PART I GENERAL PRINCIPLES

1

Introduction: why we need oxygen

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The aim of this book is not only to describe the basic functions of the respiratory systems of vertebrates, and the diversity in these functions among vertebrates, but also to examine adaptations in these systems that allow numerous vertebrates to explore more or less extreme environments in which oxygen availability is limited or in which there is no oxygen at all.

For the organism to be able to respond to variable oxygen levels, it needs to be able to sense oxygen. This can be done either directly, by monitoring the level of O_2, or indirectly, by responding to changes in the energy status of tissues or cells. Even if some oxygen-sensing structures and their functions have been examined relatively thoroughly, such as the oxygen-sensing carotid bodies in mammals, it is clear that many mechanisms related to oxygen sensing are still largely unknown, particularly when it comes to the almost mysterious ability of many (perhaps most) cells to detect and respond to changing oxygen levels. Chapter 2 will describe the present state of knowledge in this very active field of research. In Chapters 3–4, we will examine the fundamental functions of the respiratory systems of air-breathing and water-breathing vertebrates, laying out the framework for the final five chapters, which deal with adaptations to particularly challenging situations for vertebrates: life at high altitude, diving, surviving in hypoxic waters, and surviving without any oxygen at all.

Oxygen is often called the molecule of life, and we almost intuitively realize the danger of being exposed to low levels of oxygen (hypoxia) or, even worse, to an environment with no oxygen at all (anoxia). We know that it is life threatening for us to have our air supply restricted (asphyxia), with the result that our blood oxygen level falls (hypoxemia), or to have a block in the blood flow to a tissue (ischemia). But why is it that hypoxia, anoxia, asphyxia, hypoxemia and ischemia are so detrimental? This is one of the most intensively studied

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Table 1.1 *O_2 tension and content in air-saturated fresh water and sea water at 1 atm pressure*

Temperature		PO_2	Fresh water			Sea water (35 ppt salt)		
°C	°F	mmHg	mg/l	ml/l	mmol/l	mg/l	ml/l	mmol/l
0	32	158	14.6	10.2	0.457	11.2	7.8	0.349
5	41	158	12.8	9.1	0.399	9.9	7.0	0.308
10	50	157	11.3	8.2	0.353	8.8	6.4	0.275
15	59	156	10.1	7.5	0.315	7.9	5.9	0.248
20	68	156	9.1	6.8	0.284	7.2	5.4	0.225
25	77	154	8.3	6.3	0.258	6.6	5.0	0.206
30	86	153	7.6	5.9	0.236	6.1	4.7	0.190
35	95	151	7.0	5.5	0.218	5.6	4.5	0.176
40	104	148	6.5	5.2	0.202	5.3	4.2	0.165

Values are for 100% air saturation, and values at a lower percentage of air saturation are simply obtained by multiplying the partial pressure of O_2 (PO_2) or O_2 concentration given in the table by the percentage of air saturation (divided by 100).

questions in biomedical science. One main reason for this is that anoxia-related-diseases such as stroke and heart infarction are major killers of people in the developed world. In addition, hypoxia has much to do with the life and death of cancer cells and the complications caused by diabetes. Indeed, it is unlikely for anyone to die in a way that does not, at least finally, involve cellular anoxia.

Biomedical science has so far had limited success in counteracting the various detrimental effects of hypoxia-related diseases, and fresh views on these problems could be inspired by the diversity found in respiratory adaptations in vertebrates, and in the solutions that evolution has provided for survival with little or no oxygen. Indeed, hypoxia is a very common phenomenon in nature. As we shall see, it often occurs in aquatic environments (the subject of Chapter 5) and is always present at high altitude (the subject of Chapter 8). The partial pressure of oxygen at an altitude of 6000 m is less than half of that at sea level, and at the peak of Mount Everest, over which birds do fly, it has fallen to one-third. Hypoxia is common in water, because this medium holds much less oxygen than air does and is often much more stagnant than air, so oxygen can be used up readily. Even when air-equilibrated (air-saturated), one liter of water maximally holds 10.2 ml of molecular oxygen (compared with 210 ml of oxygen in 1 liter of air). Moreover, the maximal water oxygen content falls with increasing temperature and salt content (Table 1.1). For fishes, this problem comes in addition to the challenge of having to breathe in a medium that has a 50 times higher viscosity and an 800 times higher density than air, and through

