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Chapter I

The Evolution of Oxidative Stress

Nick Lane*
Department of Genetics, Evolution and Environment, University College London, Gower Street, London W1E 6BT, U.K.

1. Introduction

"Life is nothing but an electron looking for a place to rest."
Albert Szent-György

Oxidative stress has evolved in both senses of the word. It has evolved in semantic meaning. When first defined by Helmut Sies in the 1980s [1], oxidative stress was perceived as largely negative and pathological. Since then, it has become clear that the reactive oxygen and nitrogen species (ROS and RNS) that cause oxidative stress are not merely pathological, but serve as signals in many diverse circumstances (see Chapters 2 and 3). More broadly, *oxidative stress is a physiological state that elicits a decisive shift in patterns of gene expression, leading usually to its own resolution*. Blocking oxidative stress with antioxidants is rarely beneficial; and most studies of antioxidant supplements have indeed proved ineffective, if not actively damaging.

This nuanced conception makes it clear that oxidative stress has evolved in the biological sense too. No longer is it seen as a purely pathological state, over which the body or the cell has little control; it is a central part of cellular homeostasis. Cellular redox state, and specifically the activation of redox-sensitive transcription factors by oxidative stress (Chapters 24-26), is beginning to look as central to cell homeostasis as protein phosphorylation. While the interplay between protein thiol oxidation, S-nitrosylation and phosphorylation is still somewhat murky, it is plain that redox state is a critical regulator of cellular respiration, cell cycle, proliferation, differentiation, sexual development, stress-

* Email: nick.lane@ucl.ac.uk

resistance, longevity, senescence and apoptosis. All of this certainly evolved, but the evolutionary tradeoffs underlying ROS signalling are little explored. Often they seem to be in conflict, displaying Janus-like qualities. Is nitric oxide (NO) a 'good thing' or a 'bad thing'? What about mitochondrial biogenesis? Without an evolutionary narrative, the costs and benefits can be confounding.

While ROS are no longer perceived as *merely* damaging, it is far from certain how damaging they actually are, and under what circumstances. Fenton chemistry, lipid peroxidation, protein carbonyl oxidation, DNA mutation (Chapters 5-9) – the old order of free radical biology – are real enough, but many assays are equivocal, and the standard laboratory conditions are not always relevant. For example, the mutation rate (as opposed to the long term evolution rate) of mitochondrial DNA relative to nuclear DNA is unknown to within several orders of magnitude [2], as is the proportion of oxygen escaping from the respiratory chain as superoxide under physiological oxygen tensions of around 3 μM oxygen (1-2% of atmospheric tension). The frequently cited figure of 1-5% ROS leak during respiration, dating back to Britton Chance's work in the 1970s [3], is not correct at lower oxygen tensions; but the actual *in vivo* figure, or its pathophysiological relevance, is unknown. Likewise, the degree to which protein or DNA damage accumulates in aging tissues is difficult to ascertain, because apoptosis removes the evidence.

At the same time, it has become clear that free radicals are not the potent bacteriocidal agents long thought. The oxidative burst of neutrophils, which generates superoxide radicals at high levels, via NADPH oxidase, in fact alkalinizes the vacuoles, activating proteolytic enzymes inside [4]. These, not ROS, degrade bacteria. This inevitably begs the question, why use potentially toxic ROS at all? In the same context, it has also become clear that ROS leak from respiratory chains is not a simple by-product of respiration: the proportion of oxygen consumed that is converted into ROS is not fixed, but varies according to the respiratory rate, training, thyroid hormone levels and calorie intake, and between different tissues and across species [5]. Nor does ROS leak seem to be strictly necessary: cytochrome oxidase achieves the complex four-electron reduction of oxygen to water without any measurable ROS leak at all. So again: why, from an evolutionary point of view, are potentially toxic ROS used as signals?

The answer does not relate in a simple manner to oxygen toxicity and antioxidant balance, as long assumed, but in a fundamental way to the nature of energy transduction in cells. *Oxidative stress, differences in lifespan and susceptibility to age-related disease are all pleiotropic consequences of the fundamental requirement to regulate energy transduction.*

2. The Origin and Significance of Chemiosmotic Coupling

From the medical point of view, respiration is typically divided into aerobic and anaerobic. Aerobic respiration obviously requires oxygen; anaerobic respiration – or 'life without oxygen', in Pasteur's words – is typically taken to mean anaerobic glycolysis, or fermentation. The distinction, which may be reasonable enough for humans, makes a mockery of evolution. The true, meaningful, distinction is between substrate-level phosphorylations, such as fermentation, in which phosphate groups are transferred directly by

chemistry, and oxidative phosphorylation, in which electrons are transferred from an electron donor such as glucose (but which could be many other organic or inorganic donors such as Fe^{2+}) via a series of redox centres to a terminal acceptor. In aerobic respiration this acceptor is oxygen, but in anaerobic respiration a range of other electron acceptors are used, from NO to Fe^{3+} to protons. In all forms of oxidative phosphorylation, the passage of electrons from the donor to the acceptor is coupled to ATP synthesis by way of an intermediary proton gradient across a membrane – chemiosmotic coupling. Rather than chemistry, which is to say reactions between molecules, the process is basically electrical. This is, at bottom, the cause of oxidative stress.

