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Palaeoproterozoic ice houses and the evolution of oxygen-mediating enzymes: the case for a late origin of photosystem II

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Two major geological problems regarding the origin of oxygenic photosynthesis are (i) identifying a source of oxygen pre-dating the biological oxygen production and capable of driving the evolution of oxygen tolerance, and (ii) determining when oxygenic photosynthesis evolved. One solution to the first problem is the accumulation of photochemically produced H_2O_2 at the surface of the glaciers and its subsequent incorporation into ice. Melting at the glacier base would release H_2O_2, which interacts with seawater to produce O_2 in an environment shielded from the lethal levels of ultraviolet radiation needed to produce H_2O_2. Answers to the second problem are controversial and range from 3.8 to 2.2 Gyr ago. A sceptical view, based on the metals that have the redox potentials close to oxygen, argues for the late end of the range. The preponderance of geological evidence suggests little or no oxygen in the Late Archaean atmosphere (less than 1 ppm). The main piece of evidence for an earlier evolution of oxygenic photosynthesis comes from lipid biomarkers. Recent work, however, has shown that 2-methylhopanes, once thought to be unique biomarkers for cyanobacteria, are also produced anaerobically in significant quantities by at least two strains of anoxygenic phototrophs. Sterane biomarkers provide the strongest evidence for a date 2.7 Gyr ago or above, and could also be explained by the common evolutionary pattern of replacing anaerobic enzymes with oxygen-dependent ones. Although no anaerobic sterol synthesis pathway has been identified in the modern biosphere, enzymes that perform the necessary chemistry do exist. This analysis suggests that oxygenic photosynthesis could have evolved close in geological time to the Makganyene Snowball Earth Event and argues for a causal link between the two.

Keywords: Great Oxygenation Event; sterol biosynthesis; Makganyene Snowball Earth

1. INTRODUCTION

The debate about the history of atmospheric oxygen is most probably the longest-running and still unresolved controversy in the history of modern science. It began in the mid-nineteenth century with some of the earliest publications in biogeochemistry, when Ebelman (1845) and Bischof (1854), considering the balance of oxygen release by organic carbon and pyrite burial and oxygen consumption by iron and manganese oxidation, speculated that atmospheric oxygen levels might have changed over time with changes in biota (Berner & Maasch 1996). In 1856, Koene (1856/2004) argued, based on the presence of reduced matter in early rocks, that the Earth's initial atmosphere had been high in carbon dioxide and free of oxygen, and that over geological time the action of photosynthetic plants had resulted in a decline in carbon dioxide and a rise in oxygen. These early works apparently fell upon deaf ears, as few successor publications appeared until late in the nineteenth century (see Stevenson (1900), van Hise (1904) and Clarke (1924) for reviews). Koene's work had by this time vanished into obscurity; Stevenson (1900) knew of it only through a synopsis published in 1893–1894 by T. L. Phipson, one of Koene's former students.

Later, the Rhodesian geologist MacGregor (1927) complemented the chemical models with observations, both in the field and in the laboratory, of the Precambrian of southern Africa. He argued that the quantity of carbon buried in shales was sufficient to account for the quantity of oxygen in the atmosphere and therefore suggested that oxygen had accumulated over the course of the Earth's history. Consequently, he wrote, 'the assumption [that the most ancient rocks were themselves formed under an atmosphere of oxygen] can only be justified by the clearest field evidence of contemporary oxidation in the most ancient rocks themselves' (p. 158).

MacGregor turned to the rocks of Rhodesia and found evidence that the pre-Lomagundi metasediments of the Archaean Basement Schists, as he called them, were deposited under an oxygen-free atmosphere. In particular, he noted that (i) chemical analyses showed high ferrous/ferric ratios in Archaean sediments, (ii) the deposition of banded ironstones

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and their confinement to the Precambrian could be explained if they had been formed by the action of either iron-oxidizing bacteria or oxygen-producing organisms in an anaerobic ocean, and (iii) rounded pyrite-bearing clasts in Archaean conglomerates suggested fluvial transport without exposure to oxidizing conditions.

The post-World War II era saw the addition of more sophisticated chemical theory and measurements to the discussion. Urey (1952) introduced considerations of equilibrium thermodynamics and discussed some of the earliest sulphur isotope evidence. Holland (1962) expanded Urey's calculations and considered, among other lines of evidence, the conditions necessary to oxidize uranium and prevent the deposition of detrital uraninite. By the mid-1960s, many of the arguments raised in modern discussions of the timing of the rise of oxygen were in place. In 1965, the National Academies of Science hosted one of the first symposia devoted to the issue (Cloud *et al*. 1965).

The past 42 years have seen numerous revolutions in geology and biology—from the molecular revolution in microbiology, to the development of theories of plate tectonics and abrupt climate change, to the recognition of the role of non-uniformitarian events in the Earth's history—as well as the development of at least one new technique, the study of mass-independent fractionation (MIF) of sulphur isotopes, that may directly reveal anoxia in the ancient atmosphere (Farquhar *et al*. 2000). Combined with high-resolution data of critical intervals in the Earth's history from the ongoing continental drilling projects, these advances hold out the promise of a fourth stage in our understanding of the evolution of oxygenic photosynthesis and the rise of oxygen—one that can build a strong connection between the predictions of theory and specific geological observations.

In this paper, we first review a recent development that provides a solution to the long-standing 'chicken-and-the-egg' problem concerning the evolution of photosystem II (PSII): O_2-evolving processes require O_2-mediating enzymes to limit toxicity, while O_2-mediating enzymes are unlikely to evolve without a source of O_2. A similar problem arises from the fact that chlorophyll synthesis in green plants has three steps that require O_2, which is produced by oxygenic photosynthesis. Next, we review the biomarker controversy, which arises from the apparent conflict between pieces of geological evidence for planetary anoxia contemporary with lipid biomarkers interpreted as suggesting the existence of oxygenic photosynthesis. Finally, we will summarize a simple and plausible sequence of events that, in our view, accounts for all of the biological and geological constraints on the geological history of oxygenic photosynthesis.