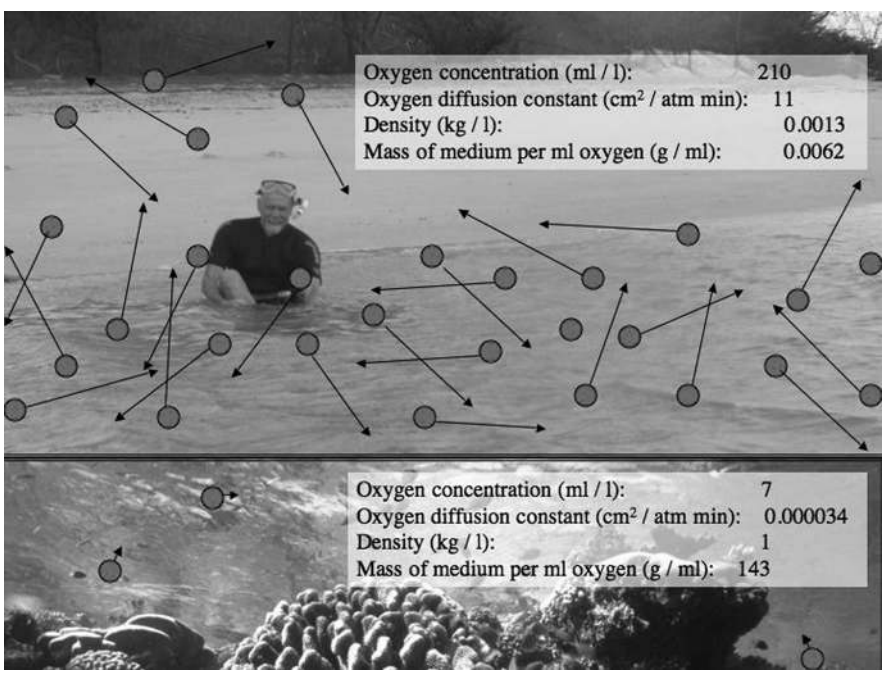

Fig. 1.1 The large differences in the physicochemical properties of water and air mean that equally different demands are put on the respiratory organs of water breathers (primarily gills) and air breathers (primarily lungs). In some respects, breathing water is much more of a challenge than breathing air. The numbers show that the oxygen content of air is about 30 times higher than that of air-saturated water and that oxygen diffuses 300 000 times faster in air than in water. Moreover, the low oxygen content and the high density of water mean that a water breather will have to move about 20 000 times more mass over its respiratory surface than an air breather to get access to the same amount of oxygen. In addition, because water contains a relatively small amount of oxygen that moves very slowly, particularly in stagnant water, hypoxic conditions can readily occur in aquatic environments, presenting an additional challenge for water breathers. However, water loss by evaporation over the respiratory surface, which is a problem for air breathers, is not an issue for water breathers.

which oxygen moves through diffusion some 300 000 times more slowly than in air (Fig. 1.1). The differences in oxygen availability in water and air are further discussed at the beginning of Chapter 6.

1.1 Oxygen and cellular energy

The immediate danger of hypoxia lies in the fact that oxygen is intimately coupled to the generation of adenosine triphosphate (ATP), which drives virtually all energy-demanding processes in cells. The generation of ATP

through oxidative phosphorylation in the mitochondrial respiratory chain requires molecular oxygen. Since so many key cellular functions need a constant supply of ATP, a fall in ATP levels is for most vertebrates immediately life threatening. For one thing, a lack of ATP will stop the activity of the Na^+/K^+ pump (also known as Na^+/K^+ ATPase) and other ion-pumping proteins, which rapidly results in a depolarization of the cell membrane. A depolarized cell without ATP has lost the means to control its volume, ion homeostasis, and intracellular environment, a situation that is highly detrimental and soon renders the cell necrotic.