Described by Leslie Orgel as 'the most counter-intuitive idea in biology since Darwin' [6], the mechanism of chemiosmotic coupling was controversial for three decades, a period known as the 'ox-phos wars', culminating in the award of the Nobel Prize to Peter Mitchell in 1978 [7]. The passage of electrons along respiratory chains – essentially the flow of electricity down electrical wires – drives conformational changes in respiratory proteins (or more simply, electro-chemical loops across the membrane, as in the Q cycle) to generate a proton gradient across the membrane. Driven by the proton-motive force (the combination of electrical potential and pH gradient), the passage of protons through the rotary motor of the ATPase in turn drives ATP synthesis. But why, and how, did such a counterintuitive system evolve?

The answer relates to the ability of a gradient to uncouple exergonic reactions from ATP synthesis. Consider the direct reaction of hydrogen with carbon dioxide, known as the acetyl CoA pathway, and probably the most ancient chemolithotrophic pathway in life. In ancient methanogens (archaea) and acetogens (bacteria), this pathway provides both the carbon and energy metabolism of life – there is no need for solar power, primordial soup, ATP or any other accoutrements. The acetyl CoA pathway is exergonic right through to pyruvate, and releases enough energy (captured as ATP) to power all intermediary metabolism. But there is one drawback: kinetics. While thermodynamically probable, the reaction between H_2 and CO_2 is slow because a kinetic barrier must be surmounted to convert CO_2 into formate (CHO_2^-). In modern organisms, this conversion requires an initial input of energy, usually in the form of ATP. The problem is that, by substrate-level phosphorylation, one ATP must be split to gain one ATP from the reaction: there is no net gain and therefore there can be no growth. Life would be impossible [8,9].

The reaction of H_2 with CO_2 is by no means alone in 'technically' not generating enough energy for growth. Many other reactions that do in fact sustain growth – such as the anaerobic oxidation of methane using nitrite (the anammox reaction) – could not do so by substrate-level phosphorylation alone. Put more broadly, if life operated by chemistry alone, it could never have got started. In the absence of oxygen or photosynthesis, there is insufficient energy available from anaerobic reactions to power both carbon assimilation and ATP synthesis by substrate-level phosphorylations alone. There are in fact only six known pathways of carbon assimilation across all life, including the Calvin cycle (used in oxygenic photosynthesis), the reverse Krebs cycle (found in many vent bacteria), and the acetyl CoA pathway. All but the acetyl CoA pathway require an input of energy, in the form of ATP or some equivalent, which is provided by sunlight in photosynthesis and oxygen in the case of chemosynthesis in vents. Because oxygen is derived from photosynthesis, the profusion of life in black smoker vents is ultimately powered by photosynthesis and could not exist without it. Only the acetyl CoA pathway, the direct reaction of H_2 with CO_2, can provide the

energy required for growth in the absence of light or oxygen; and even this pathway can only do so by way of chemiosmotic coupling.

Why does chemiosmotic coupling make such a decisive difference? Because an exergonic reaction, such as the reaction of CO_2 with H_2, can be uncoupled in both time and space from an endergonic reaction, such as the reaction of ADP with Pi to give ATP. This means that there is no direct equation between the energy released by one reaction and consumed by the other, as there must always be in substrate level phosphorylations, or indeed, in any form of chemistry. In chemiosmotic coupling, in contrast, the energy released by an exergonic reaction is used to transfer one or more protons across a membrane. So long as the energy released is sufficient to transfer a single proton at least part of the way across the membrane, the reaction can be repeated indefinitely to generate, in the end, a proton gradient. And then that gradient can be used independently to power ATP synthesis, enabling growth. In the case of the acetyl CoA pathway, it remains true that 1 ATP must be spent to overcome the kinetic energy 'hump'; but instead of reclaiming just one ATP, so precluding growth, chemiosmotic coupling makes it possible to gain about 1.5 ATPs per CO_2. The non-stoichiometric number gives it all away: stoichiometry is a property of chemistry, not gradients [10].

From this point of view it is no accident that all life on earth is powered by gradients, mostly proton gradients; and even exceptions like fermenting bacteria use their ATP to pump protons to maintain their membrane charge, used in turn to power motility, ionic homeostasis and the uptake of organic matter. Fermentation arose later, of that there can be little doubt. The glycolytic pathway evolved independently in archaea and bacteria, and no fermenters are found anywhere near the base of the phylogenetic tree. The conception of fermenting a primordial soup lacks any thermodynamic basis [9]. *Without chemiosmotic coupling, early life would have been impossible, and even today, after four billion years of evolution, the fact that chemiosmotic coupling is as universal as the genetic code goes to show that the mechanism is close to being energetically unimprovable. But the inevitable penalty, at least in the presence of oxygen, is oxidative stress.*

The daunting complexity of modern respiratory chains long concealed the conceptual simplicity of chemiosmotic coupling – the flow of electrons powers the transfer of protons over a membrane. If membranes, proton gradients or electron-transferring respiratory chains required eons of natural selection to evolve, then they could certainly not have powered early life. But in fact all are provided 'free of charge' in alkaline hydrothermal vents, which are driven by the global geological process of serpentinization [11]. In serpentinization, seawater reacts with ultramafic magnesium-rich rocks derived from the upper mantle, such a peridotite, containing the mineral olivine. Olivine is hydroxylated to metamorphose into serpentine, hence the name of the process. The dark green mottled mineral serpentinite is commonly used as a building stone, often on the facade of banks or public buildings, including the United Nations. The hydroxylation of olivine oxidises Fe^{2+} to Fe^{3+}, releasing hydrogen gas and hydroxide ions into solution, and producing enough heat to drive these alkaline fluids back up to the seafloor, where the dissolved minerals precipitate out into the colder seawater to form alkaline hydrothermal vents: porous calcium carbonate towers, riddled with microscopic, roughly cell-sized pores, lined with flimsy aragonite membranes. While their existence and periaxial location was predicted in detail two decades ago by geochemist Michael Russell [12, 13], the first such vent system was not discovered until ten years later – Lost City Vent, just off the mid-Atlantic ridge [14].