2. GLACIAL PEROXIDES AND THE EVOLUTION OF OXYGEN-MEDIATING ENZYMES

The ability of dissolved O_2 to accept an electron from a suitable donor (e.g. Fe^{+2}) and form the superoxide radical, O_2^-, is a severe threat to the organisms living in an aerobic environment. The superoxide radical reacts spontaneously with water to form the hydroxyl radical ($\cdot OH$), which attacks the sugar/phosphate backbone of DNA. The need to control this toxic process fostered the evolution of O_2-mediating enzymes, such as superoxide reductase (Jenney *et al*. 1999) and superoxide dismutase (which convert O_2^- to H_2O_2; Wolfe-Simon 2005), catalase (which converts H_2O_2 to water) and various oxygen-binding domains such as haem that help prevent superoxide formation (Niyogi 1999). Oxygen-mediating pathways had to evolve prior to the metabolic usage of O_2, including the production of oxygen by the O_2-evolving complex of PSII. Moreover, the O_2-evolving complex appears to derive oxygen from the Mn cluster of catalase, also implying that the O_2-mediating enzymes came first (McKay & Hartman 1991; Blankenship & Hartman 1998).

But while there must be a source of superoxide radicals to act as an adaptive pressure for the evolution of oxygen-mediating pathways, most geological and geochemical processes in the Earth's atmosphere and hydrosphere are relatively reducing (figure 1). Volcanic gases are buffered by redox reactions on the reducing end of this spectrum, more reducing than the ferrous–ferric redox couplet and far from the highly oxidized water/oxygen couplet. In fact, photolytic reactions involving ultraviolet (UV) radiation and water vapour provide the only known abiotic pathway for producing biologically significant concentrations of molecular O_2. However, the environments under which this process can happen are lethal to all living organisms, as the same UV radiation is destructive to complex organic molecules, including DNA.

One solution to this puzzle was provided by Liang *et al*. (2006), who noted that Antarctic ice cores preserve interannual variations in H_2O_2 concentration which reflect the history of the Antarctic ozone hole (Frey *et al*. 2005, 2006). As ozone is the major filter for short-wavelength UV, reductions in stratospheric ozone facilitate photochemical reactions involving H_2O which generate H_2O_2 and H_2 gas. H_2 diffuses away and is lost, whereas H_2O_2, with a freezing point near that of water, condenses and accumulates in the ice.

Liang *et al*. (2006) noted that the Late Archaean Pongola glaciation and Early Palaeoproterozoic glaciations occurred in atmospheres with little oxygen, lacking an ozone screen and bathed in UV radiation strong enough to produce the MIF observed in sulphur compounds (figure 2). During these glaciations, the same photochemical processes acting today in Antarctica would have acted over entire ice sheets, building up H_2O_2 concentrations. While similar photochemistry would occur in the liquid ocean, H_2O_2 produced there would diffuse away rather than accumulating. During a normal (non-Snowball) glaciation, peroxide-laced snow would follow normal glacial dynamics, being compressed into glacial ice, flowing in glaciers to the ocean and melting either there or along the wet-base portions. Upon melting, H_2O_2 would disproportionate into O_2 and water ($2H_2O_2 \rightarrow 2H_2O + O_2$), thereby producing an environment protected from lethal UV radiation but 'poisoned' with trace amounts of oxygen, in which oxygen-mediating enzymes might evolve. The small 'whiffs of oxygen' reported recently by Anbar *et al*. (2007) and Kaufman *et al*. (2007) at two nearby

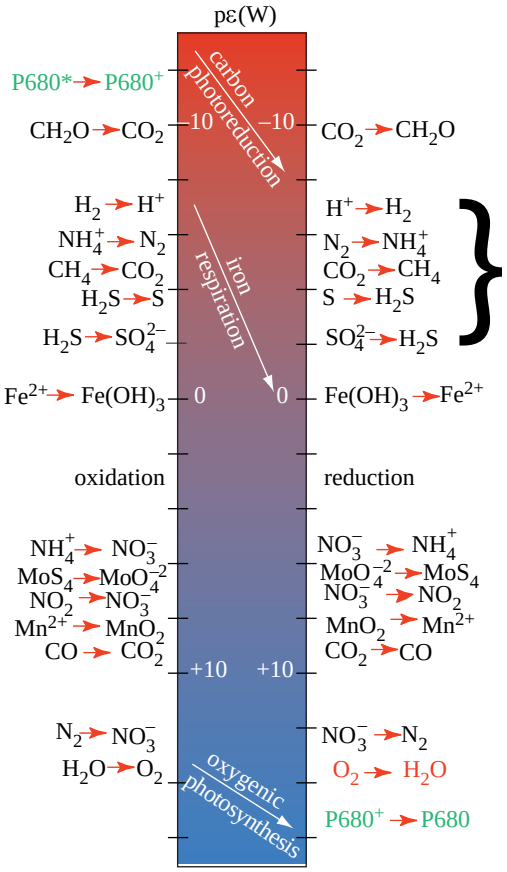

Figure 1. Electron activity (pε) of typical redox couples in water at pH 7 and 25°C (adapted from Gaidos *et al.* 1999). Half-reactions on the left couple spontaneously with those below them on the right, and most pairs are suitable for driving biological metabolism. The primary donor of PSII (P680) has the largest redox potential change known for any organic molecule, and in its oxidative (rest) state, it is capable of oxidizing water, producing oxygen. The thick bracket signifies the range of gases emitted from volcanic eruptions. Subaerial eruptions tend to be more oxidizing than subaqueous eruptions, so the shift in eruptive style towards subaerial vulcanism noted by Kump & Barley (2007) would have helped the eventual oxidation of the atmosphere, even though all volcanic gases are on the reducing side of the ferrous/ferric couple. Note that the term 'oxidizing' appears to have quite separate meanings in the geological and biological sciences. Redox couples near the quartz–fayalite–magnetite buffer in rocks are considered oxidizing by the Earth scientists, but are actually on the reducing side of the diagram and clearly faraway from the NO_3^- and O_2 couples considered oxidizing by biologists.

localities on the palaeocontinent of Vaalbarra at *ca* 2.5 Gyr ago could be the geochemical fingerprint of oxic meltwaters mixing with glacial flour (powdered rock) at the base of a polar ice field. Although the oldest confirmed glacial unit in the Palaeoproterozoic occurs sometime after 2.45 Gyr ago (Evans 2000; Young *et al.* 2001), we do not know the full duration of the Late Archaean and Palaeoproterozoic glacial epochs, as the preservation of glacial deposits depends upon having continents in the correct position, having sufficient accommodation space and the fate of deposits over geological time. The Earth may well have experienced glacial ice-house conditions, akin to those of the Late Palaeozoic and Cenozoic, during the time gap between the preserved Pongola and Huronian glacial deposits.