Another problem with hypoxia is that a halt in oxidative phosphorylation means that the respiratory chain will stop pumping H^+ out of the mitochondria. Thus, not only the cells but also their mitochondria may lose the membrane potential and become depolarized. This phenomenon has been recognized as particularly problematic relatively recently, as it leads to the activation of apoptosis ('programmed cell death' or 'cell suicide'). Thus, even if oxygen supply is restored before the cells have become necrotic, they may already be doomed to die through apoptosis within hours or days (Kakkar and Singh, 2007). One way to protect the mitochondria from depolarization, which has been found to be utilized by anoxic frogs (St-Pierre *et al.*, 2000), is to reverse the function of ATP synthase. This protein, which normally harvests the mitochondrial H^+ gradient to produce ATP, can run backwards and hydrolyze ATP while pumping H^+ out of the mitochondria. Unfortunately, this is an energy-consuming and non-sustainable mechanism that will solve the problem only temporarily and has therefore been called 'cellular treason in anoxia' (St-Pierre *et al.*, 2000).

Apart from energy metabolism, there are several other systems in the body that demand oxygen. These include detoxification enzymes, DNA synthesis, and some steps in the synthesis and catabolism of neurotransmitters. However, the arrest of these systems during anoxia is unlikely to be immediately life threatening, and is mainly of academic importance to an animal that is unable to maintain its ATP levels.

1.2 The brain: the first organ to suffer

The brain is particularly sensitive to a reduced oxygen supply. A major reason for this is the brain's high mass-specific rate of energy use. This is primarily related to its electrical activity, which demands a high rate of ion pumping. The Na^+/K^+ pump alone may be responsible for consuming at least half of the ATP used by the brain (Hansen, 1985), and the rate of ATP turnover in the brain is about ten times faster than that of the average body tissue (Mink *et al.*, 1981; Nilsson, 1996).

After oxygen stores have been depleted, the brain of most animals will rapidly suffer from falling ATP levels. In mammals, the oxygen present in the brain will last only a few seconds after blood flow to the brain has stopped (Hansen, 1985). The concentration of ATP in the mammalian brain is around 3 mmol kg^{-1} (Erecinska and Silver, 1994), and the brain ATP pool is turned over about once every 5–10 s (Lutz *et al.*, 2003). Even with the additional ATP that can be generated from the 3–5 mmol kg^{-1} of phosphocreatine (PCr) that is present in the brain, ATP levels become halved in about a minute and virtually depleted within 2 min in the mammalian brain (Erecinska and Silver, 1994). The situation is not much better for cold-blooded vertebrates such as fishes, in which brain ATP levels are generally below 2 mmol kg^{-1} (DiAngelo and Heath, 1987; Van Raaij *et al.*, 1994; DeBoeck *et al.*, 1995; Van Ginneken *et al.*, 1996; Ishibashi *et al.*, 2002), and estimated rates of ATP synthesis vary between 1.3 and 5.0 mmol kg^{-1} min^{-1} at 12–26°C (Johansson *et al.*, 1995; Nilsson, 1996). This means that the ATP pool in a fish brain is turned over about once every minute.

Thus, the brain is likely to be the first organ to lose energy charge and depolarize when an animal is exposed to severe hypoxia or anoxia. This has two consequences. First, necrotic and apoptotic processes will be initiated rapidly in the brain of an animal that has lost its oxygen supply. Secondly, the depolarized brain can no longer regulate its volume, and the brain cells will start to swell. For many vertebrates, this is a particular problem because there is simply no space in the cranial cavity to allow the brain to swell. Therefore, instead of an increase in brain volume, there will be an increase in pressure in the cranial cavity, and when this pressure rises above the blood pressure, it is no longer possible for blood to reach the brain. Even with the best health care, this is usually an irreversible situation, and consequently a lack of blood circulation in the brain is a principal legal sign of death in many countries. When it comes to brain swelling, fishes and many other cold-blooded vertebrates may be better off than mammals and birds, because they often have a brain cavity that is considerably larger than the brain, thereby allowing the brain to swell without stopping cerebral circulation: the brain of anoxia-exposed common carp (*Cyprinus carpio*) has been observed to increase in volume by 10% without impairing their subsequent recovery (Van der Linden *et al.*, 2001).