Lost City conforms to most of Russell's predictions but does not do so in every way. The two main differences are highly significant. On Earth 4 billion years ago there was virtually no oxygen, and far higher levels of CO_2 – anything up to a thousand-fold more. The absence of oxygen meant that iron was in the form of Fe^{2+}, which dissolves in water. The oceans were full of it; later, as oxygen levels rose, Fe^{2+} was oxidised to Fe^{3+}, which precipitated out as rusty minerals to form vast banded-iron formations; but 4 billion years ago, before all this iron had been precipitated from the oceans as Fe^{3+} in banded-iron formations the oceans would have been nearly saturated in dissolved Fe^{2+}. Unlike today, therefore, ferrous iron was available for incorporation into vent systems. In ancient alkaline hydrothermal vents, this ferrous iron is likely to have precipitated out with sulfide to form bubbly membranous films of iron sulfur minerals, perhaps mixed with aragonite. Fossils of such structures exist, albeit only 350 million years old (the ocean crust is constantly recycled by tectonics, making older structures rare) and they have been reproduced in the lab [15]. Critically, on the molecular scale, such minerals form into iron-sulfur clusters (Chapter 31) with an identical structure to the Fe-S clusters still found at the heart of respiratory complexes today, notably in complexes I and II.

Recent functional studies of respiration suggest that electron transfer is independent of the protein groups that embed these Fe-S clusters; what matters is the distance between clusters [16]. If the distance between successive redox centres is less than around 14 Ångstroms, electrons 'tunnel' via quantum processes down the respiratory chain. Thus, electron flow depends on a property – distance – that is geometric, and geochemical, rather than biological in provenance. Furthermore, Fe-S clusters have the important intrinsic factor of transferring single electrons, by dint of the electron configuration of the transition metal iron. Free-radical chemistry is the chemistry of single electrons, not the electron pairs characteristic of covalent bonds, and it began with Fe-S clusters precipitating in ancient alkaline vents, doing what they do today: transferring single electrons from the alkaline fluids emerging from serpentinized rocks into the acidic ocean waters above.

Acidic. That is the difference CO_2 made. When dissolved in water, CO_2 forms carbonic acid, hence the concerns today that global warming, in acidifying the oceans, is destroying the delicate reef systems that depend on slightly alkaline waters to precipitate carbonates into corals. Back then, the pH of the oceans was likely to have been in the range of 5 to 6 [17]. The pH scale, of course, is defined in terms of proton concentration, each pH unit representing a tenfold difference in proton concentration. As the alkaline fluids percolated into acidic oceans, through a labyrinth of interconnected micropores, lined with hydrophobic iron-sulfur membranes, the vent system would have developed a natural proton gradient of about 4 pH units across the mineral walls – a difference in proton concentration of about 10,000-fold, equating to a membrane potential of 200-400 mV, with the correct polarity, remarkably similar to that across bioenergetic membranes today. How the first cells began to tap the energy of this natural proton gradient is beyond the scope of this Chapter; suffice to say that polyphosphates, such as ATP and pyrophosphate, form under acidic conditions at low water activity (in hydrophobic membranes) whereas their hydrolysis is favoured under alkaline aqueous conditions [9]. It is therefore plausible that natural proton gradients could have driven the cycling between pyrophosphate and phosphate, or ATP and ADP, in the vent environment.

Whatever the mechanism, the first cells could not have left the vents without first mastering the art of chemiosmotic coupling – nothing else could have provided the necessary

energy. Luckily the vents also equipped the first cells with all the necessary tools – proton gradients, electron-conducting iron-sulfur clusters, and charged membranes. When the first prokaryotic cells did emerge, these were the tools of their trade. Without them, oxidative stress would not exist. With them, the stage was set for the second act: photosynthesis.

3. The Origin and Consequences of an Oxygen Atmosphere

Respiration, by necessity, evolved early. Not aerobic respiration, but anaerobic respiration – the transfer of electrons from an electron donor (probably hydrogen) to an acceptor (initially CO_2). At some point early in evolution, electrons would have been transferred to a range of other electron acceptors, as energy metabolism diverged from carbon assimilation. By its nature, chemiosmotic coupling splits exergonic reactions that provide energy from endergonic reactions which utilise that energy in carbon metabolism. Simply substituting alternative electron acceptors such as NO or Fe^{3+}, probably plentiful on the early Earth, required no change in membrane structure, and may have been among the earliest forms of prokaryotic specialisation [18]. As a general rule, it is fair to say that prokaryotes can be classified not by their morphology but by their metabolic capabilities. And the most significant of those was photosynthesis.

Photosynthesis reverses respiration, not only in the global chemical sense (the equation for aerobic respiration can be represented as $CH_2O + O_2 \rightarrow CO_2 + H_2O$ and oxygenic photosynthesis $CO_2 + H_2O \rightarrow CH_2O + O_2$) but also in particulars. In oxygenic photosynthesis, for example, electrons are transferred from water – split by chlorophyll photo-oxidised by the sun – via an electron-transport chain that is exactly analogous to the respiratory chain, ultimately onto CO_2 to form sugars. The flow of electrons drives the transfer of protons across the thylakoid membranes to generate a proton gradient, which in turn drives ATP synthesis. Thus, in a curious way, photosynthesis also reverses the specialisation of metabolism into carbon assimilation and energy metabolism, reuniting them in a system driven by the power of light. Of course, cyanobacteria, and later algae and plants, are also capable of normal respiration to provide energy during darkness or to bleed off excess reduction potential.

