



## " ! ... Comment ... ? "



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The Editors hope that readers will take full advantage of this section and use it to raise matters that hitherto have been confined to a limited audience.

**Jon Saunders, Editor-in-Chief**

### Primitive anaerobic protozoa: a false concept?

Since Bütschli (7), protistologists have tried to not only organize the interrelationships between the different groups of protists, but also to build phylogenetic trees to explain the evolution of the eukaryotic cell. In the last 20 years, results obtained by molecular techniques and electron microscopy have furnished general ideas on what the tree of life may look like. The base of the tree generally includes groups such as Metamonada (e.g. *Giardia*, *Hexamita* and *Trepomonas*), Microsporidia and Parabasala (e.g. *Trichomonas vaginalis*, *Pseudotrichomonas keilini*) (9).

These basal 'anaerobic' protozoa differ from aerobic cells by the absence of mitochondria and hence oxidative phosphorylation. Energy generation is derived from glycolysis with some unusual features (i.e. some enzymes are pyrophosphate-dependent) and also from alternative substrate fermentation (i.e. the arginine dihydrolase pathway). Some of them (e.g. the trichomonads) also possess unusual redox organelles known as hydrogenosomes which, as well as other products, generate H<sub>2</sub> (giving them their name).

Early anoxic Earth has been set as the

stage for the genesis of the first eukaryote and much attention has therefore centred on these 'anaerobic' protozoa. The milestone in this evolution is the endosymbiosis of the mitochondria and thus the passage from an anaerobic to an aerobic way of life. Here, we would like to briefly illustrate how this notion of a link between anaerobiosis and primitism is unfounded and how it has misled subsequent hypotheses for the evolution of the eukaryotic cell.

To discuss anaerobicity it is paramount to be clear as to its definition. To describe an organism as anaerobic because O<sub>2</sub> is not required for oxidative phosphorylation is practical in many circumstances, but may be misleading in this context. Biochemically, a true anaerobic organism should be able to carry out all its metabolic functions, such as sterol and fatty acid metabolism, anaerobically, without the need to scavenge metabolic constituents from its environment. A distinction must also be made as to whether an organism is aerotolerant or whether it benefits from low levels of O<sub>2</sub>.

### Primitive 'anaerobic' protozoa enjoy just a little O<sub>2</sub>

'Anaerobic' protozoa lack electron-linked oxidative phosphorylation. They do, however, take up O<sub>2</sub> at high rates and some have been observed to have higher affinities for O<sub>2</sub> than their aerobic counterparts containing cytochrome oxidase (4, 21). Low levels of O<sub>2</sub> have been shown to affect the carbon balance of *Giardia* and *Hexamita* (26, 5), both belonging to the diplomonads, until recently generally favoured as forming the deepest branching eukaryotic group (20). *Trichomonas vaginalis*, far from being anaerobic, requires traces of O<sub>2</sub> for optimal growth and reproduction (25). Why O<sub>2</sub> is required by these organisms is not known for certain, but part of the reason may be to recycle electron acceptors such as NAD(P)<sup>+</sup> (unpublished observations from our laboratories in fact conclude that in growth conditions where O<sub>2</sub> has been strictly omitted, some diplomonads fail to grow). Therefore, the correct term to describe these organisms should be microaerophilic. A strict anaerobe in this context will be defined as an organism which is highly susceptible to O<sub>2</sub> and which will grow optimally in an O<sub>2</sub>-free environment. To

date there are no 'ancient' protists (or Archezoa) which have been shown to be strictly anaerobic.

Strict anaerobes can however be found in the crown of the eukaryotic tree. Ciliates, such as *Metopus contortus* and *Parablepharisma* sp., do not survive for more than a few hours when exposed to aerobic conditions and require strict omission of O<sub>2</sub> for growth (15). However, even in these organisms, the capacity for anaerobic biosynthetic metabolism has not been demonstrated. Phylogenetic studies of these organisms clearly show that they have derived, on several occasions, from aerobic ciliates (12). Interestingly, examples of microaerobic ciliates behaving as facultative anaerobes have been described in the laboratory (2). Strict anaerobic ciliates also contain hydrogenosomes and in some cases, such as in *Cyclidium* and *Cristigera*, both mitochondria- and hydrogenosome-bearing species are found within the same ciliate genus (16, 14).

