base changes reported for the initial period of anaerobiosis are not consistent. In some studies an initial drop in intracellular pH has been found (Ellington 1983c), whereas others reported $pH_i$ to remain constant (Barrow et al. 1980), or even increase during transition to sustained experimentally induced hypoxia (Pörtner et al. 1984b). This difference can be readily explained if the phosphagen content and the pattern of $pH_i$ changes are compared (Pörtner et al. 1984c). Low phosphagen contents are found in tissues which exhibit an initial aci-

dosis, whereas tissues with high amounts of phosphagen become alkalotic. The residual variability may be explained by different glycolytic rates which have not been reported in all cases.

Another factor to be considered is the fluctuation in $Pco_2$. Air exposure during anaerobiosis in bivalves or restriction of water exchange for animals dwelling in a sediment promotes the build up of a respiratory acidosis during the transition period as well as during complete anaerobiosis (Fig. 14) (Toulmond 1973; Pörtner 1982; Pörtner et al. 1983; Walsh et al. 1984). In contrast, experimental hypoxia under a normocapnic atmosphere in water is linked to minor $Pco_2$ changes and, therefore, acid base disturbances are restricted to metabolic processes. In *Sipunculus nudus* a metabolic alkalosis was found during the period of phosphagen and aspartate depletion turning into a metabolic acidosis in

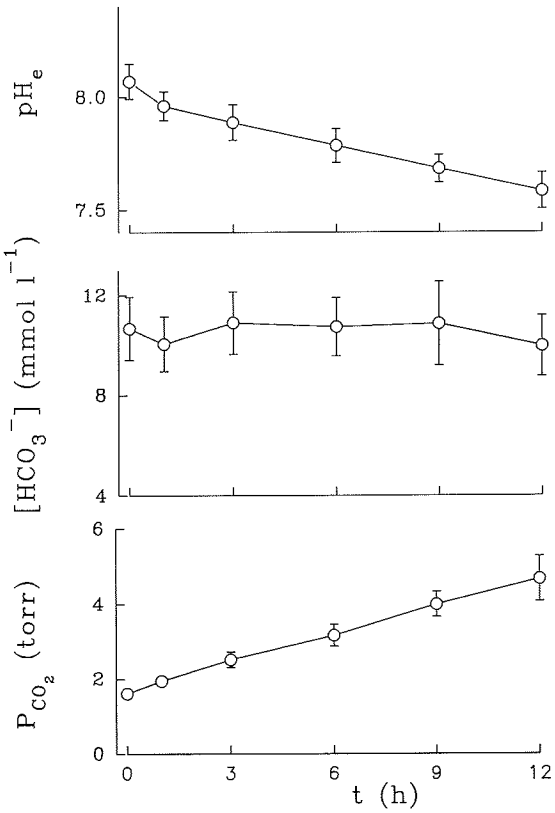


**Fig. 14.** Exposure of *Sipunculus nudus* to a low tide situation in a sandy sediment leads to the accumulation of $CO_2$ (rising $Pco_2$) and the development of a respiratory acidosis as measured in the coelomic fluid plasma. Hence $CO_2$ aggravates the acidifying influence of anaerobic metabolism and supports the shift to the long-term use of the propionate pathway (see text) (Pörtner et al. 1983)

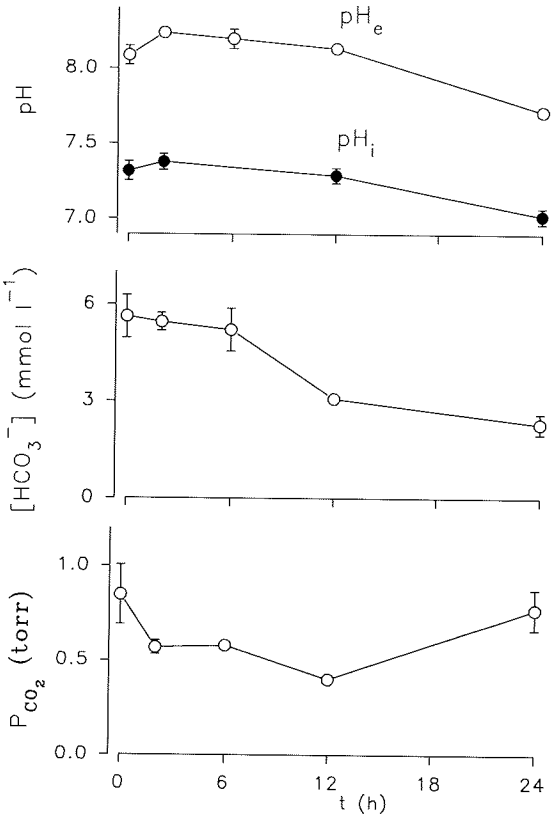


**Fig. 15.** Changes in the intracellular pH of the body wall musculature and in the extracellular acid base parameters (pH, bicarbonate concentration and $Pco_2$) of the coelomic fluid of *Sipunculus nudus* during 24 h of experimental hypoxia. Note: under these conditions the water is flushed with normocapnic nitrogen gas and the build up of $CO_2$ as observed during low tide does not occur (see text) (Redrawn after Pörtner et al. 1984b)

intra- and extracellular compartments during 24 h of anaerobiosis (Fig. 15) (Pörtner et al. 1984b).

Based on multicompartmental analyses, changes in the anaerobic acid base status of *Sipunculus nudus* could be quantitatively explained by the accumulated end products (Pörtner 1987b). In contrast to the contention of Graham and Ellington (1985) and Gnaiger et al. (1989) that acid base disturbances and metabolic $H^+$ formation are not related, this analysis provided experimental evidence for a quantitative dependence of acid base disturbances in animal tissues on anaerobic metabolism. It is also possible to state that the metabolic mechanisms considered include all those responsible for anaerobic ATP production (Hardewig et al.

1991a), and they eliminate previously large uncertainties about the potential existence and contribution of unknown metabolic pathways to anaerobic energy production and changes in the acid base status. In conclusion, during sustained anaerobiosis, metabolic rate, and rate of proton production are closely correlated (for review see Pörtner 1993).

### *6.1.1 Rates and Set Points of Acid Base Regulation*

Based on these results, an analysis of the net transmembrane movements of nonrespiratory protons revealed that proton-equivalent ion exchange between animal and ambient water is related to the changes in acid base status observed during both anaerobiosis and postanaerobic recovery (Pörtner et al. 1991). However, anaerobic ion exchange was inefficient to maintain aerobic acid base parameters in intra- and extracellular compartments (Pörtner et al. 1984b). On the background of a net proton uptake under control conditions (possibly related to the diet of the animals), a net base release during initial alkalosis turned into a net proton release during the period of progressive metabolic acidosis. This pattern as compared to the drastic increase in net proton release observed during recovery would suggest that the small exchange rates recorded during anaerobiosis could be linked to the observed metabolic rate reduction. A correlation between metabolic and ion exchange rates is also suggested by comparing animals exhibiting differences in aerobic and anaerobic capacities of metabolism and acid base regulation depending on the season (Fig. 16) (Pörtner et al. 1986a, b).

Following anaerobiosis, an increased net release of protons into the ambient water reflects an additional metabolic proton formation. However, tissue pH is no longer affected indicating an enhanced proton release from the tissues. This leads to a transient aggravation of the extracellular acidosis which is quantitatively related to the proton-producing resynthesis of the phosphagen (Pörtner et al. 1986b). The return of the metabolic rate to prehypoxic values therefore appears as a prerequisite for the drastic increase in proton-equivalent ion exchange rates. The increase in net proton transfer between animal and ambient water may reflect an increased efficiency of acid base regulation necessary to cope with the enhanced proton formation during environmental anaerobiosis.