Anoxic animals in nature have no access to emergency aid and resuscitation. For them, an energetically compromised brain will become a deadly problem before its neurons have become irreversibly damaged. This is because the brain is responsible for initiating the breathing movements necessary for moving water or air over the respiratory surfaces. In nature, having an energy-deficient brain that has stopped sending signals to the respiratory organs is a point of no return, even if ambient oxygen levels are restored.

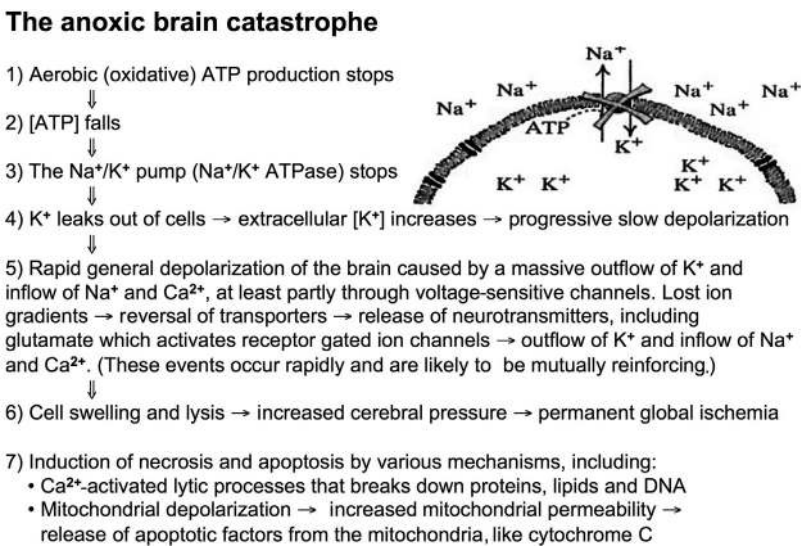

Fig. 1.2 Main disastrous events occurring in a mammalian brain exposed to anoxia.

In biomedical science, many efforts are being put into clarifying the details of the catastrophic events that affect an anoxic brain, with the ultimate aim of finding ways of interfering with the underlying mechanisms so that the detrimental effects of conditions such as stroke and circulatory arrest can be reduced. Thus, we have a relatively detailed knowledge about the catastrophe that occurs in the mammalian brain during anoxia (Fig. 1.2). In humans, unconsciousness occurs and electric activity is suppressed in the brain just 6–7 s after blood flow to the brain is halted (Rossen *et al.*, 1943). This is likely to be an initial emergency response, possibly functioning to save energy by reducing ATP-consuming electrical activity. Moreover, as a standing person faints and falls, the brain moves into a lower position in relation to the heart, which increases cerebral blood pressure and thereby cerebral blood flow.

If blood or oxygen supply to the mammalian brain is not restored within seconds, progressive changes in energy status and ion homeostasis are soon apparent (Hansen, 1985; Erecinska and Silver, 1994). Virtually immediately, there is a steady, slow rise in extracellular [K^+], probably caused by both increased K^+ permeability of the cells and a slowdown of the Na^+/K^+ pump. Within a minute, the level of ATP falls to half the normoxic level, while concentration of adenosine diphosphate (ADP) is tripled and that of adenosine monophosphate (AMP) is increased by an order of magnitude. At the same time the PCr store is virtually depleted.