Like respiration, photosynthesis is not particular about the source of electrons. Indeed, drawing on the power of the sun it has even less need to be. On the early Earth, H_2S and Fe^{2+} would have been major electron donors, as they are in many bacteria today. The principle is exactly the same: photo-oxidation of chlorophyll transforms it into an oxidant, which can strip electrons from many sources, passing them ultimately onto CO_2. Only when water itself is used as an electron donor – a difficult operation even for cyanobacteria, and never yet achieved by any other form of bacteria, or for that matter, solar scientists – is the waste product oxygen. If H_2S is the electron donor, the waste product is sulfur. While the origin of oxygenic photosynthesis is obscure, it probably arose 3 billion years ago, if not earlier, by coupling in series the two reaction centres used by anoxygenic photosynthesizers, PSI and PSII [19, 20]. But however it evolved, photosynthetic water-splitting utterly transformed the planet. Drawing on water as a fuel, rather than reduced chemicals derived from volcanic and hydrothermal processes, probably increased global biomass 10-fold [21]. The waste,

molecular oxygen, initially reacted with sulfur, iron or methane but ultimately accumulated in the atmosphere, from around 2.4 billion years ago in the Great Oxidation Event, perhaps precipitating the first global ice age – a 'snowball earth' – as it did so [22].

Oxygen changed the world. The extent to which complex life is possible at all without oxygen is an interesting question, thrown into sharp relief by the recent discovery of animals, *Loriciferans*, that complete their life cycle in the absence of oxygen [23]. In fact, oxygen produces only about an order of magnitude more power than fermentation; and the difference between aerobic and true anaerobic respiration is somewhat less than that. While this is substantial, it is orders of magnitude less than the difference made by mitochondria, as discussed later in this Chapter; and probably differences in nutrient availability or concentration gradients outweighed any metabolic advantages of oxygen, at least among bacteria. Oxygen hardly wrought a global revolution in prokaryotic physiology. Even in the presence of oxygen, no prokaryote ever came close to evolving the morphological complexity of eukaryotes. In this context, the evolution of aerobic respiration may have made a difference, but the most immediate impact of the rising tide of oxygen was its juxtaposition with the electron-transport chains of bacteria, all of which transfer single electrons.

The reactivity of oxygen, of course, is limited by kinetics in much the same way as CO_2. If it were not so, the biosphere, even the atmosphere, would spontaneously combust – as it would if it were filled with singlet oxygen. *The kinetic limitation on the reactivity of oxygen relates to its unusual electron outer orbital structure, giving molecular oxygen two electrons in parallel spin*. Unlike singlet oxygen, triplet oxygen is unable to accept a pair of electrons, and must be reduced by one electron at a time – by single-electron donors, among which iron is prominent. Because the roots of respiratory chains are in geochemistry, notably the Fe-S clusters already discussed, all these respiratory chains become badly insulated wires in the presence of oxygen. *Oxygen is hardly toxic if left to itself; but it is readily activated in the presence of the very respiratory chains that are necessary for life*.

ROS leak has more to do with the speed of electron flow down electron transport chains than it does with the concentration of oxygen itself. In general, ROS leak is lower in state III respiration (when ATP consumption is fast) than it is in state IV respiration, when electron flow is limited by ADP deficiency [5]. If the respiratory complexes become highly reduced, they become more reactive with oxygen; and the higher membrane potential can drive electrons in reverse back into complex I, again increasing the rate of ROS leak. Without compensation, then, ROS leak is largely defined by poor growth: by a low demand for ATP and highly reduced respiratory complexes. There are various ways out of this 'high-voltage' situation, from mild uncoupling to complete depolarization of the membrane, or the use of alternative oxidases, which pass electrons directly on to oxygen, without coupling to proton translocation. All of them, in effect, short-circuit the membrane potential, enabling faster electron flow, less reduced respiratory complexes and lower ROS leak. Just how important such mechanisms might be in prokaryotes is hinted at by the sheer scale of 'energy spilling' in bacteria, an apparently wasteful frittering away of up to 50% of cellular energy charge, for unknown reasons [24]. It is plausible that such energy spilling works to short-circuit overcharged membranes in the presence of oxygen, so restricting ROS leak and cellular damage.

In this context, it is revealing that the only bacteria to have evolved a form of controlled cell death analogous to apoptosis in eukaryotes (Chapters 37 and 38) – right down to the use of metacaspase enzymes, which are closely related to metazoan caspases – always deal with

oxygen. The best example is cyanobacteria, which form oceanic blooms on a similar scale to eukaryotic algae. Like algal blooms, cyanobacterial blooms often disappear overnight, in effect killing themselves in response to some stimulus, usually a viral infection. But viruses are not the only cause: light stress and iron deficiency can also literally liquefy cyanobacterial blooms – and the common denominator in all these cases is oxidative stress, brought about by ROS leak [25]. Why single-celled algae and cyanobacteria kill themselves is a complex question, and can only be interpreted in light of their differentiation within colonies – ROS leak can signal cell death or differentiation into spores or biofilms. Selection can therefore be seen as acting at the group level, where the bloom has at least some of the properties of a multicellular organism, assisted by the genetic relatedness of the clonal cells in the bloom; or in terms of selfish genes. But regardless of the genetic interpretation, perhaps the most significant factor determining the fate of the bloom is the potential for serious cellular damage in the presence of oxygen. *Only bacterial cells capable of oxygenic photosynthesis or aerobic respiration appear to undergo controlled cell death in the context of blooms* [25]. *Oxygen introduces a new penalty for failure, controlled cell death, that later played a central role in the evolution of true multicellular organisms*.

