Critical PO_2 of Euryoxic Animals

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Critical PO_2

During normoxic conditions most animals have enough oxygen available to maintain an aerobic energy metabolism. This holds especially true in animals that live in air, where a low oxygen supply is hardly ever encountered except for at high altitudes (Bouverot 1985). Animals inhabiting aquatic habitats may be more often subjected to moderately or severely hypoxic conditions, since water contains less oxygen and several ecological factors such as stratification, salinity, temperature, or plant respiration may further reduce oxygen availability. Therefore, it is not surprising that aquatic species in particular have acquired several physiological and biochemical adaptations to cope with environmental hypoxia.

Many experiments have been carried out in which the effects of a decreasing ambient PO_2 on several parameters such as the rate of oxygen consumption, blood flow, or ventilatory rate have been monitored. In an attempt to generalize the deluge of data obtained, Fry (1957) distinguished fish species with an oxygen consumption independent of a declining ambient PO_2 from those with an oxygen uptake which changes in relation to ambient oxygen tension. Prosser (1973) extended this point of view and tried to classify animals into oxyregulators (i.e., O_2-independent) and oxyconformers (O_2-dependent). As may be expected from such an oversimplified classification, several investigators have questioned this system (e.g., Mangum and van Winkle 1973). Many exceptions have been noted and attributed to, e.g., pigment affinity, level of activity, temperature, or even the means of measurements.

Oxyregulating animals decrease their oxygen consumption rapidly below a certain species-specific range of ambient PO_2. This PO_2 is traditionally called the critical oxygen tension (P_c). The physiological significance of the critical PO_2 has been a matter of controversy for a long time. Some authors have assumed that the P_c marks the point at which the respiratory pigment is not fully saturated when it leaves the gills (Walshe 1950; Redmond 1955). Others, like Hughes (1973) have speculated that at the P_c the respiratory pump is no longer able to pass sufficient oxygen over the gills to meet the aerobic demands of an animal. The latter assumption, however, implies that at the P_c at least some tissues must become anaerobic, a view which was already proposed by R. E. Young in 1973. Whichever ex-

planation of the P_c is accepted, it is obvious that it cannot be fixed at a certain range of oxygen tension even within the same species, because as the demand of the tissue for oxygen increases, e.g., due to enhanced activity, the minimum ambient PO_2 at which adequate oxygen is obtained must be higher.

Since oxygen consumption increases during enhanced activity, one could speculate that the value of the P_c is also affected. In addition is has been widely accepted that under these conditions the heavily working muscle, e.g., the white skeletal muscle of vertebrates, and some fast-twitch muscles of invertebrates become hypoxic. Some recent experiments prove, however, that one has to distinguish whether the muscle tissue itself becomes hypoxic or whether the metabolic flux through the Embden-Meyerhof pathway increases to such an extent that the mitochondria cannot consume all reducing equivalents and pyruvate for final oxidation. Reoxidation of NADH must then occur in the cytosolic compartment and, therefore, one might expect an anaerobic energy metabolism in the cytoplasm with the mitochondria still working aerobically.

In order to prove whether the P_c marks the transition from aerobiosis to anaerobiosis, first of all a detailed understanding of the anaerobic metabolism during ambient lack of oxygen as well as during enhanced activity is required. Later on the concept of the P_c is reevaluated and its applicability extended from oxyregulating to oxyconforming animals.

Anaerobic Metabolism

Anaerobiosis of course is best evoked in an animal if it is exposed to extreme hypoxia or anoxia. The experimental set up is simple although precautions have to be taken to completely eliminate oxygen. Anaerobiosis is then best reflected by the onset of anaerobic energy production, which can be assessed by monitoring several key metabolites (Gäde and Grieshaber 1988) since the tissue content of some compounds drastically changes upon the onset of anoxia. During the past 15 years several groups of investigators have dealt with this topic publishing many facets of anaerobiosis. Several reviews summarize these data and give a comprehensive view of anaerobic energy metabolism (De Zwaan 1983; Hochachka and Somero 1984; Kreutzer et al. 1985; Gäde and Grieshaber 1986).