As compared to *Sipunculus nudus*, the capability of *Mytilus edulis* to regulate intracellular pH during anaerobiosis and aerobiosis is less developed. The peanut worm's burrowing habit and adaptations to extensive digging in the substratum may require a better pH regulation than the sedentary bivalve. Conversely, the sea mussel may be able to

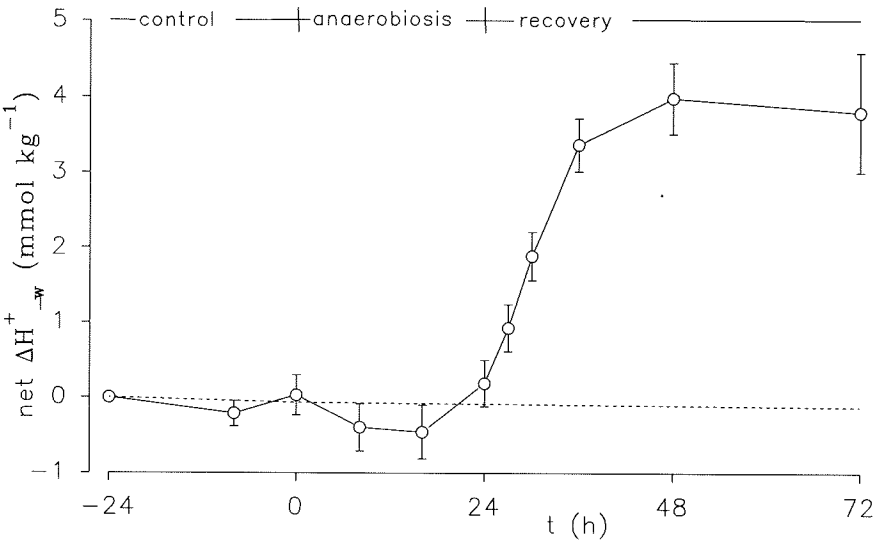


**Fig. 16.** Net proton movements observed between animals and ambient water (*w*) under control conditions, during a period of 24 h of experimental hypoxia, and during subsequent recovery. Note the drastic increase in net proton removal with the onset of postanaerobic recovery (for further explanations see text) (Based on data by Pörtner et al. 1991)

tolerate large pH deflections in some tissues (Walsh et al. 1984). Large decreases in intracellular pH, however, reduce the glycolytic rate and therefore make the shift to succinate–propionate formation necessary in order to yield more energy per glycosyl unit fermented.

During anaerobiosis acid base disturbances are not just tolerated but also part of a changing strategy of acid base regulation (Pörtner 1987b, 1993). $pH_i$ starts to be defended at a lower value than during aerobiosis. A downward shift of $pH_i$ during anaerobiosis could be useful to the cell because it induces metabolic depression. An immediate acidosis was observed during the very beginning of anaerobiosis in isolated muscles of bivalves (Ellington 1983c; Graham and Ellington 1985). In specimens of *Sipunculus nudus* dwelling in their burrows in the sand and subjected to a "low tide" situation $pH_i$ fell, but only to a limited extent. In contrast $pH_e$ continued to fall during the extended low tide period (H.-O. Pörtner, unpublished results). The defense of $pH_i$ starts about 0.2 pH units below the normoxic $pH_i$ suggesting a shift of set points to more acidic pH values during anaerobiosis (Pörtner 1993).

Extracellular acid base regulation in hypoxia-tolerant worms is also largely uninvestigated. The results obtained by Toulmond (1973) and Pionetti and Toulmond (1980) in *Arenicola marina* indicate that this

animal utilizes its extracellular compartment for the storage of protons during anaerobiosis. In *Sipunculus nudus* the extracellular compartment comprises 58% (65%, coelomic cells included) of the body (Pörtner 1982, 1987b). Despite its low buffer value, this volume can take up the major amount of protons formed in metabolism. This enables the animal to become transiently independent of acid base regulation via ionic exchange with the ambient water, which may be impeded during anaerobiosis at low tide or during digging excursions in the sediment.

Other invertebrates like crabs and bivalves possess calcium carbonate-containing shells which they can use to buffer metabolic protons during long periods of anaerobiosis (for review see Cameron 1986, 1990). This is certainly more economical during anaerobiosis than the release of protons to the environment by ATP-consuming proton-equivalent ion transport, which, moreover, has to be accomplished during the early recovery period. Shell resynthesis may occur later, after the high energy demand for the replenishment of energy stores has ceased and the animal is not stressed by extreme conditions anymore.

### *6.2 Modulation of Catabolism by Acid Base Parameters*

A regulatory drop in pH during anaerobiosis may contribute to ensure optimal flux through the propionate pathway. Low pH can be assumed to support PEP carboxylation, when an initial acidosis occurs in tissues containing little phosphagen or during respiratory acidosis. Accordingly, a rapid overshoot and excess in volatile fatty acid production over aspartate metabolization was observed during low tide simulation experiments (Pörtner et al. 1983). A low pH will also support propionate formation by increasing acyl-CoA transferase activity (Schulz et al. 1984), which again would support a shift from high to low proton production and reduce pH oscillations even further.

The overshoot observed for fatty acid production could also be related to the production and accumulation of $CO_2$ during restriction of respiratory gas exchange at low tide. The resulting metabolic acidosis will release $CO_2$ from bicarbonate stores and augment further $CO_2$ accumulation. Phosphoenolpyruvate carboxykinase utilizes $CO_2$ rather than bicarbonate as a substrate (Pörtner 1987a) and the accumulation of $CO_2$, which indeed occurs in anaerobic *Arenicola marina* (I. Hardewig, H.-O. Pörtner, and M.K. Grieshaber, unpublished results), can be expected to support a high flux through the succinate-propionate pathway.

Conversely, a reduction in bicarbonate concentration during metabolic acidification will favor the activity of propionyl-CoA carboxylase

since bicarbonate is released during this biotin-dependent decarboxylation reaction (Pörtner 1987a, 1989; I. Hardewig, H.-O. Pörtner, and M.K. Grieshaber, unpublished results). This assumption is supported by the pH optima of this enzyme being lower for decarboxylation than for carboxylation (Schulz et al. 1983). Owing to partial inhibition of the respiratory chain, mitochondrial acidification may be especially pronounced during anaerobiosis.

The intracellular pool of inorganic phosphate represents another important example of dissociation equilibria affecting substrate concentrations. For example, $HPO_4^{2-}$ is thought to be a substrate for glycogen phosphorylase (Kasvinski and Meyer 1977), and decreasing pH reduces the fraction of $HPO_4^{2-}$ in the total phosphate pool. This may be important when the phosphagen pool is depleted and cell pH falls causing an apparent decrease in phosphate concentration for glycogen catabolism. Glycogen phosphorylase activation may depend upon the accumulation of inorganic phosphate during phosphagen depletion not only in vertebrates (Wilson et al. 1981), but also among invertebrates (Kamp and Juretschke 1987). The alkalosis resulting from phosphagen transphosphorylation increases the fraction of $HPO_4^{2-}$ synergistically augmenting the provision of phosphate for the enzyme.

These few examples demonstrate how metabolism may indirectly be affected by pH when the concentration or protonation of a substrate is changed (Pörtner 1987a). Such interrelationships are often unconsidered during enzyme studies and become especially important when quantitative conclusions are drawn from *in vitro* studies for application to *in vivo* situations.