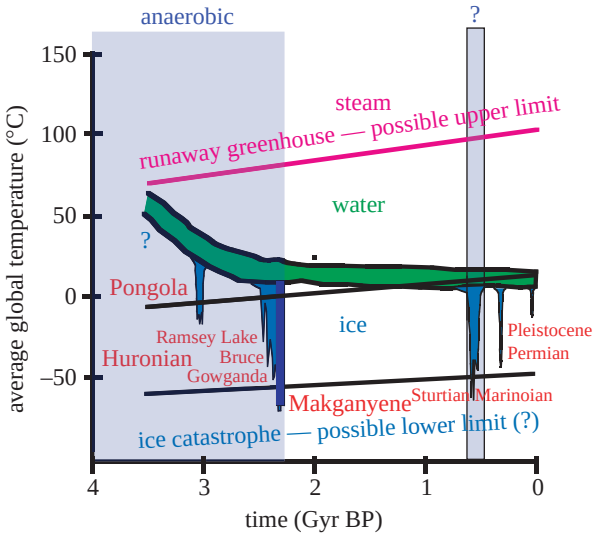

Figure 2. The Earth's glacial history, with intervals of atmospheric anoxia indicated by the light shading (adapted from a cartoon of Lovelock 1979). Major Precambrian glaciations are indicated by 'icicles' dangling from a temperature versus time curve through the Earth's history, and include the Pongola of southern Africa at *ca* 2.9 Gyr ago, the Palaeoproterozoic glaciations between *ca* 2.5 and 2.22 Gyr ago (the three major units of the Huronian series in Laurentia and the Makganyene of southern Africa), the Cryogenian glaciations in the Neoproterozoic (*ca* 800–600 Myr ago) and the Permian and Neogene glaciations in the Phanerozoic. The UV–peroxide generating mechanism of Liang *et al.* (2006) is expected to operate on any ice sheet formed in an atmosphere without an ozone or other UV screen. The question mark indicates the possibility of earlier glacial intervals, not yet recognized.

3. ARE ARCHAEAN LIPID BIOMARKERS CONTAMINANTS?

As of 2004, at least 473 biochemical reactions were known in which molecular O_2 was a substrate (Raymond & Blankenship 2004; Raymond & Segre 2006). Hence, one way to constrain the history of oxygen is to look in sediments for biomarkers that are the products of oxygen-dependent reactions. As with any geobiological tracer, one must verify that the materials being studied are of the same age as the rock; and as with many promising new techniques, the application of organic geochemistry to the Precambrian had a rocky, controversial start. During the 1960s and early 1970s, a flurry of studies reported traces of Precambrian porphyrins, fatty acids, alkanes, acyclic isoprenoids and even amino acids (Hayes *et al.* 1983; Brocks *et al.* 2003*a*). Follow-up studies revealed that virtually all the organic compounds were contaminants, usually petroleum-derived hydrocarbons from the laboratory and field environments. In response, and to their great credit, workers in the field evolved into their own most severe critics.

Despite these discouraging initial problems, great progress has been made in identifying the proper target molecules to study and in elucidating the diagenetic processes operating on them (Knoll *et al.* 2007). The lipid fraction from biological membranes is by far the best, as lipids can preserve the topology of their skeletal backbones despite various changes during

diagenesis (such as the loss and replacement of hydroxyl groups, linear side-chain degradation and saturation of double bonds). Of particular importance as potential constraints on the history of oxygen are the degradation products of polycyclic isoprenoids, especially hopanols and sterols, which preserve enough of their structural information to identify the biochemical pathways through which they have formed. Although not unknown, production of the same isoprenoid compound through different pathways is rare. The Precambrian fossil records of hopane and sterols have been the primary focus of a series of papers (Brocks *et al.* 1999, 2003*a*,*b*; Summons *et al.* 1999, 2006). For background, we recommend two excellent reviews on sterol biosynthesis and the structure and function of the family of triterpene cyclase enzymes by Lesburg *et al.* (1998) and Wendt *et al.* (2000), and on the oxygen dependence for sterol synthesis in yeast by Rosenfeld & Beauvoit (2003).

This body of work employs vastly superior techniques and internal controls for reproducibility than were used in the discredited studies of the 1960s. However, concerns about contamination have not evaporated, as Brocks *et al.* (2003*b*, p. 4322) noted: 'The age of the molecules that contain the biologic information is not fully resolved. Hence, paleobiological interpretation… should be cited cautiously and with reference to the remaining uncertainty of syngeneity.' Possible 'red flags' include the presence of characteristically Phanerozoic biomarkers (such as dinosteranes, generally associated with dinoflagellates, a Mesozoic–Cenozoic taxon), as well as the puzzling lack of any geological trace of intermediate evolutionary stages in the long sterol biosynthetic pathway. Pearson *et al.* (2003) identified one extant planctomycete, *Gemmata obscuriglobus*, which has just such an intermediate sterol synthesis pathway and is possibly on the ancestral eukaryote lineage (Fuerst 2005), but it is unknown whether the pathway is an immature relict or has been truncated. Regardless, as Brocks (2005) noted, the incremental pattern of eukaryotic radiation observed in the Proterozoic fossil record ought to be matched by similar changes in lipid biomarkers, but instead Archaean sterols with a fully modern fingerprint materialize nearly a billion years before the first fossils with possible eukaryotic affinity. New analytical methods, such as the examination of hydrocarbons in fluid inclusions (Dutkiewicz *et al.* 2006; Ueno *et al.* 2006), may soon resolve this contamination controversy.