Since some knowledge of the biochemical reactions is necessary for the understanding of the physiological meaning of P_c, a brief account of the essentials of an anaerobic energy metabolism should be presented here. This process is described in the same sequential order as it is thought to occur in hypoxic animals: (a) the utilization of a phosphagen, (b) the fermentation of glycogen as well as some amino acids, and (c) the transformation of succinate to volatile fatty acids within mitochondria if anoxia prevails. It should, however, be mentioned that volatile fatty acids are only produced in some facultatively anaerobic invertebrates and some parasites (Kluytmans et al. 1975; Saz 1981; Pörtner et al. 1984).

As soon as oxygen is not supplied sufficiently at the mitochondrial level every animal makes use of its phosphagen to buffer the content of ATP. Phosphagens are well suited for an instant supply of ATP, since they are directly connected by the reaction:

$$\text{phosphagen} + \text{ADP} + \text{H}^+ \rightleftharpoons \text{aphosphagen} + \text{ATP}$$

A wide variety of phosphagens which are all guanidine derivatives can be extracted from animal tissues (Van Thoai and Roche 1964). In vertebrates only creatine phosphate can be found. In insects, crustaceans, and molluscs phosphoarginine occurs, whereas in some annelids phospotaurocyamine or phospholombricine are present. It seems that those muscles contain a high level of phosphagen which are able to perform sudden and extreme work.

Enzymes catalyzing the transphosphorylation of a phosphagen (phosphagen kinases) usually show high activities and it is likely that phosphagen kinases catalyze close to their reaction equilibrium (Beis and Newsholme 1975). A slight increase in the ADP content will, therefore, immediately initiate ATP synthesis. It is for these reasons that phosphagens can serve as a primary energy source providing a substantial amount of ATP during the initial phase of anaerobiosis. Due to the limited amount of phosphagen available, however, it is usually rapidly exhausted.

Glycogen and some amino acids can also serve as substrates for anaerobic energy metabolism. Glycogen is a major energy source, since it can be stored in large amounts within the cell. The glycogen content in mammalian muscle cells can account for approximately 60 to 120 μmol glycosyl units $\cdot g^{-1}$ wet wt (Hultman 1967; Kepler and Decker 1969). In some marine invertebrates average gylcogen contents of about 100 μmol glycosyl units $\cdot g^{-1}$ wet wt have been reported (De Zwaan and Zandee 1972). Glycolysis via the Embden-Meyerhof-Parnas pathway leads to the formation of ATP and pyruvate which in the absence of oxygen must be reduced to balance the cytosolic redox ratio.

Most animals thus far investigated possess at least one NADH-dependent pyruvate reductase activity. In vertebrates this is lactate dehydrogenase. In a variety of marine invertebrates different pyruvate reductase activities are known which reduce pyruvate in the presence of an amino acid and NADH giving rise to an opine (Gäde and Grieshaber 1986). Specifically the following reactions can occur:

1. $\text{Pyruvate} + \text{NADH} \rightleftharpoons \text{D,L-lactate} + \text{NAD}^+$
2. $\text{Pyruvate} + \text{glycine} + \text{NADH} \rightleftharpoons \text{strombine} + \text{NAD}^+ + \text{H}_2\text{O}$
3. $\text{Pyruvate} + \text{L-alanine} + \text{NADH} \rightleftharpoons \text{alanopine} + \text{NAD}^+ + \text{H}_2\text{O}$
4. $\text{Pyruvate} + \text{L-arginine} + \text{NADH} \rightleftharpoons \text{octopine} + \text{NAD}^+ + \text{H}_2\text{O}$
5. $\text{Pyruvate} + \text{taurine} + \text{NADH} \rightleftharpoons \text{tauropine} + \text{NAD}^+ + \text{H}_2\text{O}$

Lactate and the four opines are considered to be end-products of anaerobic glycolysis which are accumulated in the cytoplasm during the early phase of long-term anaerobiosis, as well as during enhanced activity (Grieshaber and Kreutzer 1986).