## 7 Functional Anaerobiosis During Enhanced Muscular Activity

Functional anaerobiosis which results from enhanced muscular activity is often experienced by marine, limnic, and terrestrial invertebrates. Animals escaping from predators, in pursuit of prey, or during burrowing activity in substrata use specific muscles with extreme performance. The energy turnover of these muscles is too high to be met by the aerobic scope of the energy metabolism. The sudden increase of ATP consumption relies on substrate phosphorylations from a phosphagen and from anaerobic glycolysis. Both metabolic possibilities are characterized by high flux rates which are obtained almost instantaneously. The concomitant increase in NADH production from the Embden–Meyerhof–Parnas pathway cannot be balanced by mitochondrial oxidation

because the transport capacity for reducing equivalents into the mitochondria or the flux rate of the Krebs cycle are limiting. Although these rapidly working muscles derive ATP from anaerobic glycolysis, they need not necessarily have an anaerobic mitochondrial compartment, since oxygen supply for the respiratory chain may still be sufficient. In many species, however, strong muscle contraction can impede the circulatory system and thereby cause a restricted oxygen supply.

Functional anaerobiosis has been extensively studied in many species. The body wall muscle of burrowing annelids, the adductor muscle of valve-clapping bivalves, the pedal retractor muscle of jumping gastropods, the mantle muscle of cephalopods swimming by jet propulsion, or tail-flipping crustaceans have gained particular interest because of the peculiar locomotory movements in these species. Even animals which are buried in the substratum and are usually considered very sluggish are of interest. Although these animals are quite well protected from predators, they can nevertheless be preyed upon by wading birds, fish, or carnivorous invertebrates. Parts of the larger species projecting near or above the surface may be bitten off or the entire animal pulled out of the burrow. Buried species try to avoid attacks by rapidly retreating into the depth of the burrow. If caught, they attempt to anchor the body against the burrow wall via strong isometric muscle contractions. Species living in sandy or muddy marine sediments may also be washed out when the surface layers are suspended by strong wave action, or they may translocate themselves within the sediment or even migrate, as proposed for *Arenicola marina* when the winter water temperature at low tide drops to $+1$ to $-1$°C (Werner 1954, 1956). During reburrowing strong and repeated muscle contractions require a high rate of ATP production which is accomplished in part by an anaerobic metabolism.

### *7.1 Rapid Formation of ATP from Phosphagens*

Phosphagens not only serve as storage compounds of phosphate-bond energy during environmentally induced severe hypoxia, they are also utilized during functional hypoxia. The importance of phosphagens for immediate energy provision has been demonstrated in the body wall musculature of *Arenicola marina* and *Sipunculus nudus* which were forced to dig into the substratum until exhausted. Digging movements lasted for 65 and 45 min, respectively. In lugworms, the phosphotaurocyamine content was depleted from 8.9 to 2.6 $\mu$mol·g$^{-1}$ wet weight (Siegmund et al. 1985) and phospho-L-arginine was diminished in the body wall musculature of digging *Sipunulus nudus* from 30.6 to 17.2 $\mu$mol·g$^{-1}$ wet

weight (Pörtner et al. 1984a). The degradation of phosphotaurocyamine in lugworms was prominent during the first three digging cycles which are usually faster (5 min each) than the remaining cycles. The rate of phosphagen depletion in digging lugworms amounted to 0.42 $\mu$mol·min$^{-1}$·g$^{-1}$ wet weight during the first three cycles. Conversely, lugworms kept in nitrogen-saturated sea water degraded phospho-L-arginine at a rate of 0.014 $\mu$mol·min$^{-1}$·g$^{-1}$ wet weight (Table 2). A rapid phosphagen usage is characteristic for the flare up of the metabolic rate during functional anaerobiosis and a low phosphagen turnover distinguishes environmental anaerobiosis.

In order to analyze the interdependence of the transphosphorylation of phospho-L-arginine with the development of muscle tension, the disappearance of phospho-L-arginine and the isometric force development were measured in isolated and directly stimulated (0.1 Hz at 15°C) introvert retractor muscle of *Sipunculus nudus* (Kreutzer 1987; Kreutzer et al. 1985). Relative force development was reduced to about half the initial amount within 10 min and during the same period approximately 13 $\mu$mol·g$^{-1}$ wet weight of the phospho-L-arginine pool was depleted, corresponding to a rate of 0.65 $\mu$mol·min$^{-1}$·g$^{-1}$ wet weight. After 20 min, force development was low and the remaining content of phospho-L-arginine was not further degraded (Fig. 17). Quiescent introvert retractor muscles, which are anoxically incubated for 5 h, however, degraded phospho-L-arginine only at a rate of 0.03 $\mu$mol·min$^{-1}$·g$^{-1}$ wet weight.

The importance of phospho-L-arginine as an instantaneously available energy source is also well documented in mollusks. The phosphagen shows high resting steady-state levels of around 30 or more $\mu$mol·g$^{-1}$ wet weight in the muscles of highly active species such as scallops, file shells, and cephalopods, whereas cockles and whelks have resting steady-state levels of phospho-L-arginine around 10 $\mu$mol·g$^{-1}$ wet weight. If the common whelk *Buccinum undatum* is attacked by a starfish (for example, *Marthasterias glacialis*), it extends its food and tries to escape by rapid, jerky contrarotations of the foot and shell around the longitudinal axis of the visceral mass (Fig. 18). This type of escape movement is powered by contractions of the columellar muscle (Mackie et al. 1968) which degrades phospho-L-arginine from 8.8 to 5.2 $\mu$mol·g$^{-1}$ wet weight (Koormann and Grieshaber 1980). The initiation of escape movements in *Nassa mutabilis* was accompanied by a dramatic decrease in the level of phospho-L-arginine. The rate of phosphagen transphosphorylation amounted to 5.4 $\mu$mol·min$^{-1}$.g$^{-1}$ wet weight during functional anaerobiosis, but only to 0.02 $\mu$mol·min$^{-1}$·g$^{-1}$ wet weight during nitrogen exposure (Gäde et al. 1984). Little phospho-L-arginine degradation was