### Problems with current mitochondrial hypotheses

The first misconception regarding anaerobicity and eukaryotic antiquity arose from the somewhat artificial establishment of the amitochondriate protozoan group, the Archezoa (9). By various phylogenetic means [but mainly by nuclear small subunit (SSU) rRNA analysis], the Archezoa were inferred

#### ► GUIDELINES

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as being primitive and anaerobic, urging the notion that the organism which first harboured the mitochondrial endosymbiont was a primitive anaerobic protozoan. This hypothesis, however, has now generally been rejected. Over the past few years, genes of mitochondrial origin have been shown to be present in these Archezoa (namely the microsporidia, *Giardia* and *Trichomonas*; see recent review, 11). Furthermore, there are growing concerns as to the use of single genes to confer phylogenetic relationships. Significantly, recent studies now favour the 'Archezoan' microsporidia species to be more closely related to fungi than protists (e.g. 10, 19). It has been suggested that the primitive status enjoyed by the diplomonads is also proving increasingly tenuous (e.g. 6).

The demonstration of an anaerobic origin for eukaryotes was again perpetuated in the recent 'hydrogen hypothesis' (23). This hypothesis argues that the original eukaryote was derived from an association between a H<sub>2</sub>-producing proteobacterium and an autotrophic archaeon, describing how in some circumstances this association gave rise to hydrogenosomes whilst in others it gave rise to mitochondria. This prediction, however, is contrary to the simplest explanation that hydrogenosomes are an adaptation of pre-existing mitochondria (3, 13).

The hydrogenosome until recently enjoyed an enigmatic reputation. Since its discovery some 25 years ago, the origin of this organelle has been under debate. Characterization of key enzymes such as pyruvate:ferredoxin oxidoreductase led early workers to hypothesize that hydrogenosomes derived from an endosymbiosis with *Clostridia*-like bacteria (24). Unlike protozoan hydrogenosomes which are bound by two membranes, fungal hydrogenosomes (e.g. chytrid fungi) appeared to possess only one membrane and were argued to have derived from peroxisomes (8). Finally, free-living ciliate hydrogenosomes which possess characteristic mitochondria-like folding of the inner membrane, prompted the notion of a mitochondrial origin (17). In the last few years, there has been overwhelming evidence (reviewed in 3 and 13) in molecular (e.g. presence of mitochondrial chaperonin genes in hydrogenosome-bearing protozoa), physiological (e.g. hydrogenosomes possess a membrane potential and act as calcium stores) and morphological (e.g. the reassertion that fungal hydrogenosomes possess two membranes) studies to strongly support a mitochondrial origin for hydrogenosomes.

The lack of a detectable hydrogenosomal genome and therefore the lack of an evolutionary molecular fingerprint has been responsible for the speculation about ancestry. The loss of respiratory function (i.e. lack of detectable cytochromes) would

explain the absence of DNA in hydrogenosomes. However, it has been shown recently that the hydrogenosome-bearing symbiotic ciliate *Nyctotherus* contains DNA (as confirmed by immunocytochemistry) (1). Furthermore, this recent study revealed that rDNA from *Nyctotherus* hydrogenosomes shows high sequence similarity to mitochondrial SSU rRNA genes from aerobic ciliates: surely, the nail in the coffin for the die-hard sceptic of a mitochondrial origin for hydrogenosomes.

Taking into account the probable monophyletic origin of mitochondria, there is increasing evidence to suggest that the origin of the eukaryotic cell and its mitochondrial component came into being simultaneously rather than sequentially as the serial endosymbiosis hypothesis would predict (18). There is no compelling evidence, however, that this phenomenon occurred in the absence of O<sub>2</sub> or that the symbiont which gave rise to the mitochondrion was anaerobic. It is our interpretation of the available evidence that (i) the primitive status of Archezoa is precarious, (ii) 'ancient' anaerobic protozoa described to date are not true anaerobes, but more likely have adapted to an anaerobic lifestyle (parasitic or free-living) and probably at some stage contained mitochondria, and (iii) there is more substantial evidence to suggest that hydrogenosomes are a secondary modification of mitochondria, therefore refuting (at least in part) the recent 'hydrogen' and 'syntrophy' hypotheses (23, 22).

A vision of prebiotic Earth as a mass of bubbling rock and toxic fumes is perhaps too tantalizing for evolutionists to ignore when dealing with the origin of eukaryotic life. However, closer examination of aerobic as well as anaerobic protozoa may, in our opinion, be just as fruitful.

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## Primitive anaerobic protozoa: the wrong host for mitochondria and hydrogenosomes?

Since Mereschkowsky (22), thoughts on the nature of the most primitive kinds of cells that we now call eukaryotes have entailed the notion that some cytoplasmic organelles arose from free-living bacteria through endosymbiosis. Endosymbiotic hypotheses have fared extremely well when it comes to pinpointing the similarities between organelles and free-living bacteria as evidence in favour of their endosymbiotic origin, both in the case of chloroplasts (22) and mitochondria (30). But for organelles to take up residence in a cytoplasm, there had to be a host. Endosymbiotic hypotheses for the origin of mitochondria have had much more success explaining the origins of the organelle than they have had with explaining the origin of the host. So what was the host?