Some amino acids can serve as another fuel for the anaerobic energy production (Fig. 1). Generally, several amino acids show particularly high concentrations in marine invertebrates. During the early phase of environmental anaerobiosis a decrease in the aspartate content and concomitant increase in the alanine level has been established. Aspartate is metabolized in the cytosol via the following reactions:

1. $\text{Aspartate} + \text{2-oxoglutarate} \rightleftharpoons \text{glutamate} + \text{oxaloacetate}$
2. $\text{Glutamate} + \text{pyruvate} \rightleftharpoons \text{alanine} + \text{2-oxoglutarate}$
3. $\text{Oxaloacetate} + \text{NADH} \rightleftharpoons \text{malate} + \text{NAD}^+$

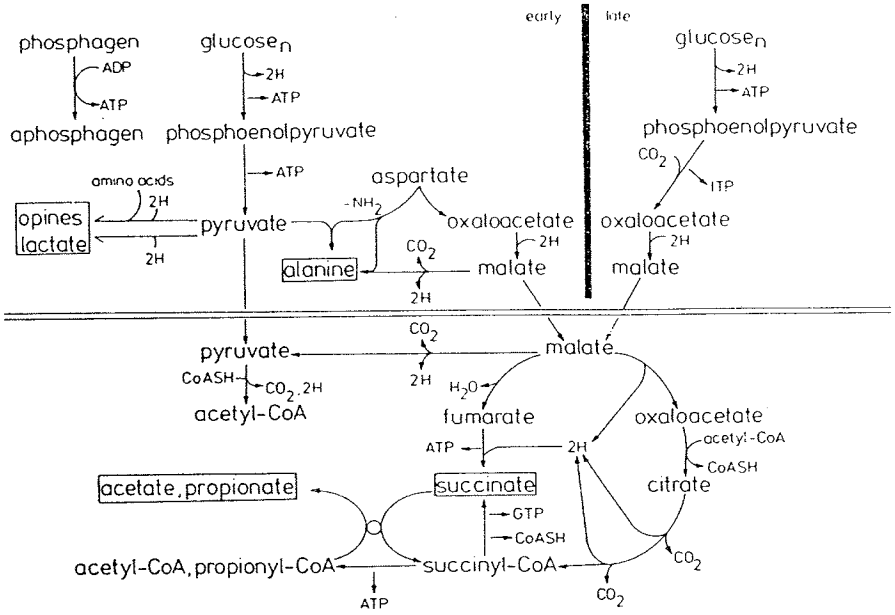

Fig. 1. Scheme of anaerobic energy metabolism as found in facultatively anaerobic marine invertebrates starting from aspartate and glycogen in the early phase and solely from glycogen in the late phase of anaerobiosis. End-products and some intermediary products (outlined in *boxes*) are formed in the cytosol and inside the mitochondria. The mechanism indicated for the release of acetate and propionate from the CoA esters is based on Schöttler (1986)

This reaction sequence which is catalyzed by glutamate oxaloacetate transaminase (1), glutamate pyruvate transaminase (2), and malate dehydrogenase (3) integrates aspartate and glycogen fermentation resulting in alanine and malate formation (Fig. 1).

The cytosolic end-products of the early phase of anaerobiosis are lactate, opines, alanine, and malate. In most animals, however, the malate content only slightly increases compared with other end-products. Malate, in contrast, serves as the metabolite which links the anaerobic energy metabolism of cytoplasm and mitochondria not only during the early phase of anaerobiosis but also during prolonged deprivation of oxygen.

In the good anaerobes such as many annelids and molluscs the degradation of glycogen to lactate and opines ceases after 3–5 h because the major route of the metabolic flux does not lead to pyruvate. Instead, it deviates from the gylcolytic pathway at the phosphoenolpyruvate branchpoint where phosphoenolpyruvate is now carboxylated to oxaloacetate with the concomitant production of a nucleotide triphosphate. Redox balance in the cytosol is achieved by the reduction of oxaloacetate to malate.