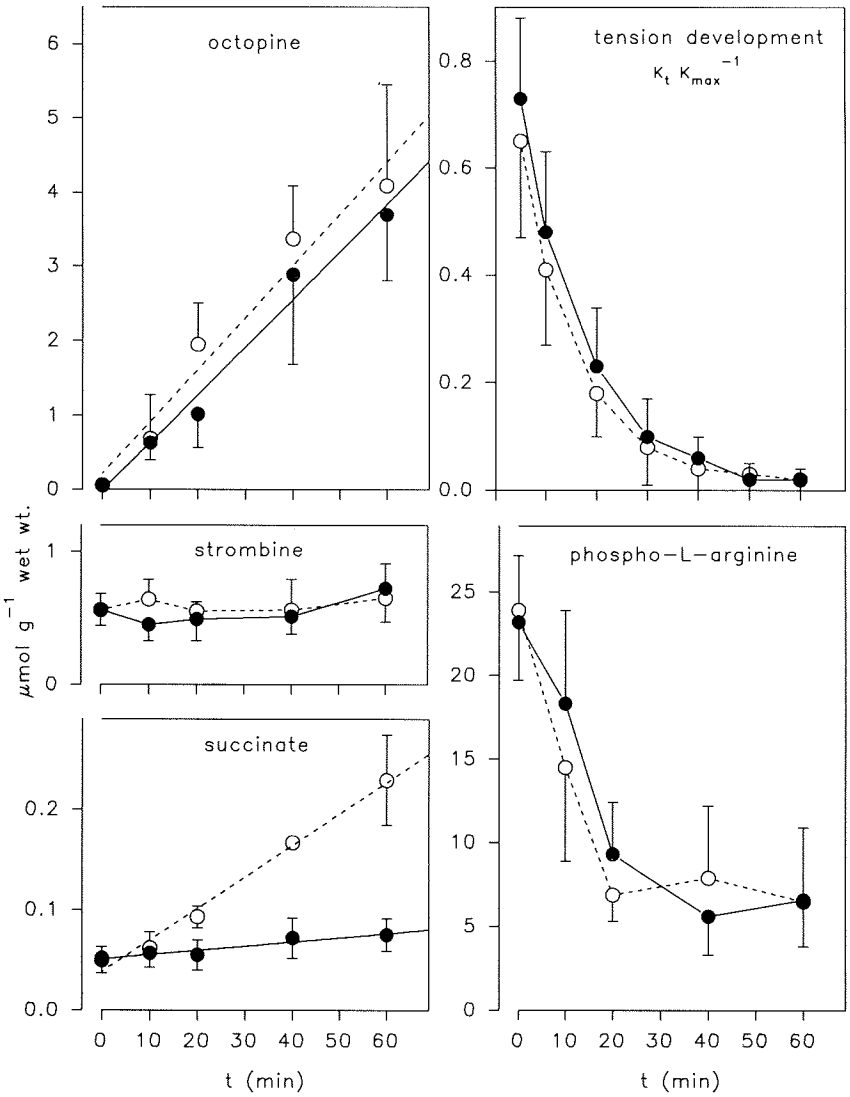


**Fig. 17.** Relative tension development, the contents ($\mu$mol·g$^{-1}$ wet weight) of octopine and succinate, as well as the phosphagen content in aerobically (*solid circles*) and anaerobically (*open circles*), isotonically contracting introvert retractor muscle of *Sipunculus nudus* (Kreutzer 1987)

observed in the potassium-stimulated radula retractor muscle of *Busycon contrarium*. This muscle usually maintains a rhythmic contractility during feeding and the energy is probably derived from an aerobic metabolism. The force developed during potassium-induced contracture might be too small to cause a drastic reduction of the phosphagen (Ellington

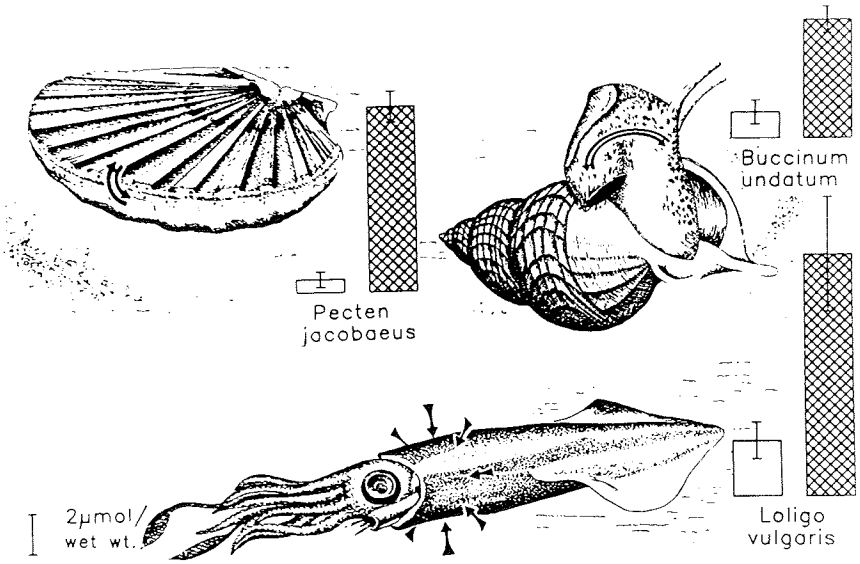


**Fig. 18.** Tissue contents of octopine in quiescent (*open bars*) and escaping mollusks after functional anaerobiosis (*cross-hatched bars*). Swimming by valve clapping in the scallop *Pecten jacobaeus* and foot twisting in the whelk *Buccinum undatum* were induced with an exudate obtained from a sea star (Makkie et al. 1968). The squid *Loligo vulgaris*, which was chased by hand, performed jet propulsion swimming (Trueman 1975). (Data on octopine formation from Grieshaber and Gäde 1977; Koormann and Grieshaber 1980; Grieshaber and Gäde 1976a,b)

1982). Zange et al. (1989) investigated the energy provision from phospho-L-arginine in the anoxic anterior byssus retractor muscle (ABRM) of *Mytilus edulis* during a single phasic contraction which lasted for 2 min. The muscle performed work of 62 $mJ \cdot g^{-1}$ wet weight and the phosphagen level decreased by 2.98 $\mu mol \cdot g^{-1}$ wet weight, corresponding to 1.49 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight or 0.05 $\mu mol \cdot m\,J^{-1}$. The phosphagen consumption of resting anoxic ABRM amounted to 0.12 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight which is in fair agreement with the phosphagen consumption of 0.5 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight in the severely hypoxic posterior adductor muscle of this species (Ebberink et al. 1979). High turnover rates of phospho-L-arginine were found in Pectinidae and Cephalopoda which were forced to swim until exhaustion, but phosphagen depletion is low during exposure to severe hypoxia (Table 2). Thus phosphagens provide ATP at a high rate during the initial phase of functional anaerobiosis, but only a low phosphagen supply is necessary during environmental hypoxia.

Arthropods are highly mobile animals and they usually rely on an aerobic energy metabolism. But in many species burst activity is powered

by an anaerobic energy metabolism providing ATP at high rates. Again, phospho-L-arginine is the main energy source during the first few powerful muscle contractions. Escape swimming in macruran decapods is achieved by powerful contractions of the abdominal muscle which accelerate, for example, lobster or crayfish backwards. The first vigorous tail flips in the Australian yabbie (*Cherax destructor*) are almost exclusively powered from the transphosphorylation of phospho-L-arginine which decreases from 25 to 7 $\mu mol \cdot g^{-1}$ wet weight. During further escape swimming, the phosphagen is almost completely used up, and only then lactate does start to rise (see below). But at this stage the series of tail flips are slower and less powerful than in the beginning (England and Baldwin 1983). In the tail muscle of the shrimp *Crangon crangon* the phospho-L-arginine content was found to decrease after ten tail flips, which occurred within 10 s, from 20.7 to 10.5 $\mu mol \cdot g^{-1}$ wet weight. Further stimulation of the shrimp caused rapid fatigue and the phosphagen level in the tail muscle fell to 2.9 $\mu mol \cdot g^{-1}$ wet weight after 50 s of work (Onnen and Zebe 1983). Similar data were published by Gäde (1984) for the crayfish *Orconectes limosus* which used phospho-L-arginine at a rate of 23 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight, during exhaustive tail flipping, which strongly contrasts to 0.4 $\mu mol \cdot g^{-1}$ wet weight during sustained lack of oxygen.

Many arachnids are predators and they try to overwhelm their prey during sudden attacks. The energy production in working muscle is probably mainly anaerobic as was already deduced by Zebe and Rathmayer (1968) who found only very few mitochondria in the leg muscles of spiders. As a corollary, very low activities of the enzymes involved in aerobic energy production, but high glycolytic capacities were reported from the muscles of the lycosid spider *Cupiennius salei* (Linzen and Gallowitz 1975). Prestwich (1983, 1988) investigated burst activities in *Lycosa lenta* and *Filistata hibernalis*. Both spiders could maintain maximal running speed for 20 s before their power output was reduced and after 2 min the animals could not move at all. The immediate burst activity draws energy almost exclusively from phospho-L-arginine. The pool was almost completely transphosphorylated within 20 s.

Although the presence of glycolytic enzymes in different insect muscles has been shown (Zebe and McShan 1957), only limited data on the energy contribution of phospho-L-arginine are available. A prime candidate for functional anaerobiosis is the femoral muscle in the hind legs of locusts, because it is responsible for the sudden backward movement of the tibia eliciting the jump. *Locusta migratoria*, which can be stimulated to perform 15 to 20 leaps, used phospho-L-arginine from

19.6 to 6.2 $\mu$mol·g$^{-1}$ wet weight during the first five leaps. The remaining leaps became shorter and no further degradation of phospho-L-arginine was found (Kirsten et al. 1963; Schneider et al. 1989).