Anaerobic protists play an important role in this issue because the host for the origin of mitochondria has traditionally been envisaged, either explicitly or implicitly, as a heterotrophic cell with a glycolytic pathway that did not depend on molecular oxygen for ATP synthesis, like contemporary protists that have such a lifestyle. In some versions of the endosymbiont hypothesis, the host is envisaged as a prokaryote, but in most versions it is envisaged as a eukaryote that either arose autogenously (without symbiosis) or as the result of a symbiosis of cells that occurred prior to the origin of mitochondria (for a random sample see 3, 6, 9, 11, 12, 15, 18, 19, 22). But in all versions of the 'classical' endosymbiont hypothesis that make a statement on the issue (e.g. 3, 9, 15), the host that acquired the mitochondrion is envisaged as a heterotroph, one that satisfied its ATP needs with the help of the glycolytic pathway.

At present, the endosymbiont hypothesis has three problems. First, it no longer has a host. This is because available data indicate that contemporary amitochondriate protists investigated thus far – organisms which were thought to be descendants of the host – possessed a mitochondrion in their evolutionary past, but subsequently lost the organelle through reduction (summarized in 9, 10, 12, 20, 25). These findings indicate that such cells can therefore hardly be descendants of the host in the endosymbiont hypothesis and they furthermore intertwine the issues of the origin of mitochondria and the origin of eukaryotes even more tightly than before (9, 10, 12). Second, there is no clear evidence to suggest that the host was even a heterotroph. This is because nuclear genes for enzymes of the glycolytic pathway in the eukaryotic cytosol – the backbone of heterotrophy in eukaryotes and the pathway that feeds mitochondria – appear themselves to be acqui-

sitions from the ancestors of organelles, also in amitochondriate protists (5, 8, 20, 21). Eukaryotes thus seem to have acquired the heterotrophic lifestyle that they now use, raising the question of how they synthesized their ATP prior to that acquisition. Third, formulations of the endosymbiont hypothesis that focus on the role of oxygen in the origin of mitochondria (e.g. 2) cannot directly account for data indicating a common ancestry of mitochondria and hydrogenosomes – the double-membrane-bound, ATP-producing organelles of many protists that shun oxygen-containing habitats (23, 24). Indeed, hydrogenosomes (and ATP synthesis in anaerobic mitochondria in general) have been a thorn in the side of the endosymbiont hypothesis, having been difficult to incorporate into traditional views (16, 23, 27) or having been disregarded (19). Given these newer findings, it is not unreasonable to question the view that the host may have been a hypothetical, heterotrophic, primitively amitochondriate eukaryote, and to pursue alternative avenues of thought.

The hydrogen hypothesis (20) is such an alternative, one that specifically addresses the compartmentalization and the ancestral state of eukaryotic ATP synthesis. It avoids the need to assume that primitively amitochondriate protists ever existed, because it entails the premise that the host was an Archaeobacterium, not a eukaryote. It avoids the need to assume that the host was a heterotroph because it posits that the host was an autotroph, one that was strictly dependent upon molecular hydrogen as an energy and electron source. Through explicitly formulated selective pressures for gene transfer from symbiont to host chromosomes, it directly accounts for the finding that eukaryotes studied to date tend to possess a eubacterial rather than an archaeobacterial glycolytic pathway. It also directly accounts for findings that indicate a common ancestry of mitochondria and hydrogenosomes, because it posits that the symbiont was a facultatively anaerobic  $\alpha$ -proteobacterium, one that was able to synthesize ATP either through oxygen-consuming electron transport like mitochondria or through hydrogen-producing fermentations like hydrogenosomes, dependent upon environmental conditions. The hydrogen hypothesis thus differs from previous views both on the origins of mitochondria and on the nature of the host. It generates some of the same predictions as the endosymbiont hypothesis does, for example that the respiratory pathways of oxygen-consuming mitochondria and  $\alpha$ -proteobacteria should be homologous, but it generates different predictions about the origins of pathways of ATP synthesis in hydrogenosomes than the endosymbiont hypothesis does. It furthermore generates very different predictions about the host, for example that the host ultimately should descend from the lineage of Archaeobacteria

that today contains methanogens, the only prokaryotes currently known to contain histones (26).

Is the view that hydrogenosomes are a 'secondary modification' of mitochondria at odds with the hydrogen hypothesis? No, because this is just a roundabout way of saying that hydrogenosomes and mitochondria share a common ancestor, since, obviously, no contemporary hydrogenosomes can have arisen through modification of contemporary mitochondria. Rather, they must have arisen from mitochondria that existed in the past. So what were those ancient mitochondria like in terms of biochemical properties? Were they like the mitochondria of the fungus *Fusarium* that perform nitrate and nitrite respiration, just as many facultatively anaerobic bacteria do (16)? Were they like plathelminth mitochondria that perform fumarate respiration as many facultatively anaerobic bacteria do (27)? Were they like the mitochondria of the ciliate *Nyctotherus* that produce H<sub>2</sub> with the help of a hydrogenase like hydrogenosomes do (1)? Were they like kinetoplastid mitochondria, which use acetate:succinate CoA transferase to regenerate CoASH like hydrogenosomes do (29)? Or were they like the mitochondria of *Euglena* that, instead of the pyruvate dehydrogenase complex, use ferredoxin-dependent pyruvate:NADP oxidoreductase in a reaction that is very similar to that of pyruvate:ferredoxin oxidoreductase (PFO) in hydrogenosomes (14)?