The basic problem, which is central to eukaryotic evolution too, is that the rates of photo-oxidation and electron transfer, being essentially quantum events, differ from the rates of chemical reduction and carbon assimilation. This means that conditions such as high light intensity (which rapidly photo-oxidizes chlorophyll), low temperatures (electron transfers are barely slowed, but chemical reactions are much slower), and iron deficiency (leading to poor respiratory stoichiometry) all cause high ROS leak. If this high ROS leak is not brought under control quickly, the caspase enzymes are activated – significantly by the loss of the respiratory carrier cytochrome c, in plants as well as animals – and the cell is eliminated. Controlled cell death offers the advantage of recycling scarce nutrients, and so can be beneficial to the larger grouping, whether an organism, a colony, or selfish genes.

Much the same problems affect the respiratory chains of non-photosynthetic aerobic bacteria, such as some α-proteobacteria, among them the free-living ancestors of mitochondria, which likewise are capable of controlled cell death using metacaspase enzymes. The later development of apoptosis in metazoans makes use of enzymes that are bacterial in ancestry, notably the caspases, but also the Bcl-2 family and other mitochondrial apoptotic proteins [26]. The point is that the evolution of metazoan cell death – and with it the complex developmental programs that require apoptosis – all build on a system that evolved in relatively complex clonal bacteria capable of an apoptotic-style of cell death in response to oxidative stress. The single greatest danger is the failure to pass electrons on swiftly down respiratory chains, resulting in highly reduced complexes in an aerobic atmosphere. The way in which these factors played out in the respiratory chains of eukaryotes may have been one of the most significant selective forces in eukaryote evolution.

4. Oxidative Stress and the Chimeric Origin of Eukaryotes

All truly complex life on Earth is composed of eukaryotic cells. All eukaryotes are closely related in cellular structure, and this structure is totally unlike bacteria or archaea. All

by definition share the nucleus, with its double membrane, pitted with large protein pore complexes. All have straight chromosomes, telomeres, centromeres, chromatin structures, introns and exons, mitosis, meiosis, reciprocal sex, dynamic cytoskeleton, endoplasmic reticulum, lysosomes, mitochondria and so on: undoubtedly these complex traits were all inherited from a common ancestor, which must already have been a complex cell quite unlike any known prokaryote. These detailed similarities reach into the deepest structure of genes, with the same introns occupying the same site in the same gene in fungi, algae, plants, animals and protists. The deep unity of eukaryotic cells is arguably evidence for the evolution of sex in the earliest eukaryotes; but whatever the cause, the sheer number of shared traits testifies to the common and *unique* origin of eukaryotic cells. Unique, because if complex cells had arisen more than once, then there should be various disparate types of eukaryote today, each with a spectrum of different traits, unless all fell extinct without trace. While it is not possible to rule out this possibility, there is no evidence in its favour, and much to suggest a genuinely unique origin.

The most significant piece of evidence testifying to the unique origin of eukaryotes is the realization, over the last two decades, that all known eukaryotes either currently possess, or once had and later lost, mitochondria. There are in fact around a thousand species of apparently primitive single-celled eukaryotes that do not have mitochondria – a paraphyletic grouping described as 'archezoa' by the cell biologist Tom Cavalier-Smith [27]. These species – mostly parasites such as *Giardia* and entamoeba, albeit with free-living relatives – were hypothesized to be representatives of the earliest eukaryotes, primitive phagocytes that had never acquired mitochondria, but which survived in marginal niches. But closer scrutiny of their cell structure (all contain double-membraned structures derived from mitochondria, known as hydrogenosomes or mitosomes) and their genomes (which contain genes derived from mitochondria) betrayed the fact that these apparently primitively amitochondriate groups had once possessed mitochondria, but had later lost them in the course of specializing into anaerobic environments [28]. This discovery has two major implications. First is the fact that the niche itself is perfectly viable: it is occupied by *bona fide* amitochondriate eukaryotes, and from their cell structure and diverse habitats there is no obvious reason why they should have driven all genuinely primitively amitochondriate eukaryotes to extinction. Positing extinction as an explanation for the unique origin of eukaryotic cells is therefore, at the least, counter to Occam's razor. Second, the fact that all eukaryotes once possessed mitochondria pushes the origin of mitochondria and the origin of the eukaryotic cell back to the same time frame, and plausibly the same event, as first proposed by evolutionary biologist Bill Martin [29].