The control of the relative flux through the carboxylation of phosphoenolpyruvate to oxaloacetate versus the transphosphorylation of phosphoenolpyruvate to pyruvate is exerted via the regulation of the activity of pyruvate kinase. Holwerda et al. (1981) as well as Holwerda et al. (1983) demonstrated the existence of two in-

terconvertible forms of pyruvate kinase in the adductor muscle of *Mytilus edulis*. They found that with progressive hypoxia one variant of the pyruvate kinase became predominant. It is characterized by a lower enzymatic activity, decreased affinity for phosphoenolpyruvate, and an increased sensitivity to inhibition by L-alanine and protons. In *Busycotypus canaliculatum* the changes in the catalytic properties of pyruvate kinase could be assigned to the phosphorylation of a threonine residue in the protein. This may lead to conformational changes resulting in kinetic differences between normoxic and hypoxic forms of pyruvate kinase. As a consequence, phosphoenolpyruvate could be metabolized preferentially to oxaloacetate (Plaxton and Storey 1984).

Malate arising from the metabolization of either aspartate or glycogen enters the mitochondria (Fig. 1). If the intramitochondrial redox potential is highly negative as can be expected during oxygen deprivation, malate is dehydrated to fumarate which is further converted to succinate with the concomitant rephosphorylation of one mole ADP per mole of succinate formed. This reaction sequence which represents a reversal of some intermediary steps of the normoxic citric acid cycle immediately leads to an increase of the succinate concentration as soon as oxygen is limiting. An elevated succinate level will also persist during prevailing lack of oxygen. Thus, succinate is a true indicator of anaerobically working mitochondria.

Particular interest has focussed on the reduction of fumarate to succinate since the aerobic and anaerobic function of the respiratory chain meet in this reaction. During normoxia reducing equivalents are transferred from succinate via FAD to oxygen, whereas during anoxia electrons are transferred from NADH via site 1 of the respiratory chain to fumarate. A stoichiometric synthesis of approximately one mole of ATP per mole of reduced fumarate has been verified by several investigators (Schöttler 1977; Schroff and Schöttler 1977). Unfortunately, determinations of the lowest oxygen partial pressure at which a reversal of the succinate dehydrogenase reaction commences have not yet been obtained. It can, however, be expected that the P_{50} of fumarate reduction is in the order of magnitude of the P_{50} of cytochrome oxidase, i.e., that fumarate reduction can only occur if the electron transfer to oxygen is completely inhibited. This corroborates again the view that an increase in succinate content should provide the most sensitive tool for monitoring mitochondrial hypoxia and many experiments have indeed proven that the tissue content of succinate increases as soon as oxygen is limiting (Table 1).

For a long time it was also assumed that an increase in lactate concentrations within the tissues (in particular muscle) or blood signals lack of oxygen. This view was derived from the fact that hypoxia which was experimentally imposed on an animal provokes lactate accumulation. Experiments with exercising vertebrate muscles, however, have indicated lactate accumulation while cytochrome aa_3 was still oxygenated (Jöbsis 1963). Aerobic lactate formation not only occurs in white muscle but also in submaximally exercising red muscles of vertebrates where mitochondria are abundant (Connett et al. 1984).