In rodents, the whole brain depolarizes after 1–2 min of ischemia. This is an event that is characterized by a massive outflow of K^+, and influxes of Na^+ and

Ca^{2+}. In larger mammals, this anoxic depolarization may take a few minutes longer due to their lower mass-specific metabolic rate. In cold-blooded vertebrates, the anoxic depolarization is further delayed. Thus, in rainbow trout (*Oncorhynchus mykiss*), the brain depolarizes after about 15–30 min of anoxia at 10–15°C (Nilsson *et al*, 1993; Hylland *et al*., 1995). Still, the mechanisms behind the anoxic depolarization are probably similar for most vertebrates, and time differences can be fully explained by differences in metabolic rate.

Following the anoxic depolarization, there is a massive outflow of neurotransmitters from the intracellular to the extracellular compartment, where these neurotransmitters can activate their receptors. Contrary to expectations, Ca^{2+}-mediated vesicular release of neurotransmitters probably plays a minor (if any) role in this event, because vesicular transmitter release is ATP dependent, and as already discussed, very little ATP is available in this situation. Instead, the release of the neurotransmitters appears to be primarily caused by a reversal of neurotransmitter transporters. These normally harvest the ion gradients over the cell membranes to take up neurotransmitters and keep their extracellular levels low (Danbolt, 2001). However, as the ion gradients collapse, the transporters start to run backwards and release transmitters. At this point, the worst problem for the brain appears to be the release of glutamate, the major excitatory neurotransmitter in the vertebrate brain. In this uncontrolled situation, glutamate functions as an excitotoxin. In particular, the activation of two major glutamate receptor types, called NMDA and AMPA, are thought to play key roles in excitotoxic cell death in the brain. These receptors let large amounts of Ca^{2+} and Na^{+} into the neurons. The resultant massive rise in intracellular [Ca^{2+}] wreak havoc on the cells by, for example, activating proteolytic and lipolytic processes, as well as DNA-degrading mechanisms (see Lipton, 1999; Lutz *et al*., 2003 for reviews). The result is either immediate necrotic cell death, which in the case of brain ischemia (stroke) will affect cells in areas completely devoid of blood flow, or slow cell death through autophagocytotic and apoptotic mechanisms. The latter two occur hours to days after blood flow has been restored and in an ischemic brain affect many cells in the so-called ischemic penumbra (the zone of suppressed blood flow surrounding the central ischemic area). There appear to be several mechanisms involved in anoxia- or ischemia-induced apoptotic cell death. One of these was recently termed ‘parthanatos,’ and it appears to be particularly common in ischemic/post-ischemic brain tissue. The name is derived from the death signal in this pathway, poly(ADP-ribose) (‘Par’) polymer, and Thanatos, the Greek personification of death and mortality. Parthanatos is biochemically and morphologically distinct from the normal (caspase-dependent) apoptosis (Harraz *et al*., 2008).

1.3 Boosting oxygen uptake: the first option

In this book, we will not only describe how oxygen is normally handled by the organism, but also how the organism can regulate its oxygen uptake and defend itself against hypoxic conditions. The first option for an animal that experiences a fall in ambient oxygen levels is to boost the extraction of oxygen from the environment. This is primarily done by increasing the ventilation of the lungs or gills and by increasing the blood perfusion through these respiratory organs. As we shall see in Chapter 6, some animals even switch from breathing with gills to breathing with lungs. Through such adjustments, most vertebrates become what is known as 'oxygen regulators' (Prosser and Brown, 1961), i.e. they are able to regulate their oxygen extraction capacity so that oxygen uptake ($\dot{V}O_2$) is maintained at a steady level over a more-or-less wide range of ambient oxygen concentrations. There is a great species-to-species variability in how well vertebrates can do this, a variability that has its origin in how evolutionary processes have shaped the organism in response to its environment or lifestyle. For example, animals that are adapted to hypoxic habitats can typically maintain their $\dot{V}O_2$ at much lower water oxygen levels than can species that are unlikely to encounter hypoxia. The lowest level at which an animal can maintain its $\dot{V}O_2$ is denoted the critical oxygen concentration ($[O_2]$crit), or critical oxygen tension ($P O_2$crit) if the oxygen level is recorded as the partial pressure of oxygen (Prosser and Brown, 1961). The $P O_2$crit is a common measure of hypoxia tolerance in fishes (mechanisms of hypoxia tolerance in fishes are the subject of Chapter 5).