These questions are intended to make the point that there are a number of anaerobic (but not necessarily strictly anaerobic) mitochondria known that possess and use enzymes and/or pathways that are common to facultatively anaerobic bacteria and/or hydrogenosomes. As the simplest alternatives to explain this observation, either (a) mitochondria arose from strictly aerobic bacteria that did not possess genes for enzymes of anaerobic ATP-producing pathways as found in hydrogenosomes, in which case the enzymes germane to hydrogenosomal metabolism in the various eukaryotic lineages that possess hydrogenosomes can be predicted to have been acquired through independent lateral gene transfers, or (b) the genetic starting material for this biochemical diversity was present in a facultatively anaerobic bacterium that was the common ancestor of mitochondria and hydrogenosomes (20). Under the first alternative, the homologous enzymes in hydrogenosomes of different eukaryotic lineages should be shown to stem from independent lateral transfers involving different prokaryotic donors. Under the second alternative, the homologous enzymes in hydrogenosomes of different eukaryotic lineages should be shown to stem from a single eubacterial source (13). More molecular data from eukaryotes that possess hydrogenosomes and 'atypical' mitochondria are needed.



None of this is to detract from the importance of the observation that many eukaryotes commonly designated as 'anaerobic' do grow more prolifically when a bit of oxygen is present and can be designated as microaerophilic. In the example of the kinetoplastids, this appears to have to do with the maintenance of redox balance, i.e. the regeneration of NAD<sup>+</sup> (7). When oxygen is present, bloodstream forms of trypanosomes can regenerate additional NAD<sup>+</sup> from glycolytic NADH with the help of a glycerol-3-phosphate oxidase, such that one mole of water and two moles pyruvate per mole of glucose are produced as waste products. When no oxygen is available, one mole each of glycerol and pyruvate are excreted as end products to maintain redox balance (7). In other 'anaerobic' or 'microaerophilic' protists such as trichomonads and diplomonads, which generate ATP solely through extended glycolysis (24), oxygen-consuming systems to regenerate NAD<sup>+</sup> for glycolysis, hence to maintain redox balance, have also been reported (4, 17). Of course, oxygen also has important roles in eukaryotic fatty acid and sterol biosynthesis, but traditional formulations of the endosymbiont hypothesis are at a loss to explain why eukaryotes (also amitochondriate ones) possess eubacterial rather than archaeobacterial lipids in the first place, whereas the hydrogen hypothesis directly accounts for this finding as well (20). Clearly, more work is needed on the evolution and metabolism of eukaryotes that can grow with little or no oxygen.

The hydrogen hypothesis invokes the observable phenomenon of anaerobic syntrophy (one organism gleaning a living from the waste products of the fermentations of another) to infer how the patterns of compartmentalized energy metabolism observed among contemporary eukaryotes might have come to be. It can readily account for a number of observations, including the mitochondrial remnant recently described in *Entamoeba histolytica* (28), an organism that uses a homologue of hydrogenosomal PFO in its cytosol (13) for ATP synthesis (24). The syntrophy hypothesis (18) also invokes the principle of anaerobic syntrophy, but derives a heterotrophic, primitively amitochondriate eukaryotic host and does not address the origin of hydrogenosomes or their relationship to mitochondria. That the hydrogenosomes of the ciliate *Nyctotherus* (1) possess a remnant

of a hydrogenosomal genome bears out one of the predictions of the hydrogen hypothesis, rather than refuting it (in part or otherwise).

A vision of the host as a primitive eukaryote that never possessed mitochondria is perhaps too tantalizing for evolutionists to ignore when dealing with the origin of this ATP-producing organelle in its various aerobic and anaerobic manifestations (20, 23–25, 27). The hydrogen hypothesis predicts that upon closer examination of aerobic as well as anaerobic protozoa, none will be found that are primitively amitochondriate. Indeed, if eukaryotes are ever found that can be conclusively shown to have never possessed a mitochondrial symbiont in their evolutionary history, the hydrogen hypothesis would be falsified. The endosymbiont hypothesis is currently in search of a formulation that is sufficiently explicit as to generate similarly falsifiable predictions about the host that acquired mitochondria. That search may or may not be fruitful.

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