There is now large-scale genomic evidence that this is exactly what happened: the eukaryotic cell originated in some kind of chimeric endosymbiotic event between two prokaryotes, the host cell being an archaeon and the endosymbiont the ancestor of the mitochondria [30, 31]. Exactly what benefit mitochondria brought is not obvious, despite 40 years of research since Lynn Margulis marshalled the evidence to demonstrate the bacterial origin of mitochondria [32]. The advantage was not aerobic respiration, as many prokaryotes respire aerobically, and many mitochondria are anaerobic, notably among protists and fungi. Nor did mitochondria protect against oxygen toxicity: mitochondria, being full of respiratory chains, are among the most potent free-radical generators known (Chapter 27). They were part of the problem, not the solution. Mitochondria did not enable anaerobic host cells to adapt to rising oxygen levels as anaerobic environments disappeared; on the contrary, rising oxygen levels actually gave rise to sulfidic 'Canfield' oceans, in which the oceans stratified in

the same manner as the Black Sea today, with the subphotic zone remaining anoxic for more than a billion years, from 1.8 to 0.7 billion years ago – the period during which eukaryotes almost certainly evolved [33]. And while it is true that mitochondria compartmentalized respiration within the cell, many prokaryotes internalize their bioenergetic membranes in circumscribed regions of the cell, including cyanobacteria, nitrosococcus and nitrosomonas, so compartmentalization *per se* could not have been the decisive advantage either.

That decisive advantage probably lies in the mitochondrial genes. All eukaryotes that have retained the capacity for oxidative phosphorylation have also retained a small genome – a core set of genes encoding predominantly integral inner membrane proteins essential for respiration. Mitosomes and hydrogenosomes have lost the capacity for oxidative phosphorylation and almost invariably also lost the complete mitochondrial genome, although in one single case the retention of a hydrogenosome genome was instrumental in establishing the mitochondrial ancestry of hydrogenosomes . While the reasons for the retention of a mitochondrial genome are beyond the scope of this Chapter, the likely answer, as argued by biochemist John Allen, is that the mitochondrial genes form part of a necessary regulatory loop that enables highly hydrophobic proteins to be transcribed and translated quickly and specifically, immediately adjacent to the bioenergetic membranes they service, in response to sudden changes in membrane potential, substrate availability, or oxygen tension [34, 35]. A large body of data shows that the rate of cell respiration depends on the copy number of mitochondrial DNA (mtDNA), with active cells having more copies of the genome, and *vice versa*. Cells depleted in mtDNA have a low respiratory capacity, while mutations that cause mtDNA depletion are typically associated with mitochondrial diseases [36]. Likewise, the rate of respiration is controlled directly by transcription of the ND5 subunit of complex I, encoded by mtDNA [37] (the rate of transcription depending in part on mtDNA copy number). *Thus there is compelling evidence that mtDNA is a necessary component of the regulatory loop that controls respiration in all eukaryotic cells capable of oxidative phosphorylation.*

The requirement for genes to control respiration explains why prokaryotes do not usually expand up to eukaryotic size. While certain bacteria do internalise their respiration, as already noted, they only do so across a relatively restricted membrane area – no larger than the cristae area of mitochondria, or the thylakoid area of chloroplasts. But eukaryotic cells contain thousands of mitochondria, indeed hundreds of thousands in the case of large amoebae, so the surface area of bioenergetic membranes is four or five orders of magnitude greater than most prokaryotes. A simple and powerful hypothesis is that prokaryotes cannot expand up to eukaryotic size (on average five orders of magnitude larger than bacteria) without colocalizing cytoplasmic genes with their bioenergetic membranes [38, 39]. If correct, this hypothesis predicts that giant bacteria, of which there are a number of examples, must control respiration by colocalizing genes with their bioenergetic membranes. On the evidence of a few cases this does seem to be true. *Epulopiscium* for example, a parasite of the surgeonfish gut, is a true giant that, with a length of about 0.5 mm, dwarfs even ciliates such as paramecium. *Epulopiscium* exhibits extreme polyploidy, with as many as 600,000 copies of its full genome [40]. Other giant bacteria, such as *Thiomargarita* (even larger, but filled with a massive, metabolically inert vacuole) has between 6,000 and 17,000 copies of its full genome (H. Schultz-Vogt, personal communication). There is as yet no proof that polyploid genomes are necessary to control respiration in giant bacteria; but their tight association with

the bioenergetic membranes is suggestive, at least. The available evidence is therefore consistent with a requirement for genes to control respiration.

But the really significant point about giant bacteria, with enormous implications for the evolution of oxidative stress, is the nature of their polyploid genomes: they are invariably full genomes. This has an intractable energetic cost [39]. The significant fact about mitochondria is that they have lost the vast majority of their genes, leaving only the handful needed for the control of respiration across a wide area of membranes. Gene loss is the normal outcome of reductive evolution in intracellular bacteria and is characteristic of all endosymbionts, obligately parasitic bacteria and organelles. Because all of them started out as autonomous cells with their own cell division apparatus (and even mitochondria retain this apparatus) they are not dependent on the cell division machinery of the host cell and can replicate semi-autonomously. There is inevitably competition within populations of endosymbionts for succession to the next generation, and the long-term outcome is genome reduction, because the fastest replicators tend to have the smallest, most streamlined genomes, and leave more progeny.