## *7.2 Formations of Lactate and Opines During Functional Anaerobiosis*

### *7.2.1 Lactate*

The presence of high enzymatic activities in the Embden–Meyerhof–Parnas pathway already indicates the use of anaerobic rather than aerobic metabolism during periods of high work. The exploitation of glycolytic substrate level phosphorylations in addition to phosphagen transphosphorylation is best assessed by monitoring the end products of anaerobic glycolysis–lactate and opines.

Lactate was found to accumulate in many fatigued muscles. Three different species of Nereis synthesized approximately 5 $\mu$mol·g$^{-1}$ wet weight of D-lactate when stimulated electrically (Schöttler 1979). In the leech *Hirudo medicinalis*, which was induced to swim for various time periods, L-lactate rose continuously in the body wall tissue to levels between 8 to 14 $\mu$mol·g$^{-1}$ wet weight (Zebe et al. 1981). Among mollusks lactate formation is probably limited to only a few species during functional anaerobiosis. This end product was found to accumulate at low concentrations in the electrically stimulated foot of the common cockle *Cardium edule* (Meinardus and Gäde 1981).

In arthropods lactate seems to be the only end product synthesized during exhaustive muscular activity and subsequent recovery. Early reports on lactate synthesis documented a significant accumulation of this metabolite in the stimulated abdominal muscle and in the heart of *Palinurus vulgaris* and *Homarus vulgaris* (Boyland 1928). The tetanically stimulated muscles of the chelipeds of the crayfish *Astacus fluviatilis* also showed an increase of L-lactate of 8.7 $\mu$mol·g$^{-1}$ wet weight (Meyerhof and Lohmann 1928).

These early results have been repeatedly confirmed for other crustacean species during vigorous movements such as burst activity by tail flipping, swimming, or crawling. The lobsters *Homarus gammarus* (Phillips et al. 1977) and *Cherax destructor* (England and Baldwin 1983), the shrimp *Crangon crangon* (Onnen and Zebe 1983), or the crayfish *Orconectes limosus* (Gäde 1984) accumulate L-lactate in the heavily working muscle. With the exception of *Homarus gammarus*, lactate builds up towards the end of excessive work or during subsequent recovery.

In most crustaceans L-lactate accumulated during excessive work is transported to the hemolymph where the concentrations can reach different levels (Phillips et al. 1977). In *Gecarcinus lateralis*, a terrestrial air-breathing crab, lactate release into hemolymph is slow during strenuous running, and peak concentrations of only 2 $\mu$mol·ml$^{-1}$ are reached 20 min after exhaustion (Smatresk et al. 1979). Severe exercise on a treadmill of another species of land crab, *Cardisoma carnifex*, resulted in lactate accumulation of approximately 6 $\mu$mol·ml$^{-1}$ hemolymph (Wood and Randall 1981). When the Dungeness crab *Cancer magister* was kept in near continuous locomotory activity over 20 min, a lactate build up of 8 $\mu$mol·ml$^{-1}$ hemolymph was found. Thus, the anaerobic scope of the land crab *Cardisoma carnifex* is similar to that of the marine Dungeness crab (McMahon et al. 1979). However, the latter species continues to accumulate L-lactate up to approximately 12 $\mu$mol·ml$^{-1}$ hemolymph during the early phase of aerobic recovery (McDonald et al. 1979). Similar lactate concentrations were found in the two intertidal crabs *Pachygrapsus crassipes* and *Carcinus maenas* when forced to excessive running (Burke 1979). Steady-state swimming for 60 min of the Blue crab *Callinectes sapidus* resulted in an increase of the lactate concentration in hemolymph from 0.7 to 9.8 $\mu$mol·ml$^{-1}$ within 25 min, even though prebranchial hemolymph remained well oxygenated. Unfortunately, lactate was not repeatedly measured during the 60min swimming period. Therefore it is difficult to decide whether lactate accumulation was a result of a delayed release of lactate originating from a functional anaerobiosis occurring during the beginning of exercise or of sustained functional anaerobiosis in the muscles of the continuously beating swimming legs (fifth pereiopods) (Booth et al. 1982).

The metabolic fate of hemolymph lactate is still uncertain during recovery. Phillips et al. (1977) investigated the reoxidation of lactate in two lobster species, but found no clear evidence for a metabolization of lactate in the midgut gland or in any other tissue. Further investigations would be of interest.

Amongst Chelicerata, D-lactate formation was found in *Limulus polyphemus*. This species is capable of extensive locomotion with its walking legs and small animals often swim by rhythmically moving their legs and gill leaflets (Shuster 1982). Exercise caused a formation of approximately 4 $\mu$mol·g$^{-1}$ wet weight. D-lactate in the muscles that move the gill leaflets, and some of the anaerobic end product is transported to the hemolymph where it rises to similar levels as in muscle tissue (Carlsson and Gäde 1986). Prestwich (1983) investigated functional anaerobiosis in several species of spiders and found that after

maximal activity D-lactate levels are greatest in legs and prosoma and can be as large as 15 $\mu mol \cdot g^{-1}$ wet weight. Accumulation of D-lactate in the opisthosoma averaged near 2.5 $\mu mol \cdot g^{-1}$ wet weight.

Insects, commonly considered as highly aerobic animals, have received only little attention with respect to functional anaerobiosis. Bishai and Zebe (1960) measured L-lactate formation in the femoral muscle of the hind legs of Locusta migratoria and found an increase of approximately 6 $\mu mol \cdot g^{-1}$ wet weight of L-lactate in animals jumping until exhausted.

### *7.2.2 Opines*

Octopine is a typical end product of functional anaerobiosis in most molluscan species. In vivo, the formation of octopine was demonstrated in the mantle muscle of the squid *Loligo vulgaris* (Fig. 18) as well as in *Octopus vulgaris* which were forced into jet propulsion swimming until exhausted. In both species, tissue levels of octopine rose by 17 $\mu mol \cdot g^{-1}$ wet weight and 15 $\mu mol \cdot g^{-1}$ wet weight, respectively (Grieshaber and Gäde 1976a, b). Similar data were obtained from indirectly stimulated mantle preparations of *Loligo vulgaris* (Grieshaber and Gäde 1976a) and the directly stimulated spadix retractor muscle of *Nautilus pompilius* (Hochachka et al. 1977). Later, Storey and Storey (1978, 1979) and Baldwin and England (1980) confirmed these results for the mantle muscle of squid and cuttlefish. Recently Pörtner et al. (1993) reported tissue levels of octopine of 24 $\mu mol \cdot g^{-1}$ wet weight in the ommastrephid squid *Illex illecebrosus.*

Comparative studies verified octopine synthesis in the collumelar muscle of the whelk *Buccinum undatum* (Koormann and Grieshaber 1980), the pedal retractor muscle of several species of Strombidae (Baldwin and England 1982a, b), as well as in *Nassarius coronatus* (Baldwin et al. 1981) and *Nassa mutabilis* (Gäde et al. 1984) which use these muscles extensively during leaping and twisting behavior while escaping predatory starfish or *Conus* species (e.g., Weber 1926).