With succinate as an easily measurable indicator of mitochondrial anoxia at hand, it should be possible to distinguish between an anaerobic metabolism in the cytosol (as it may occur either during enhanced muscular activity or during ambient lack of oxygen) versus mitochondria synthesizing ATP anaerobically. For an

Table 1. Levels of succinate found during anaerobiosis in invertebrate and vertebrate tissues (in μmol g^{-1} fresh weight, $\bar{x} \pm SD$)

Species	Tissue	Succinate content		
		Control	Anaerobiosis	(h)
Sipunculus nudus[a]	Body wall musculature	0.10 ± 0.01	1.46 ± 0.08	(24)
Mytilus edulis[b]	Post. add. muscle	0.07	3.04	(24)
Arenicola marina[c]	Body wall musculature	0.05 ± 0.10	2.60 ± 0.20	(24)
Scrobicularia plana[d]	Whole animal	0.14 ± 0.03	3.17 ± 0.27	(24)
Felis[e]	Papillary muscle in vitro	0.13 ± 0.08	0.77 ± 0.52	(1)

Data are from [a] Pörtner et al. (1984); [b] DeZwaan et al. (1982); [c] Schöttler et al. (1984); [d] Brinkhoff et al. (1983); and [e] Wiesner et al. (1986)

invertebrate nonstriated muscle a segregation of an anaerobically working cytoplasmic energy metabolism from mitochondrial respiration could be proven. The isolated introvert retractor muscle of the peanut worm *Sipunculus nudus* was stimulated to contract isometrically under normoxic and under hypoxic conditions (Kreutzer et al. 1985). Under either experimental procedure energy is derived from the transphosphorylation of phospho-L-arginine as well as from anaerobic gylcolysis terminating in the formation of octopine. The kinetics of phosphagen degradation and octopine formation are almost indentical under normoxia and hypoxia, respectively (Fig. 2). At first sight this could be attributed to a poorly developed system for oxygen transport causing intracellular hypoxia even in normoxic muscles. Figure 3, however, shows that succinate production as the indicator of mitochondrial anoxia is barely detectable in normoxic muscles, while it is prominent in muscles working in a nitrogen atmosphere. Thus, the stimulation of anaerobic ATP production in the cytoplasm appears to be independent of mitochondrial oxygenation. In the described example (a white muscle) aerobic energy production might not be limited by the mechanism of oxygen transport, but rather by the overall capacity of the mitochondria to regenerate ATP.

Succinate, however, is only a transient end-product of anaerobiosis. If anoxic conditions prevail the new steady-state concentrations of succinate are maintained. Some of it is released from the tissue into the body fluid. Moreover, in the good anaerobes among invertebrates succinate is further metabolized to propionate (Fig. 1). As soon as the succinate pool is sufficiently high, succinate is transformed to succinyl-CoA. In the early phase of mitochondrial anoxia acetyl-CoA serves as donor of the CoA moiety, but when the propionyl-CoA content in the mitochondria increases, the latter compound activates succinate to succinyl-CoA. This reaction, which is catalyzed by a CoA transferase, does not require any additional energy. Succinyl-CoA is carboxylated via L-methylmalonyl-CoA to D-methylmalonyl-CoA which in turn is decarboxylated by the propionyl-CoA carboxylase to propionyl-CoA. The reaction also renders one mole ATP per mole of propionyl-CoA formed. This metabolite again swaps the CoA moiety with succinate leaving propionate as the final end-product of anaerobiosis. The latter is released into the medium, and therefore, does not disturb the internal milieu of the