Contrast this situation with extreme polyploidy in giant bacteria. Here, the polyploid genomes are non-autonomous copies of the host cell genome, and without some form of cytoplasmic inheritance there is no way in which they can be reduced in size over generations. Competition does not and cannot exist. Each generation passes on a fraction of its polyploid genomes, and the daughter cells are then obliged to amplify the number of copies of this genome in proportion to cell size – large cells have greater polyploidy, presumably at least in part to retain control over the wider area of bioenergetic membranes. The energetic cost is colossal. The genomic weight of 200,000 copies of the *Epulopiscium* genome, for example, is around 760,000 Mb of DNA, whereas the equivalent cost of 200,000 copies of mtDNA is just 6 Mb [39]. But if the two cells could support, energetically, the same amount of DNA, then eukaryotes could support the difference, more than 750,000 Mb of DNA, in the nucleus, as genes that can be segregated at low copy number. In other words, the fact that the mtDNA genome is so small means that eukaryotic cells can support five or six orders of magnitude more DNA *in the nucleus* (and equally, that many more genes) than their prokaryotic counterparts. There are strong arguments to suggest that this massive increase in genomic capacity is the critical difference between eukaryotes and prokaryotes, explaining why only the eukaryotes have evolved true morphological complexity [39].

But there is a significant cost to this arrangement, which explains much about the evolution of oxidative stress [41]. If large complex cells *are not possible at all* without tiny mtDNA genomes, then there is a necessary interaction between mtDNA and the nuclear genes encoding mitochondrial proteins. In other words, mosaic respiratory chains, whose protein subunits are encoded by two separate genomes, are a *strictly necessary feature* of eukaryotic cells; eukaryotes could not exist with any other arrangement. The trouble is that the proteins encoded by the two genomes must interact with nanoscopic precision, or electron flow down respiratory chains will be blocked. Any blockage of electron flow in an aerobic world leads to a high rate of ROS leak, a collapse in energy charge (which is to say, an irreversible fall in ATP levels), the oxidation of membrane lipids such as cardiolipin, and the release of cytochrome c. Put another way, any failure of the two genomes to work together correctly leads directly to apoptosis. The surprising involvement of cytochrome c in apoptosis, greeted with ‘general stupefaction’ when first reported in the mid 1990s [42], emerges as an explicit prediction of the hypothesis that eukaryotic cells must undergo functional selection for the

compatibility of mtDNA and nuclear genes encoding adjoining respiratory chain subunits [41].

There is abundant evidence that selection for genomic coadaptation does indeed take place, ranging from the high proportion of neutral mutations and the concordance of nucleotide substitution rates in the nuclear and mitochondrial genomes, to the loss of respiratory function, fitness and fertility in introgressed populations [43]. The outstanding questions are where and how such selection takes place. What is certain is that *selection for mitonuclear coadaptation necessarily involves oxidative stress*.

5. Why Oxidative Stress Is Central to the Evolution of Eukaryotes

The speed of electron transfer down respiratory chains depends on the distance between redox centres, and slows down by about an order of magnitude per Ångstrom additional distance, for reasons that relate to the probability of transfer by quantum tunnelling [16]. A likely consequence of even single nucleotide mutations or polymorphisms in mtDNA would be small misalignments in subunit juxtaposition, slowing electron transfer. Slower electron transfer increases the reduction state of respiratory complexes, making them more reactive with oxygen and therefore increasing ROS leak and susceptibility to apoptosis. Thus, any mismatch between mtDNA and nuclear genes encoding respiratory-chain subunits should increase the risk of apoptosis, with manifest costs in terms of embryonic development and aging [41].

Mitonuclear mismatch is unavoidable. The only question is how much can or 'should' be tolerated. The basic problem is that the tempo and mode of evolution of the two genomes are quite distinct. Nuclear genes evolve by sex, being recombined every generation, but the underlying mutation rate is low [38]. In contrast, mitochondrial genes evolve asexually, passing down the maternal line, but the underlying mutation rate is substantially higher, in animals and fungi at least. In yeast, the petite mutation, which deletes a large part of the mitochondrial genome, occurs at around 10,000 times the frequency of nuclear mutations [44]. Because yeast can survive by fermentation alone, selection against the petite mutants is low, hence the difference in mutation rate is observable. In animals, which usually depend on their mitochondria for oxidative phosphorylation, such mutations would necessarily be eliminated by selection. Thus, in animals, selection for mitochondrial function means that the long-term evolution rate is much lower than the mutation rate. Only the evolution rate is known with any certainty; and this is around 10-20 times faster than the evolution rate of nuclear genes [45]. Thus, again, there is a major discrepancy between the evolutionary rates of the nuclear and mitochondrial genomes. The mtDNA mutation rate is much slower in plants, for unknown reasons, but the principle stands, as there is still a large discrepancy in tempo and mode of evolution.

These divergent rates of evolution inevitably generate mitonuclear mismatch, which is evidently eliminated by selection over time. The open questions are how much time, and how much selection? The nuclear background changes every generation, following sexual reproduction, so some kind of selective filter must be imposed each generation. Conceptually, there are only three places where selection could operate – during oocyte maturation, after

fertilization, or after birth. Selection for mitochondrial function could operate at all three points, but selection for mitonuclear coadaptation cannot take place during oocyte development, because at this time the new nuclear background is not known. Selection for mitonuclear coadaptation must therefore take place during development, or after birth. Presumably, if coadaptation is poor, electron flow will slow down in the new mosaic respiratory chains: ROS leak rises, ATP levels fall and cytochrome c is released, triggering apoptosis. Compromised bioenergetics, combined with high cell loss due to apoptosis, undermines embryonic development, leading to miscarriage or mitochondrial disease [41].