Among the more vivid bivalves, octopine synthesis was investigated during escape in several swimming Pectinidae, in *Lima hians* as well as in the jumping cockle *Cardium tuberculatum.* All species of Pectinidae investigated form octopine in varying amounts within their valve adductor, while *Cardium tuberculatum* produces octopine in the foot (Gäde 1980b). Octopine accumulation is most pronounced in *Argopecten irradians concentricus* and *Lima hians* showing end product levels of 8 and 7 $\mu mol \cdot g^{-1}$ wet weight, respectively (Chih and Ellington 1983; Gäde 1981). The queeny, *Chlamys opercularis* (Grieshaber 1978) as well as the

giant scallop *Pecten maximus* (Gäde et al. 1978) and *Placopecten magellanicus* (de Zwaan et al. 1980; Livingstone et al. 1981) synthesize octopine either completely or to a major part during the recovery period following exhaustive swimming.

In the foot muscle of *Cardium tuberculatum* octopine formation is linear with the number of leaps performed, thus probably with the amount of work performed (Meinardus-Hager and Gäde 1986a, b).

The anaerobic energy metabolism in the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* during contraction and catch was investigated by Zange et al. (1989). This muscle is known for its reduced energy turnover during catch (Rüegg 1971). Repeated phasic contractions and the initial period of catch in severely hypoxic ABRM caused a small but significant increase of octopine levels. During sustained catch no further octopine formation occurred, confirming the low energy demand of this muscle during this mode of contraction.

The only nonmolluscan species known to synthesize octopine is the peanut worm *Sipunculus nudus*, which accumulates an average of 8 $\mu$mol·g$^{-1}$ wet weight of octopine during digging cycles repeatedly performed for approximately 45 min (Grieshaber and Zebe 1978; Pörtner et al. 1984a) and the nemertean *Cerebratulus lacteus* where small changes in octopine levels were found during swimming in hypoxic water (Gäde 1983b).

Exact and specific determinations of alanopine and strombine synthesized during muscular activity have been delayed somewhat, since the tissue levels of these metabolites were not as easily accessible as those of lactate and opines due to the unspecificity of the alanopine/strombine dehydrogenase-dependent photometric assay. Baldwin and England (1982b) as well as Chih and Ellington (1983) nevertheless made use of the optical assay and reported small increases of alanopine and/or strombine contents in *Strombus* species and in *Argopecten irradians concentricus*, respectively, after exercise and subsequent recovery.

Using a more specific analysis (Siegmund and Grieshaber 1983), Siegmund et al. (1985) demonstrated alanopine as a major end product of anaerobic glycolysis in the lugworm *Arenicola marina* (Fig. 19). This opine accumulated to a level of 8 $\mu$mol·g$^{-1}$ wet weight in the body wall musculature of digging animals, whereas the level of strombine increased only to 1.6 $\mu$mol·g$^{-1}$ wet weight.

From the many investigations on opine formation it can be concluded that octopine and alanopine are glycolytic end products which are predominantly synthesized during functional anaerobiosis (see, however, *Glycera dibranchiata*, Fig. 10). Octopine is mainly restricted to mollusks while alanopine occurs in a few annelids.

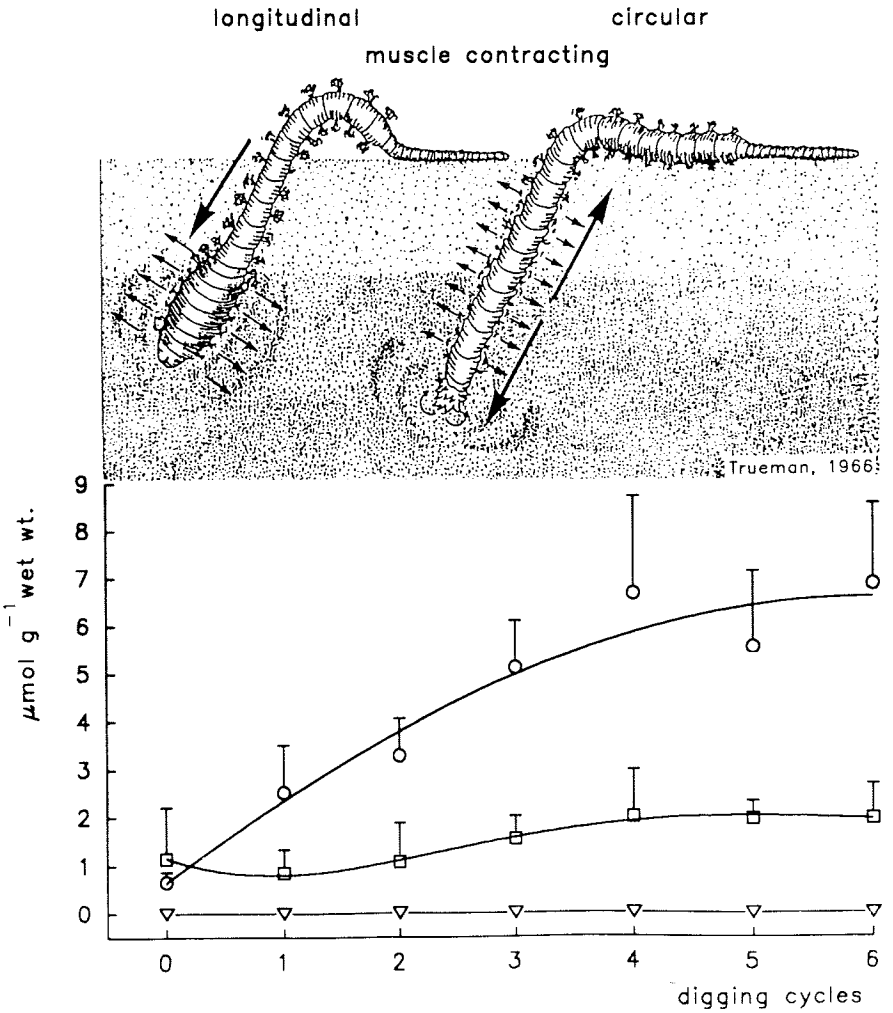


**Fig. 19.** Digging movements of the lugworm *Arenicola marina* L. *Upper panel* depicts alternating contractions of circular and longitudinal musculature (Trueman 1966). *Lower panel* shows the contents of alanopine (*circles*), strombine (*squares*) and lactate (*triangles*) in the body wall musculature of *Arenicola marina* during repeated digging cycles. (Modified from Grieshaber et al. 1992)

From some of the data compiled, the amount of end product formed per escape movement or per muscle contraction can be calculated. For instance the crayfish *Orconectes limosus* synthesizes between 0.01 and 0.15 $\mu$mol·g$^{-1}$ wet weight L-lactate per single tail flip within the tail muscle (Gäde 1984). In *Cardium tuberculatum* (Gäde 1980b), *Argopecten irradians concentricus* (Chih and Ellington 1983), and *Nassa mutabilis* (Gäde et al. 1984) synthesis of octopine averages 0.1 $\mu$mol·g$^{-1}$ wet

weight per contraction. Introvert retractor muscle of *Sipunculus nudus* stimulated to contract isometrically at 6, 3, and 1 Hz for 60 min showed an average octopine synthesis of 0.014, 0.007, and 0.005 $\mu mol \cdot g^{-1}$ wet weight per single contraction. Since both types of experiments also demonstrate a linear correlation between end product formation and the number of contractions, it can be assumed that anaerobic glycolysis contributes a small but constant amount of ATP.

The production rates of various glycolytic end products ($\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight), however, differ considerably between various species, usually being highest in those animals which can perform best. *Orconectes limosus* can produce between 1 and 17 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight L-lactate in the abdominal muscle (Gäde 1984). The mantle muscle of *Loligo vulgaris* and *Sepia officinalis* synthesize 8 and 17 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight octopine, respectively (Grieshaber and Gäde 1976a, b; Storey and Storey 1979). Escape swimming of the scallop *Placopecten magellanicus* draws 2.3 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight ATP from octopine formation (de Zwaan et al. 1980). Many other molluscan species, however, show much lower production rates, ranging between 0.1 and 2.0 $\mu mol \cdot min^{-1} \cdot g^{-1}$ wet weight.