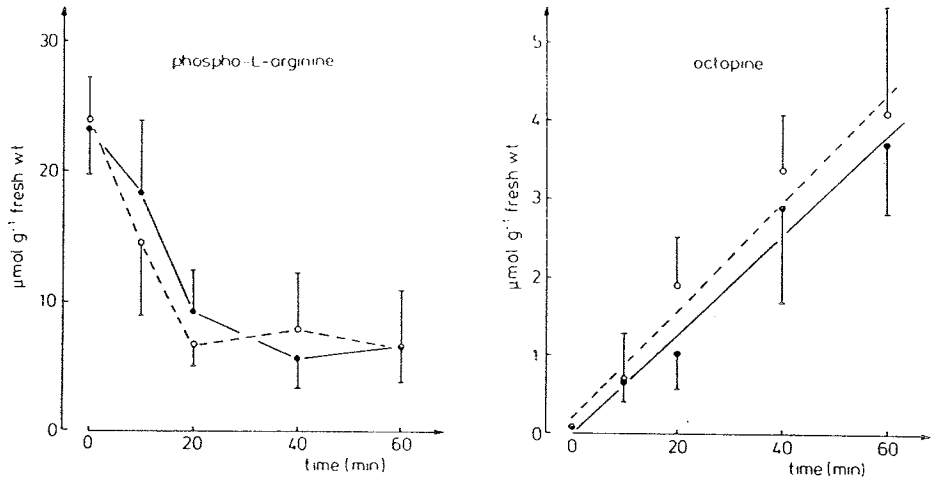

Fig. 2. Phospho-L-arginine and octopine content (µmol·g^{-1} fresh weight; $\bar{x}\pm$SD, $n=4$) of isolated introvert retractor muscle (IRM) of *Sipunculus nudus* contracting isometrically at a frequency of 6 min^{-1} (modified after Kreutzer et al. 1985). ●—● contraction under normoxic conditions ($PO_2=150$ torr), O-----O contraction under hypoxic conditions ($PO_2<10$ torr)

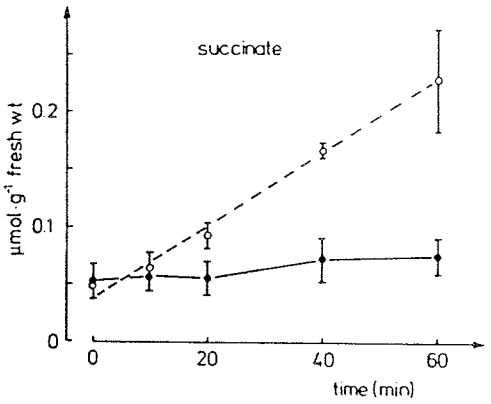

Fig. 3. Succinate content of isolated IRM contracting isometrically under normoxia and hypoxia (legend see Fig. 2)

cell even during prolonged anoxic conditions (Schultz and Kluytmans 1983; Schöttler 1986).

Using this knowledge of metabolic reactions occurring during anaerobiosis, we can determine at which ambient PO_2 an animal becomes anaerobic simply by using the cytosolic end-products of anaerobic glycolysis and succinate as probes.

Both mitochondrial and cytosolic anaerobiosis were investigated in the oxyconformer *Sipunculus nudus* (Pörtner et al. 1985). Specimens of *Sipunculus nudus* were incubated for 24 h at various ambient PO_2, followed by the determination of anaerobic end-products in extracts of the body wall musculature. As shown in Fig. 4, there is a significant increase in opine and succinate levels at a PO_2 of 20 Torr. The onset of end-product accumulation occurs below the same PO_2 value