4. IS O_2 A FUNDAMENTAL REQUIREMENT FOR HOPANE AND STEROL FORMATION?

For organic biomarkers to provide strong constraints on palaeo-oxygen levels, it is important to verify that the fundamental chemistry involved requires oxygen, precluding any possibility of anaerobic mechanisms, and that the basic processes on which the inferences are based have not changed over geological time. Even if biosynthetic pathways leading to hopanol and sterol synthesis evolved during Hadean or Archaean time, we argue that recent work has weakened the case considerably linking them to the presence of oxygenic photosynthesis. Brocks *et al.* (2003*b*, p. 4331) summarized their four lines of molecular evidence for free O_2 in the surface waters as follows.

> 1. The bitumens contain molecular fossils of bacteriohopanoids. Although hopanoid biosynthesis does not require oxygen, these lipids have never been isolated from strict anaerobes (Ourisson *et al.* 1987).
>
> 2. The Archaean shales also contain high relative concentrations of cyanobacterial 2-methylhopanes. These biomarkers are indirect evidence for oxygen release within the photic zone.
>
> 3. The side-chain degradation pattern of the 2-methylhopane series (Fig. 5) indicates oxic conditions during earliest diagenesis of cyanobacterial organic matter.
>
> 4. The bitumens contain molecular fossils of sterols. Sterol biosynthesis in extant eukaryotes requires dissolved oxygen in concentrations equivalent to 1% PAL (Jahnke and Klein 1983).

Recent work suggests that all of these can be explained by mechanisms that do not require free oxygen in the environment; each will be considered in turn.

(a) *Bacteriohopanoids and 2-methylhopanes*

As Brocks *et al.* (2003*b*) noted, the production of hopanoids does not involve O_2, and subsequent work has identified multiple anaerobic bacteria that produce hopanoids. Fischer *et al.* (2005) found that at least one bacterium, *Geobacter sulphurreducens*, can synthesize diverse hopanoids (not including 2-methylhopanoids) when grown under strictly anaerobic conditions. Moreover, Rashby *et al.* (2007) reported that copious quantities of 2-methylhopanoids are produced under anaerobic conditions mimicking those presumed to exist during Archaean time by *Rhodopseudomonas palustris*. As they state (p. 15 102), '… because 2-MeBHPs may be produced by organisms that do not engage in oxygenic photosynthesis and because their biosynthesis does not require molecular oxygen, 2-methylhopanes cannot be used as *de facto* evidence for oxygenic photosynthesis'.

(b) *Side-chain degradation pattern*

Brocks *et al.* (2003*b*, p. 4330) stated:

> The side-chain degradation pattern of the C30 to C36 2-methylhopane homologous series relative to the corresponding C30 to C36 3-methylhopanes suggests that late Archaean cyanobacteria lived in an oxygenated micro-environment. The C30 to C36 2-methylhopane series has in all analyzed samples a characteristic even-over-odd carbon number predominance. The elevated abundance of the C32-homologue relative to C31 and C33 indicates oxidative side-chain cleavage of a bacteriohopanetetrol to a C33 carboxylic acid and subsequent decarboxylation under non-reducing conditions.

The even-over-odd carbon number predominance of 2-methylhopanes relative to 3-methylhopanes can also be explained by anaerobic processes. So & Young (1999) isolated an anaerobic sulphate-reducing bacterial strain from a petroleum-contaminated sediment that metabolizes straight-chain saturated alkanes with a similar two-carbon removal process.