1.4 Oxygen-independent ways of making ATP

If ambient oxygen levels fall below $P O_2$crit, the animal has to start making ATP anaerobically. PCr can rapidly regenerate ATP from ADP, but as PCr levels are usually quite limited, ranging from about 0.5 to 5.0 mmol kg^{-1} in the brain of vertebrates (e.g. DiAngelo and Heath, 1987; Erecinska and Silver, 1994; Van Raaij *et al.*, 1994; Van Ginneken *et al.*, 1996), this pathway can maintain ATP levels only for one or a few minutes. To be able to maintain ATP levels longer in anoxia, anaerobic glycolysis is the only viable option. Sources of fuel other than glucose, i.e. fat and protein, are virtually useless in the absence of oxygen, because these demand a functional citric acid cycle. Without oxygen, the intimate connection between the citric acid cycle and the respiratory chain rapidly makes the citric acid cycle come to a halt (Hochachka and Somero, 2002).

Unfortunately, for most vertebrates, the anaerobic capacity of the brain is not high enough to allow it to compensate for more than a fraction of its aerobic rate of ATP production. The reason for this is that most of the chemical energy stored

in glucose is left in the glycolytic end product, which in vertebrates is normally lactate. Thus, from every molecule of glucose, only two molecules of ATP are generated (an additional ATP is produced in the breakdown of glycogen to glucose). In the presence of oxygen, the complete breakdown of glucose to CO_2 and H_2O can yield up to 36 molecules of ATP, although for various reasons (including partly uncoupled mitochondria), a yield of 29 molecules of ATP is probably a more realistic figure (Brand, 2003). Thus, aerobic metabolism is able to produce about 15 times more ATP per molecule of glucose than can anaerobic glycolysis. Another serious problem with generating ATP anaerobically is that it normally leads to the production of lactate and equimolar amounts of H^+. The H^+ is actually formed during the hydrolysis of ATP rather than through glycolysis, but the net effect is that lactic acid is produced (see Hochachka and Somero, 2002 for review). The hydrogen ions can cause life-threatening acidosis and the lactate causes osmotic disturbances. Nevertheless, for many vertebrates, generating ATP through glycolysis during hypoxic or anoxic conditions prolongs the survival time considerably, and in some cases even allows anoxic survival for days or months (see Chapter 9).

It may be that the initial cause of anoxic death in vertebrates varies to some degree between species of vertebrate groups. Although it is clear that the rapid and severe drop in the brain ATP level initiates the anoxic catastrophe in mammals, some fishes may die from lactic acidosis rather than an inability to produce enough ATP. A study of anoxic rainbow trout and brown bullhead (*Ameiurus nebulosus*) found that ATP levels were relatively well maintained at the time when they ceased to breathe, but lactic acid levels had risen to 12–20 mmol kg^{-1}, which may have been too high for the brain to tolerate (DiAngelo and Heath, 1987; Van Raaij *et al.*, 1994). By contrast, in severely hypoxic common carp and Nile tilapia (*Oreochromis niloticus*), considerable falls in brain ATP levels have been detected (Van Raaij *et al.*, 1994; Ishibashi *et al.*, 2002), although the possibility remains that the falling ATP levels seen in some fish brains is caused by metabolic dysfunction caused by lactic acidosis or a rundown of the glycogen stores. Nevertheless, there are also striking similarities in the anoxic death process between mammals and fish. Measurements of extracellular K^+ and glutamate levels in the brain of anoxic rainbow trout reveal an anoxic depolarization and an outflow of K^+ and glutamate from the cells that is very similar to that observed in mammals (Nilsson *et al*, 1993; Hylland *et al.*, 1995).

In addition to boosting oxygen uptake and activating glycolytic ATP production in hypoxia or anoxia, some animals have evolved a third survival strategy: metabolic depression. Thus, during oxygen shortage they are able to lower their use of ATP so that consumption can be matched by production and ATP levels