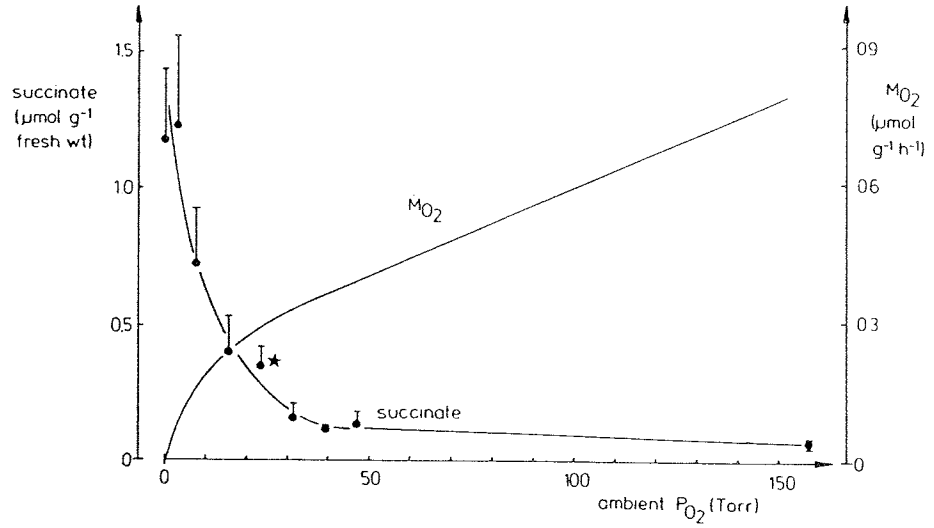

Fig. 4. Succinate contents in the introvert retractor muscles of *Sipunculus nudus* after 24 h of exposure to different ambient oxygen tensions ($\bar{x} \pm SD$; modified after Pörtner et al. 1985) compared with the oxygen consumption curve. Under progressive hypoxia the introvert retractor muscles as an "inner" tissue are first affected by a reduction in oxygen supply. The onset of anaerobiosis in these tissues is reflected by a concomitant drop in the rate of oxygen consumption decline for the whole animal

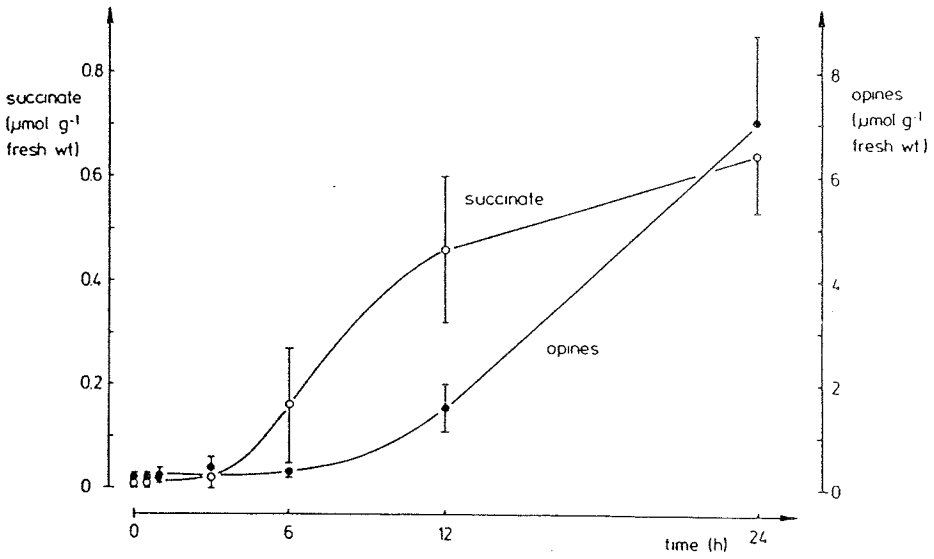

Fig. 5. Succinate and opine (sum of octopine, strombine, alanopine) contents in the body wall musculature of *Sipunculus nudus* after different periods of hypoxic exposure at $PO_2 = 7.5$ torr ($\bar{x} \pm SD$; modified after Pörtner et al. 1985). The significant onset of succinate accumulation occurs before the formation of opines becomes apparent

below which the oxygen consumption of the worm decreases rapidly. These data already indicate that the P_c can not only be defined for an oxyregulator, but also for an oxyconforming animal (see below).

After having determined the PO_2 below which anaerobiosis commences, an even lower PO_2 was chosen to evaluate whether the cytosolic end-products or the mitochondrial succinate levels increase first. Since almost quiescent animals were used muscular work was minimal. Under these circumstances the mitochondria should be able to accept all the pyruvate resulting from the Embden-Meyerhof-Parnas pathway as long as oxygen is available. When the mitochondria gradually become deprived of oxygen, then succinate levels should increase. Since not all of the pyruvate can be metabolized by the mitochondria anymore, an increase of opine levels should follow, depending on the equilibrium of the respective opine dehydrogenase reaction. Figure 5 demonstrates that the content of succinate increases already after 6 h, while opine levels are increased after 12 h. The results of this experiment lead to the assumption that below the P_c the onset of cytosolic and mitochondrial metabolism is interdependent, whereas during muscular activity no such interrelated control is found.