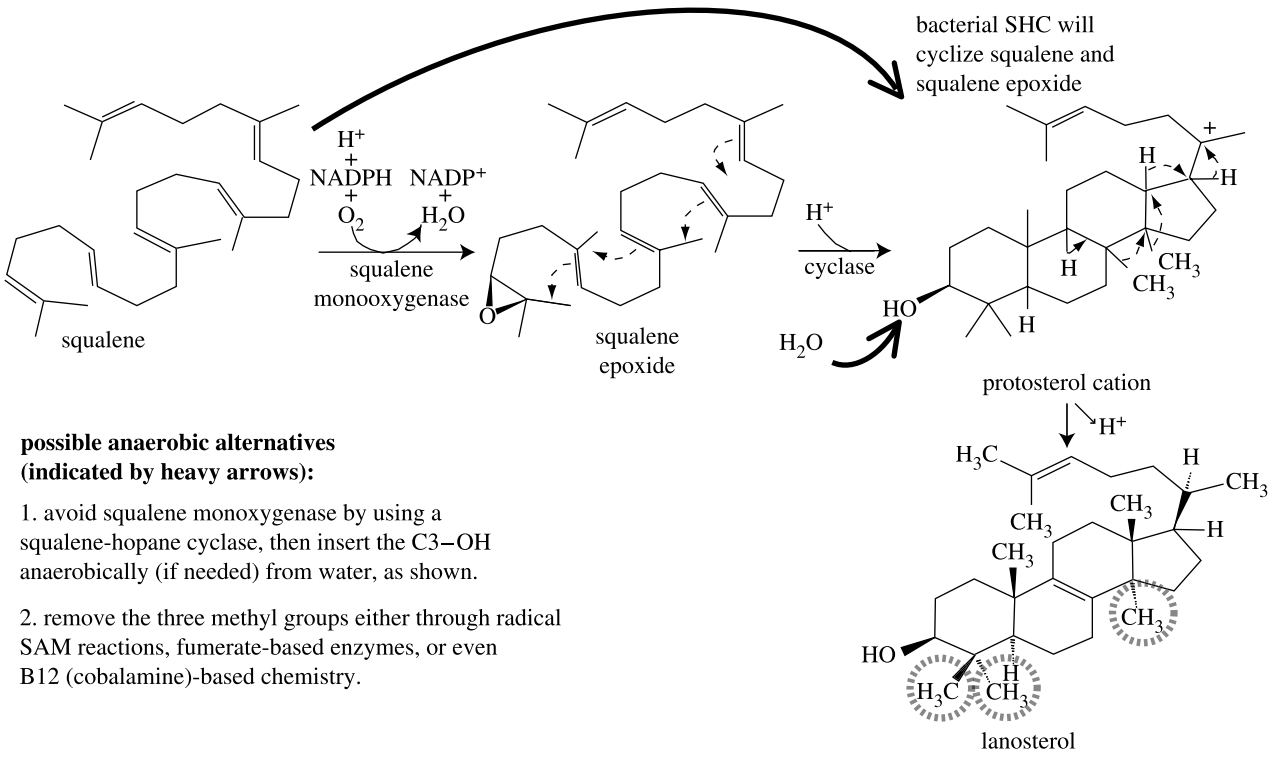

Figure 3. Sterol biosynthetic pathway, with proposed modifications for anoxic operation. In eukaryotes, the oxygen-dependent steps include the conversion of squalene to squalene epoxide, and the removal of two methyl groups on the C4 carbon and one on the C14 carbon (indicated by faint circles). As indicated by the faint dotted circles, we suggest that an ancestral anaerobic eukaryote may have directly done the initial cyclization reaction on squalene rather than its epoxide, in the fashion of many bacterial squalene–hopane cyclase (SHC) enzymes that will cyclize either form. The C3 hydroxyl (if it was really there in Archaean time) could be added after the cyclization reaction as described in the text. Similarly, a variety of demethylation reactions are known from sulphate-reducing organisms that could, in principle, remove the three methyl groups from lanosterol to form ergosterol (not shown), which is the simplest sterol from which eukaryotes can grow anaerobically.

If grown on pure C_{even} alkanes, their total fatty acids were predominantly even in carbon number, while if grown on C_{odd} alkanes, their total fatty acids were predominantly odd in carbon number. Although this example is from the degradation of alkanes, similar chemistry ought to work for the degradation of isoprenoid side chains. While post-depositional degradation would affect side chains of the 2- and 3-methylhopane fractions equally, it is clear that anaerobic organisms have a variety of biochemical pathways involving the addition of small carbon compounds that can produce such even/odd patterns, the complexity and mechanisms of which have yet to be resolved (see Aeckersberg *et al.* (1998) and Berthe-Corti & Fetzner (2002) and references therein).

(c) *Sterols*

Sterols and their diagenetic derivatives, steranes, are potentially more relevant to the oxygen question. In yeast, the modern biosynthetic pathway to ergosterol (the simplest sterol required for strictly anaerobic growth; Andreasen & Stier 1953) is a long chain of biochemical reactions that use the following three O_2-dependent enzymes: (i) squalene monooxygenase, (ii) lanosterol 14-α-methyl demethylase cytochrome P450, and (iii) sterol-4-methyl oxidase (Risley 2002). Figure 3 shows the first biosynthetic steps of this pathway, from the linear squalene molecule to lanosterol. Molecular oxygen is first used by enzyme (i) to form an epoxide bridge between the C2 and C3 carbons in the linear squalene molecule, which is then cyclized and converted to lanosterol as shown, leaving the oxygen as an hydroxyl on C3. To convert lanosterol to ergosterol, enzymes (ii) and (iii) use oxygen to remove three methyl groups, two on C4 and one on C14.

Owing to the inertness of the C–H bond in hydrocarbons and the resonance energy of aromatic compounds, it is often assumed that oxygen must be involved in the activation, rearrangement and metabolism of molecules like these. For demethylation, oxygen is used to introduce hydroxyl groups by replacing one of the carbon-bound hydrogens, after which other enzymes can proceed through a series of steps including dehydrogenation and eventual decarboxylation (see Darnet & Rahier (2003) for a recent overview of the oxidative mechanism for enzyme (iii)). It was long assumed that a direct hydrogen atom transport (HAT) reaction, where an activated radical removed a stably bound H atom from a stable carbon substrate leaving a reactive intermediate, was impossible under anaerobic conditions (see Suflita *et al.* (2004) for an excellent review). As noted below, this is no longer the case.

Goldfine (1965) recognized over 40 years ago that many anaerobic enzymes have been replaced by aerobic equivalents. Raymond & Blankenship (2004) discovered that, of the 473 reactions using O_2 as a substrate, there were more than 80, in at least 20 metabolic pathways, for which there was a direct anaerobic-to-aerobic

substitution. Hence, the substitution of an O_2-dependent enzyme for an anaerobic one appears to be a common evolutionary occurrence. In at least one lipid pathway, that of haem/chlorophyll biosynthesis, 3 out of 17 steps were replaced on a one-to-one basis with oxygen-dependent enzymes; two of these are in the nine-step haem portion of the pathway. If three swaps happened in only 17 steps in the chlorophyll pathway, it is not unreasonable to suggest that four swaps might have happened in the more than 25-step sterol pathway.

Most enzymes involved in the sterol synthesis pathway are membrane bound. Rather than being handed directly from one enzyme to another, substrates passively diffuse within this membrane layer between enzymes, which do not need to interact directly with each other. Hence, from an evolutionary perspective, it is easy to modify intermediate steps within a long biosynthetic pathway of this sort without 'rebuilding' the entire chain from scratch. Squalene monooxygenase might have been a late addition to this pathway, and the cyclase enzyme could have evolved its specificity to 2,3-oxidosqualene sometime *after* the Great Oxygenation Event.

The question then focuses on whether or not there are plausible anaerobic substitutes for the steps catalysed by the oxygen-dependent enzymes (i), (ii) and (iii) listed above, particularly the HAT reactions. The answer appears to be yes, and the relevant discoveries were triggered from two surprising sources: economic geology and the discovery of anaerobic methane oxidation. Prior to 1990, it was thought that aerobic processes were the major cause of petroleum degradation in nature, yet it has since become clear from the study of deep oil reservoirs that extensive biological degradation occurred when sulphate-bearing (but anoxic) waters made contact with the hydrocarbons. A variety of sulphate-reducing bacteria were eventually implicated (Aeckersberg *et al.* 1991; Rueter *et al.* 1994; Aitken *et al.* 2004), and a series of previously unknown but powerful biochemical pathways were discovered (Widdel & Rabus 2001; Boll *et al.* 2002). Despite the stability and inertness of C–H and C–C bonds, these bacteria are able to perform all structural rearrangements needed to oxidize saturated and aromatic hydrocarbons to CO_2 anaerobically, including demethylation. Although, owing to environmental and health concerns, the major biochemical work has been focused on the aromatic hydrocarbons such as benzene, fully saturated hydrocarbons such as alkanes and isoprenoids such as pristane are also degraded and altered (Bonin *et al.* 2004; Suflita *et al.* 2004). A comparison of relevant bond energies for the first hydrogen atom extraction in the radical formation process $RH \Rightarrow R\cdot + H\cdot$ is illuminating; removal of an H atom from benzene requires approximately 113 kcal mol^{-1}, compared with only 105 and 101 kcal mol^{-1} from methane and ethane, respectively. The example of anaerobic methane oxidation, done by an archaeon working in concert with a sulphate-reducing bacterium (Orphan *et al.* 2001, 2002), shows that these direct HAT reactions are indeed possible even without the participation of electrons in adjacent π orbitals, as may happen in aromatic compounds.