The reality of such selection is unveiled by introgression between different populations of the same species, or between species. In the case of the marine copepod *Tigriopus californicus*, for example, introgression between neighbouring but reproductively isolated populations leads to a fall in ATP synthesis of about 40%, along with similar (c.a. 40%) reductions in fertility, developmental time and survival, even in the F2 generation: a serious, global fitness penalty [46]. Backcrossing to the maternal population completely eliminates the fitness reduction, proving that these effects are mitonuclear incompatibilities [46]. Such changes may well be responsible for the first stages of speciation in some populations, especially where the mtDNA mutation rate is high [45, 47]. Because these genes evolve faster than the nuclear average (and because nuclear genes encoding mitochondrial subunits are obliged to co-adapt, and so evolve faster themselves) the bioenergetic/apoptotic axis evolves more quickly than most nuclear genes. This drives the rate of change, and so the rate at which incompatibilities accumulate between populations, leading first to hybrid breakdown, and ultimately to speciation. It is not a long stretch to imagine that the hybrid breakdown in copepods is the first step to speciation. *The mechanism of speciation, in this case, can be ascribed entirely to mitonuclear incompatibilities, which is to say the consequences of poor electron flow down respiratory chains, leading to a higher rate of ROS leak.*

But if poor coadaptation leads to directly to apoptosis and high rates of infertility, what constitutes 'poor coadaptation'? The answer is likely to vary among species. Species that need a high aerobic capacity, such as flighted birds or bats, could not get airborne at all if they did not have an aerobic capacity several-fold higher than even fast runners like the cheetah. On the other hand, rats do not require a high aerobic capacity, and so could presumably tolerate a poorer mitonuclear match. Put another way, *there must be an adjustable threshold, above which ROS leak stimulates apoptosis and developmental failure, and below which ROS leak is tolerated, or might even be beneficial as a redox signal* [45]. This leads to an eminently testable hypothesis. Birds should be highly sensitive to ROS leak from their mitochondria, whereas mammals like rats should be more tolerant of ROS leak, and also mitochondrial heteroplasmy (a mixture of different mtDNA genotypes in the same cell) [45]. This is because heteroplasmy undermines selection for mitonuclear coadaptation, as there is no means of selecting an optimal match. Given a mix of different mtDNAs, some must function against the nuclear background better than others. The overall aerobic capacity depends on the average function of mtDNA, and this average is necessarily lower than the optimum, which is, again necessarily, homoplasmic. Mitochondrial heteroplasmy is normally eliminated by the combination of uniparental inheritance and a mitochondrial bottleneck during oocyte development – which is to say, by the existence of two sexes, one of which (the female) bequeaths a homoplasmic population of mitochondria, and the other (the male) has his mitochondria destroyed [38]. Whether the requirement for mitonuclear coadaptation is

partly responsible for the maintenance, perhaps even the origin, of two sexes, is an interesting, unanswered question; but the consequences are equally pervasive.

A variable apoptotic threshold has profound implications for fertility, fecundity, adaptability, fitness, aging and age-related disease. The reason is simple. Setting the apoptotic threshold high – meaning a high tolerance of ROS-leak before apoptosis is triggered – enables high fertility and fecundity. Poor mitonuclear match is overlooked, and embryos that would fail to develop in more discerning animals develop full term. Some degree of heteroplasmy is tolerated and indeed can be beneficial, as a range of mtDNA enables greater adaptability to changing environments. However, the offspring are less fit, and more likely to suffer from mitochondrial diseases. They will have lower aerobic capacity. Worst of all, they will leak ROS from their mitochondria at a faster rate, without triggering apoptosis. The outcome is a shorter lifespan, and a greater tendency to oxidative stress and chronic inflammatory conditions linked with aging, such as diabetes, cardiovascular disease and cancer. In short, there is a trade-off between fertility, fecundity and adaptability, on the one hand, and aerobic capacity, life-span and susceptibility to age-related disease on the other [45]. The trade-off is mediated by sensitivity to oxidative stress.

In birds, the apoptotic threshold is low: they are sensitive to ROS leak from mosaic respiratory chains and quickly trigger apoptosis. A poor mitonuclear match leads to slow electron flow, high ROS leak and swift apoptosis, translating into infertility and low fecundity. An intolerance of heteroplasmy means a low incidence of mitochondrial disease but also a low adaptability to changing conditions. On the positive side, birds have a high aerobic capacity, a long lifespan and low susceptibility to the chronic inflammatory conditions characteristic of old age in mammals. The difference is not trivial. Pigeons and rats have a similar body size and similar metabolic rate, even a similar foraging lifestyle, to the point that pigeons are often dismissed as flying rats. Far from it. Rats live for 3 – 4 years, pigeons for 35, ten times longer. Their mitochondrial ROS leak is nearly 10-fold lower [5]. While this difference makes no sense in terms of the efficiency of respiration (the proportion of ROS leak as a fraction of total oxygen consumption is too small) it makes a big difference in terms of functional selection for mitonuclear match, and it makes a big difference in terms of lifespan and healthspan [45].

“Nothing in biology makes sense except in the light of evolution”, wrote the evolutionary theorist Theodore Dobzhansky. The same applies to medicine. For decades, researchers have wrestled with oxidative stress, and found it surprisingly refractory. *It is time to replace the simplistic notion that ROS leak is merely a trivial by-product of respiration, or that aging and age-related disease can be blocked with antioxidants* [48]. An evolutionary perspective makes it clear why antioxidants don’t work: they must not be allowed to interfere with sensitive ROS signals that affect everything from fertility to speciation. But equally, the evolutionary perspective reveals the costs and benefits of oxidative stress, many of them hitherto unsuspected [45]. Aging and age-related diseases are the outcomes of flexible evolutionary trade-offs, with oxidative stress at the heart of it all. But if birds can solve the aging problem, surely we can too.

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