Critical Oxygen Tension(s), Metabolic Rate, and Anaerobiosis

The variety of responses to declining oxygen tensions described in the literature leads us to search for a concept which allows the explanation of similarities and differences in these observations. A general parameter which would allow such a comparison is the lowest possible rate of aerobic metabolism which has to be defined for both oxyregulators and oxyconformers. For oxyregulators the lowest rate of oxygen consumption at a given PO_2 is defined as the standard metabolic rate (Beamish 1964; Ultsch et al. 1980). Since several oxyregulators (e.g., among crabs and fish) have been shown to maintain this rate down to PO_2 values below which anaerobiosis starts (Teal and Carey 1967; Pamatmat 1978; Pelster et al. 1988), SMR would represent the metabolic rate closest to the lowest aerobic metabolic rate.

SMR is evaluated in long-term measurements (Ultsch et al. 1980) or by extrapolation to zero activity (Beamish 1964). Ideally, the measurement in a flow-through system is required in order to make sure that the lowest rate of metabolism at a given PO_2 is considered. For extrapolation to zero activity, anaerobic metabolism must be excluded as a source for energy during the different degrees of activity. Since only the influence of locomotory activity is considered by the extrapolation procedure, the calculated rate of oxygen consumption still includes the influence of ventilation. The methodological problems involved in the evaluation of SMR may be one reason why this analysis has not yet been done systematically for many species throughout the animal kingdom. Considering the variety of experimental procedures applied, it may very well be that some animals presently seen as oxyconformers will then be identified as oxyregulators with a low standard metabolic rate.

In oxyconformers, the lowest possible rate of aerobic metabolism cannot easily be derived from oxygen consumption curves. Considering, however, the same

methodological concerns as described for oxyregulators, the PO_2 can be evaluated, below which anaerobic metabolism becomes involved in energy production (Pamatmat 1978; Schöttler et al. 1983; Pörtner et al. 1985). The rate of oxygen consumption found at this PO_2 can be seen as the lowest aerobic metabolic rate or, as the standard metabolic rate (SMR) of an oxyconforming animal. Below this critical PO_2 a more rapid drop in oxygen consumption is very likely to occur. This change in the rate of oxygen consumption decline could be demonstrated to coincide with the onset of anaerobic metabolism in the oxyconformer *Sipunculus nudus* (Pörtner et al. 1985; Fig. 3). The rapid fall of the rate of oxygen consumption reminds of the pattern of oxygen uptake variation seen below the P_c in oxyregulators.

In *Arenicola marina* two critical PO_2 values can be distinguished, Pc_I which indicates the shift towards anaerobic metabolism (Schöttler et al. 1983) and a higher Pc_{II} which is characterized by the transition from oxyconformity towards oxyregulation (Toulmond and Tchernigovtzeff 1984).

It is diffusion limitation which very likely leads to the onset of anaerobiosis below the Pc. The predominant view is that diffusion limitation of oxygen in the cytoplasm together with the rate of mitochondrial respiration and the clustering of mitochondria in areas of high energy needs define the point at which the oxygen concentration finally becomes limiting for the rate of oxygen uptake of a cell (Jones 1986). This PO_2 may vary between cells and tissues. It depends on the rate of perfusion of a tissue and also on the density of capillaries and on SMR. For the whole organism, it may depend on the structure of the circulatory system, on the O_2 affinity of the pigment and its regulation, and on the function and structure of the gas exchange system.

Respiratory control of mitochondria in a wide range of ambient PO_2 above the Pc very likely does not depend on diffusion limitations for O_2, since they only require a PO_2 of approximately 0.05 torr to saturate their oxidative capacity (Chance 1976). O_2 measurements at the site of O_2 consumption are required to substantiate this hypothesis. In a more detailed approach it has to be analysed, whether the critical PO_2 of some mitochondria is reached at the same PO_2 in vivo as the critical PO_2 of the whole animal.

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