We will consider possible anaerobic precursors for the squalene monooxygenase (i) and demethylation (ii) and (iii) enzymatic steps separately.

(d) *Squalene monooxygenase*

Brocks *et al.* (2003*b*) referred to the kinetic work of Jahnke & Klein (1983) on the squalene monooxygenase from the yeast *Saccharomyces cerevisiae* to argue that O_2 concentrations equivalent to 1% of the present atmospheric level would have been required for Archaean sterol synthesis; these levels would either demand the presence of photosynthetic oxygen or operate at the bottom of a melting, peroxide-rich ice sheet noted earlier.

However, the evidence permits alternative interpretations. First, the putative Archaean steranes do not have a hydroxyl group on the C3 carbon, which is the oxygen fingerprint of the squalene monooxygenase enzyme. Diagenesis would have removed the hydroxyl group had it originally been present, but without it there is no physical evidence that the squalene monooxygenase enzyme was ever involved in the formation of the particular sterol biomarkers in question. Although a clever organic geochemist might be able to infer indirectly whether or not a functional group was once present on the C3 carbon, to our knowledge no evidence of this sort has yet been reported. Using the Jahnke & Klein (1983) O_2 constraint for squalene monooxygenase as a palaeoenvironmental indicator is an example of extrapolating the modern biosphere back *ca* 3 Gyr, and must be done with caution.

Nevertheless, it is worth considering the possibility that the Archaean steranes might have originally been sterols, and to determine if there are plausible anaerobic alternatives to get the hydroxyl on the C3 carbon. As noted by Fischer & Pearson (2007), it is not possible to hydroxylate the linear squalene molecule prior to cyclization and have the OH group wind up on the proper carbon, so an anaerobic hydroxylation step of this sort most probably came after the cyclization reaction. Conversion to the present epoxide-based sterol synthesis pathway would have happened after O_2 became abundant by an enzyme swap. This scenario is made more plausible by the fact that the oxidosqualene cyclase in eukaryotes evolved from a larger class of less substrate-specific bacterial squalene–hopane cyclase enzymes (Pearson *et al.* 2003; Fischer & Pearson 2007). Squalene cyclization reactions are highly exothermic, and the presence or absence of the oxygen in the epoxide moiety has little to do with the reaction kinetics (Wendt *et al.* 1997; Fischer & Pearson 2007). It also appears that the resulting stereochemical conformation of the product is controlled by how a methyl group on C8 is positioned (Fischer & Pearson 2007), not by the configuration of the epoxide moiety as was once thought. These bacterial enzymes will even today cyclize either squalene or 2,3-oxidosqualene, whereas the more specific eukaryotic version accepts only the 2,3-oxidosqualene.

For this scenario to work, there must have been an anaerobic hydroxylase capable of removing a C3 hydrogen from the sterene and replacing it with hydroxyl. There are several possibilities for this. As

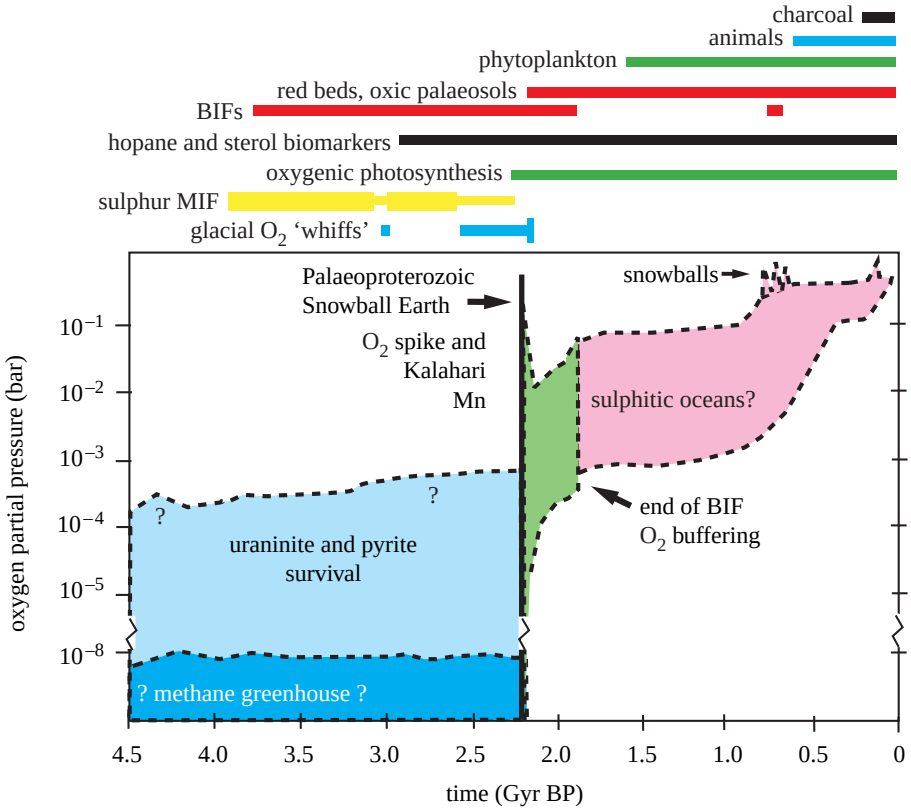

Figure 4. History of the Earth's oxygenation, showing important constraints described in the text. Adapted from Kirschvink & Weiss (2002). Question marks indicate uncertainty in the relative levels of oxygen present.

noted above, this could be done by one of the direct sulphate-driven hydrogen atom extraction processes ($RH \Rightarrow R\cdot + H\cdot$; Bonin *et al.* 2004; Suflita *et al.* 2004). Extraction energies for this would be well below that required for benzene (approx. 113 kcal mol^{-1}), a reaction that does proceed anaerobically. Alternatively, Kniemeyer & Heider (2001) purified and characterized a molybdenum/iron–sulphur/haem enzyme, ethylbenzene dehydrogenase, that catalysed the anaerobic hydroxylation of the $-CH_2-$ of ethylbenzene. The reaction puts an oxygen atom from water onto the substrate as a hydroxyl while reducing an electron acceptor. Kniemeyer and Heider reported similar activity for other related substrates, indicating that this is simply the first of a potentially large family of dehydrogenase enzymes that can activate otherwise stable hydrocarbons without the use of O_2.