Different rates of end product formation also reflect the involvement of anaerobic glycolysis relative to the transphosphorylation of the animals's phosphagen in the total supply of ATP during functional anaerobiosis. Disregarding any aerobic energy contribution, anaerobic glycolysis provides 64% of total ATP in the whelk *Buccinum undatum*, which incidently has a very low phospho-L-arginine pool (Koormann and Grieshaber 1980), and between 25% and 45% in *Loligo vulgaris* (Grieshaber and Gäde 1976), *Pecten alba* (Baldwin and Opie 1978), *Argopecten irradians concentricus* (Chih and Ellington 1983), and in the crayfish *Orconectes limosus* (Gäde 1984). In *Placopecten magellanicus* a 58-fold increase of the glycolytic rate contributes only 10% of the total ATP required during escape swimming (de Zwaan et al. 1980; Livingstone et al. 1981) and in *Chlamys opercularis* anaerobic glycolysis is not involved at all during valve clapping but becomes important during subsequent recovery (Grieshaber 1978).

In most species capable of vigorous muscle movements during flight and fight reactions, the transphosphorylation of the phosphagen pool is the most important and immediately available energy source. Usually high concentrations of a phosphagen and pronounced activities of phosphagen kinases guarantee the rapid anaerobic supply of ATP. Only when the phosphagen pool has been depleted does anaerobic glycolysis become the main energy source which can support but a minor development of work. ATP production from the Embden–Meyerhof–Parnas

pathway, however, averts a pronounced drop of the ATP/ADP ratio and thus mitigates the influence of a low energy status on the metabolite flux of anaerobic glycolysis.

### *7.3 Dualism of Anaerobic End Product Formation*

Investigations on the distribution of opine and lactate dehydrogenases in different animal phyla (Livingstone 1983; Livingstone et al. 1990), as well as determinations of the tissue contents of the corresponding end products, revealed that vertebrates possess only lactate dehydrogenase while in many invertebrates several pyruvate oxidoreductase activities coexist within the same tissue (Table 3). As a corollary, some annelids and mollusks that are able to exploit glycolytic energy production during ambient lack of oxygen as well as during bursts of muscular activity revealed the interesting phenomenon that even within the same species and the same muscles the pattern of glycolytic end product formation changes with the mode of anaerobiosis. Therefore, one might ask which parameters regulate the different metabolic responses to burst activity and environmental severe hypoxia.

The mollusks *Cardium tuberculatum* and *Ensis directus*, the lugworm *Arenicola marina*, and the peanut worm *Sipunculus nudus* are typical examples for what has been termed the "dualism" of anaerobic glycolysis. Both bivalves accumulate primarily octopine if stimulated to burst activity, while during long-term hypoxia the dominating end product is D-lactate in these two species (Meinardus-Hager and Gäde 1986b; Schiedeck and Zebe 1987). Similarily, in *Arenicola marina* and *Sipunculus nudus*, alanopine and octopine, respectively, are end products during muscular activity, but both species accumulate strombine during environmental anaerobiosis (Pörtner et al. 1984a; Siegmund et al. 1985).

Approaches to elucidate the regulatory aspects of this phenomenon first concentrated on the properties of opine and lactate dehydrogenases, but none of the catalytic characteristics reported could fully account for the production of different end products during the two modes of anaerobiosis. Extending an approach by Fields and Quinn (1981), Meinardus-Hager and Gäde (1986b) suggested that the equilibrium state of the different pyruvate oxidoreductase reactions is an important determinant in glycolytic end product formation. Concluding from studies on enzyme kinetics and on glycolytic end product formation in the jumping cockle *Cardium tuberculatum*, the latter authors postulated that in the foot muscle of the cockle, which has D-lactate dehydrogenase as well as octopine dehydrogenase and alanopine/strombine dehydro-

genase activities, D-lactate formation is favored over opine synthesis by the more favorable apparent equilibrium situation of the D-lactate dehydrogenase reaction, thus explaining why the jumping cockle produces predominantly D-lactate during ambient lack of oxygen. This effect is thought to be of minor consequence during jumping activity when octopine is the predominant end product, since the relatively low D-lactate dehydrogenase activity cannot compete with the high capacity of octopine dehydrogenase in a situation of high glycolytic flux.

This view was applied to the tauropine/D-lactate system in the adductor muscle of the ormer *Haliotis lamellosa* (Gäde 1988) and extended to other pyruvate oxidoreductase systems in comparative investigations on a wide variety of marine invertebrates as well as studies on isolated introvert retractor muscle of *Sipunculus nudus* (Kreutzer et al. 1989).

Data on amino acid and opine contents in over 20 species subjected to environmental hypoxia and/or vigorous muscular activity showed a correlation between the end product predominantly produced during sustained environmental hypoxia and the amino acid present in the highest level in the tissues (Table 6). If several pyruvate oxidoreductases

**Table 6.** Contents ($\mu mol \cdot g^{-1}$ wet weight) of amino acids and glycolytic end products in different species of marine invertebrates after environmental hypoxia. Data taken from Kreutzer et al. 1989 and Pörtner et al. 1984a

| Species | AA<br>EP | Lactate | Glycine<br>Strombine | L-Alanine<br>Alanopine | L-Arginine<br>Octopine |
|---|---|---|---|---|---|
| *Arenicola* | AA | | 178.1 | 43.5 | 0.1 |
| *marina* | EP | 0.2 | 6.4 | 1.0 | b1 |
| *Glycera* | AA | | 2.9 | 25.5 | 0.2 |
| *convoluta* | EP | b1 | b1 | 3.2 | b1 |
| *Nephtys* | AA | | 36.9 | 5.6 | 0.2 |
| *hombergi* | EP | 0.3 | 6.5 | 0.3 | b1 |
| *Sipunculus* | AA | | 185.0 | 7.6 | 16.0 |
| *nudus* | EP | b1 | 3.9 | 0.1 | 0.7 |
| *Ensis* | AA | | 154.1 | 13.2 | 6.6 |
| *siliqua* | EP | 1.4 | 5.6 | 0.2 | 0.2 |
| *Solen* | AA | | 206.0 | 37.0 | 20.5 |
| *marginatus* | EP | b1 | 5.6 | 0.9 | 0.6 |
| *Pharus* | AA | | 240.1 | 15.0 | 40.0 |
| *legumen* | EP | 0.4 | 13.1 | 0.3 | 2.3 |
| *Mytilus* | AA | | 59.0 | 15.6 | 16.3 |
| *edulis* | EP | 0.6 | 3.3 | 0.4 | 1.0 |
| *Littorina* | AA | | 5.4 | 25.7 | 8.3 |
| *littorea* | EP | 3.0 | 0.4 | 3.9 | b1 |

AA, amino acids; EP, glycolytic end products; b1, below limit of detection.

are present, species with a high glycine content, such as *Mytilus edulis*, *Solen marginatus*, or *Arenicola marina*, accumulate predominantly strombine; species with a high alanine level but low glycine content, such as the gastropod *Littorina littorea* or the polychaete *Glycera convoluta*, form predominantly alanopine during environmental anaerobiosis.