(e) *Demethylation steps (enzymatic reactions (ii) and (iii) above)*

To understand the mechanism for the oxidative removal of methyl groups, Darnet & Rahier (2003) developed a micelle-based system for characterizing the enzymological properties of the yeast sterol-C4-methyl-oxidase (enzyme (iii)), and using various mutants and information from the genome were able to confirm the reaction scheme. O_2, NADH and cytochrome b_5 are used in the first step, converting the C4-α-methyl groups into the alcohol, $-CH_2OH$, which they were able to isolate from the micelles as a stable intermediary product. The same enzyme then catalyses the removal of two more hydrogens to form the acetate, –COOH, presumably by the addition of the remaining oxygen not used in the first step. The subsequent removal of the carbon by the enzyme 4αCD does not require oxygen, but it does involve the temporary conversion of the hydroxyl on C3 mentioned earlier into a ketone, making its presence essential for this particular O_2-dependent demethylation pathway. In yeast, this same enzyme removes both the C4 methyl groups.

In contrast, Boll *et al.* (2002) reviewed some completely anaerobic pathways for demethylation in sulphate- and nitrate-reducing bacteria. The best characterized of these is for the metabolism of toluene, which involves the initial addition of a fumarate co-substrate to the methyl group. A novel glycyl radical enzyme, (*R*)-benzylsuccinate synthase, removes a hydrogen from the methyl group and adds the fumarate to the methyl radical to yield benzylsuccinate. (This is the difficult activation step that avoids the use of molecular oxygen.) Following this, the benzylsuccinate is converted to benzoyl CoA and succinate via β-oxidation, and the succinate is recycled to fumarate via another enzyme, succinate dehydrogenase. As Boll *et al.* noted, 'Thus, the overall pathway affords an oxygen-independent six electron oxidation of the methyl group of toluene to the carbonyl-CoA group of benzoyl-CoA.' These are representatives of a family of previously unknown enzymes that can activate internal carbons by the addition of products that can be oxidized by conventional β-oxidation pathways, sidestepping the need for activation by molecular oxygen (Boll *et al.* 2002). They also observed further that variants of the fumarate pathway appear to be 'everywhere' in the anaerobic metabolism of hydrocarbons in a large variety of sulphate- and nitrate-reducing organisms. Hence, a similar mechanism tailored to the removal of the C4 (and C14) methyl

groups of lanosterol is not unreasonable, and in addition would not need an adjacent hydroxyl group on C3 to proceed, which the oxidative pathway of sterol-C4-methyl-oxidase (enzyme (iii) above) apparently does require. As the complete genome sequences are known for several of these sulphate-reducing organisms as well as the yeast, *S. cerevisiae*, it may be possible to recreate completely anaerobic sterol biosynthesis by reintroducing these fumarate pathways into yeast or other model eukaryotes.

An additional category of powerful anaerobic enzymes capable of doing structural rearrangements on hydrocarbon skeletons includes the radical *S*-adenosylmethionine (SAM) reactions (Imlay 2006; see figure S1 in the electronic supplementary material). This superfamily of enzymes can produce SAM radicals energetic enough to catalyse the removal of a single hydrogen atom from a carbon backbone. In all these enzymes studied to date, the reaction mechanism appears to proceed by causing an exposed iron in a [4Fe–4S] cluster to move from tetrahedral-like coordination to the octahedral geometry. In the process, the sulphur atom bound to the adenosylmethionine moiety is released in a highly activated form, and can be focused enzymatically to perform dehydrogenation, demethylation or other structural rearrangements. Oxygen, however, poisons the Fe–S clusters by oxidizing the iron atoms in all these enzymes.

In summary, although enzymes with the proper protein scaffolding have not yet been discovered, the biosphere has preserved the chemistry necessary to perform anaerobic sterol synthesis. The absence of the enzymes themselves is unsurprising given the rapidity with which the scaffolding portion of enzymes evolve compared with the functional groups and the selection pressure to swap entire enzymes for ones that are O_2 tolerant. Hence, we remain sceptical that the removal of these methyl groups can be used as a firm constraint on the existence of abundant oxygen supplies in the Archaean oceans.

5. WHEN DOES GEOLOGICAL EVIDENCE DEMAND THE PRESENCE OF OXYGENIC PHOTOSYNTHESIS?

The question of when oxygenic photosynthesis and environmental free oxygen first arose can be addressed from two directions. One could, on the grounds of uniformitarianism, assume that the Earth's system processes resembled modern processes as far back as the slightest bit of geological evidence suggests a kinship; one could assume that any oxidation or biochemical process that involves oxygen today has always involved oxygen, and any evidence of such a process is therefore evidence for oxygenic photosynthesis. This has been done many times (e.g. Rosing & Frei 2004), and a great deal of elegant intellectual effort has been spent trying to explain how oxidation of the Earth's surface could be delayed many hundreds of million years after the advent of oxygenic photosynthesis (e.g. Canfield 2005; Catling & Claire 2005; Fennel *et al.* 2005).

Alternatively, following MacGregor (1927), one could take a sceptical approach and, working forward from the Earth's origin, question the essence of oxygenic photosynthesis until the rock record permits no alternative explanation. As with all dichotomies, the most correct answer probably lies in-between. The sceptical approach is a fruitful one for identifying the limits of current scientific understanding and generating new hypotheses to direct testing of those limits, but a strictly sceptical approach would render it nearly impossible to investigate many historically contingent events.

Our sceptical hypothesis to explain the Great Oxygenation Event (figure 4) rejects, for the reasons discussed above, the uniformitarian interpretation of the biomarker record and accepts the direct geological indicators of the redox transition (using the redox tower indicated in figure 1). In the Archaean, the presence of detrital uraninite, detrital pyrite and sulphur MIF argue for a dominantly anaerobic surficial environment, kept clement by a combination of greenhouse gases including H_2O, CO_2, CH_4 and perhaps SO_2. The redox levels present in the environment were governed by reducing gases such as CH_4, sulphate produced by photo-oxidation (Farquhar *et al.* 2001) and microbial disproportionation (Philippot *et al.* 2007) of S^0, volcanogenic SO_2 and ferric iron produced by anaerobic iron-oxidizing photosynthetic bacteria (Walker 1987; Widdel *et al.* 1993; Kappler *et al.* 2005).

During glacial intervals, such as the Pongola at *ca* 2.9 Gyr ago and the Huronian at *ca* 2.5–2.3 Gyr ago, H_2O_2 would accumulate in ice and be released in the basal meltwaters. O_2 released by H_2O_2 disproportionation could then act as a local agent for oxic poisoning and drive the evolution of oxygen-mediating enzymes. Observed decreases in sulphur MIF associated with these glacial intervals may be the products of oceanographic changes. Both enhanced physical mixing, associated with the larger pole–equator thermal gradient present in ice-house conditions (Kopp *et al.* 2005), and the presence of more oxidizing electron acceptors, associated with the glacial peroxide source, would lead to greater mixing of oxidized and reduced sulphur reservoirs and thus diminish the MIF preserved in the sedimentary record.

Several redox-sensitive trace elements have been used in an attempt to constrain palaeoredox conditions, notably U, Mo and Re (e.g. Rosing & Frei 2004; Anbar *et al.* 2007). Uranium is soluble in its oxidized form and insoluble in its reduced form, so evidence for uranium mobility has been taken as evidence for oxidizing conditions. The redox potential of the U(VI)/U(VIII) couplet is, however, comparable to that of the ferrous/ferric couplet, and so does not provide much additional constraint on oxygen levels. Mo(IV) similarly forms insoluble sulphide minerals in its reduced form and is soluble in its oxidized Mo(VI) state. Owing to the stability of MoS_2, increased Mo input through oxidative weathering provides a better constraint on redox conditions, but the redox potential of the MoS_2/Mo(VI) couplet is still below that of other redox-sensitive metals, such as Mn(II)/Mn(IV), below that of the P_{870} photosystem complex of purple bacteria, and well below that of H_2O/O_2 (see table S1 in the electronic supplementary material).

The oldest evidence for an environment containing massive amounts of free molecular oxygen of which we are aware is the *ca* 2.22 Gyr ago Kalahari Manganese

Member of the Hotazel Formation, Transvaal Supergroup, South Africa (Cairncross *et al.* 1997). As indicated in figure 1 and in the electronic supplementary material, table S1, nitrate and molecular O_2 are the only environmentally significant oxidants capable of converting soluble Mn^{2+} into insoluble Mn^{+4}, and nitrate itself requires oxygen to form in the modern oceans. Hence, the deposition of the BIF-hosted manganese in this unit is a firm oxygen constraint. The Hotazel Formation was also deposited in the aftermath of the Makganyene Snowball Earth Event (Kirschvink *et al.* 2000; Kopp *et al.* 2005), the only confirmed low-latitude glaciation in the Palaeoproterozoic (Evans *et al.* 1997; Hilburn *et al.* 2005).

It is hard to imagine a more dramatic step in biochemical evolution than the final tinkering with the manganese–calcium cluster in the oxygen-evolving complex of PSII that led to the splitting of water and the release of molecular O_2. Novel innovations that confer large selective advantages to a species lead to their rapid, exponential growth, and the creation and dominance of ecological niches. Such a profound evolutionary innovation ought to have left a clearly legible mark on the planet. Two dramatic events punctuate the Earth's history in the critical period *ca* 2.3 Gyr ago, when our sceptical interpretation suggests the first appearance of oxygenic photosynthesis: the Makganyene Snowball Earth and the deposition of the Kalahari Manganese Field. As we have argued elsewhere (Kopp *et al.* 2005), the simplest explanation of these occurrences is that the sudden release of oxygen from the evolution and radiation of oxygenic phototrophs destroyed reduced greenhouse gases such as methane and initiated Snowball conditions.

An additional implication of this scenario is that the formation of glacial peroxides is an essential step in the development of oxygen-mediating enzymes, and ultimately oxygenic photosynthesis. The Earth-like planets that orbit too close to their parent star for ice to form are therefore unlikely to evolve the aerobic metabolism essential for animal life.

6. CONCLUSIONS

As noted by Liang *et al.* (2006), production and accumulation of hydrogen peroxide on the surface of polar ice caps provides a straightforward mechanism for generating trace but substantial concentrations of H_2O_2 and free O_2 in the ocean below melting glaciers, away from the lethal influence of UV radiation. This environment is capable of driving the evolution of oxygen-mediating enzymes, and paving the way for the evolution of the oxygen-evolving cluster of PSII.

We find no chemical requirement for molecular oxygen in the biosynthesis of the lipid biomarkers of presumed Archaean age, which cannot be met by known anaerobic biochemical mechanisms. As the anaerobic enzymes that perform analogous chemical steps to oxygen-requiring ones use redox-sensitive metal cofactors that are poisoned by oxygen (Imlay 2006), there is intense evolutionary pressure to swap them out for those that are not oxygen sensitive. As three such substitutions in 17 enzymatic steps are documented in the biosynthesis of chlorophyll, we argue that four substitutions in a more than 25-step sterol synthesis pathway are not unreasonable.

The evolution of the oxygen-releasing complex of PSII as measured by environmental redox indicators is correlated with the Makganyene Snowball Earth and the deposition of the Kalahari Manganese Field. We suggest that the destruction of reduced greenhouse gases such as methane (Pavlov *et al.* 2000) did not take 400 Myr, but was rapid enough to trigger the Makganyene Snowball (e.g. Kopp *et al.* 2005).

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