The reaction equilibrium of any opine dehydrogenase (A) can be expressed by the equation

$$K'eq\,(A) = \frac{[\text{opine (A)}] \times [NAD^+]}{[\text{amino acid (A)}] \times [\text{pyruvate}] \times [NADH] \times [H^+]} \quad (1)$$

Comparing two different opine dehydrogenase reactions (A and B), the resulting equations can be solved for their common parameters [NAD], [NADH], [$H^+$] and [pyruvatel] to

$$\frac{K'eq\,(A)}{K'eq\,(B)} = \frac{[\text{amino acid (B)}] \times [\text{opine (A)}]}{[\text{amino acid (A)}] \times [\text{opine (B)}]} \quad (2)$$

For 12 of the species investigated the expected ratios of tissue concentrations of amino acids and the corresponding opines were calculated according to Eq. 2 using different combinations of opine dehydrogenase reactions. Data for environmental anaerobiosis yielded similar values regardless of the animal species considered. Since K'eq is a thermodynamic constant, this should be expected if both reactions are close to equilibrium.

This hypothesis was further tested taking the isolated introvert reactor muscle of *Sipunculus nudus* as an example. In hypoxic and contracting muscle all substrates and products of the octopine and strombine dehydrogenase reactions were measured, and the mass action ratios (Rolleston 1972) were determined according to Eq. 1. The muscles accumulated 1 $\mu mol \cdot g^{-1}$ wet weight, strombine, but only 0.1 $\mu mol \cdot g^{-1}$ wet weight octopine when incubated in a nitrogen atmosphere. When stimulated to contraction, however, octopine content increased by 4 $\mu mol \cdot g^{-1}$ wet weight, but insignificant amounts of strombine were accumulated. Mass action ratios for the octopine dehydrogenase reaction yielded almost identical values for control, hypoxic, and contracting muscle and indicated that octopine dehydrogenase catalyzes near equilibrium in both modes of anaerobiosis. Mass action ratios for the strombine dehydrogenase reaction varied twofold in hypoxic muscles, but showed a pronounced ninefold decrease in contracting muscles, indicating that this reaction shifts away from near equilibrium in a situation of high glycolytic flux. With 228 $U \cdot g^{-1}$ wet weight, the octopine dehydrogenase activity is 18-fold higher than the strombine dehydrogenase activity (13 $U \cdot g^{-1}$ wet weight) in this muscle. It seems

reasonable that under physiological substrate conditions a near equilibrium state can only be established for the octopine dehydrogenase reaction. Similar relationships hold for other invertebrates (Table 4) where in most cases the glycolytic end product synthesized during muscular activity is linked to the highest pyruvate reductase activity.

The results above can generally explain the diversity of glycolytic end product formation. During environmental hypoxia, when the glycolytic flux is relatively low, the different pyruvate oxidoreductases present in a tissue catalyze close to their reaction equilibrium and end product formation is controlled by thermodynamic effects. In the case of opine dehydrogenases, the relative amount to which a specific opine accumulates depends on the concentration of the corresponding amino acid. During bursts of muscular activity, when the glycolytic flux is high, only those opine dehydrogenases present in sufficiently high activities can catalyze near their reaction equilibrium. In this situation the capacity of the enzyme determines which end product will primarily accumulate (Kreutzer et al. 1989).

The glycolytic end products remain generally within the tissue. Lactate is an exception since it can be transported to other tissues for

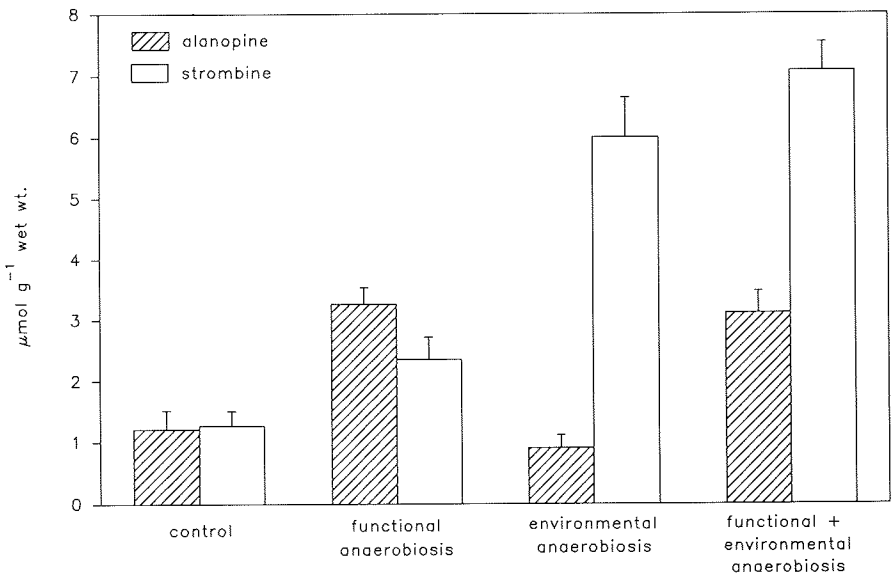


**Fig. 20.** Contents ($\mu$mol·g$^{-1}$ wet weight) of alanopine and strombine in the body wall musculature of *Arenicola marina* after functional and environmental anaerobiosis. The last bars show the opine contents of specimens which were consecutively forced to functional and environmental anaerobiosis. (Based on data from Kreutzer and Grieshaber in Grieshaber et al. 1992)

further oxidation. Since anaerobic glycolysis is terminated with a dead-end reaction, the accumulated end product inhibits pyruvate reduction and thus the regeneration of $NAD^+$ from NADH. If for some reason an animal is first forced to vigorous muscular activity while burrowing in the sediment (functional anaerobiosis) and then has no access to oxygen for a limited period because it is buried (environmental anaerobiosis), it can draw energy from anaerobic glycolysis during both situations by consecutively using two different terminal pyruvate oxidoreductases. When the first one is inhibited by its own end product, the second one can be used for further NADH oxidation, thus leading to the accumulation of two different end products. This dualism of anaerobic end product formation is, therefore, particularly pronounced in those species which can perform intensive flight and fight reactions and are able to sustain limited periods of environmental hypoxia. Figure 20 demonstrates the end product accumulation in *Arenicola marina*. Specimens were first forced to dig until exhausted and then incubated anoxically. Digging and anoxic treatment indeed led to the consecutive accumulation of both glycolytic end products.

## 8 Conclusions

Despite the abundant presence of oxygen in the telluric atmosphere and hydrosphere, many habitats exist which are characterized by continuously (high altitude, intestine of vertebrates) or temporarily low concentrations of oxygen. Nevertheless, these habitats are densely populated by many different invertebrate species. They show a rich diversity of functional adaptations which allow an aerobic metabolism during moderate hypoxia. As soon as a critical $Po_2$ is reached, many species resort to a state which is termed environmental anaerobiosis. An anaerobic energy metabolism is exploited to power a metabolic rate which is considerably lower during low ambient oxygen than during normoxia.

Energy is derived from various sources. In the beginning of environmental anaerobiosis different substrates such as phosphagens, aspartate, and glycogen are used. ATP is derived from the transphosphorylation of phosphagens, substrate level phosphorylations of the Embden–Meyerhof–Parnas pathway and the reduction of fumarate to succinate. Reoxidation of NADH is achieved by the lactate dehydrogenase or pyruvate oxidoreductase (opine dehydrogenase) catalyzed reduction of pyruvate leading to the accumulation of lactate and opines. Transphosphorylation of a phosphagen and opine formation also support elevated energy requirements during functional hypoxia.

The mitochondrial compartment which becomes involved in the energy provision during sustained environmental anaerobiosis increases the yield of ATP from glycogen fermentation by exploiting the fumarate reductase complex and the acetate–propionate pathway. Thereby the cytoplasmic yield of 3 mol ATP per glycosyl unit is increased to approximately 7 mol ATP per glycosyl unit. The definitive end products acetate and propionate are released into the surrounding medium, thus avoiding too strong a perturbation of the intracellular milieu. Using these physiological and biochemical mechanisms, many invertebrates are able to adapt to even extreme conditions of low ambient oxygen concentrations for prolonged periods.

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