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INTRODUCTION

The presence and function of mitochondria in eukaryotes that inhabit anaerobic environments was long a biochemical and evolutionary puzzle. Major insights into the phylogenetic distribution, biochemistry, and evolutionary significance of organelles involved in ATP synthesis (energy metabolism) in eukaryotes that thrive in anaerobic environments for all or part of their life cycles have accrued in recent years. Underpinned by many exciting advances, two central themes of this progress have unfolded. First, the finding that all known eukaryotic groups possess an organelle of mitochondrial origin has mapped the origin of mitochondria to the origin of known eukaryotic groups. Second, the phylogeny of eukaryotic aerobes and anaerobes has been found to interleave across the diversity of eukaryotic groups, erasing what was once

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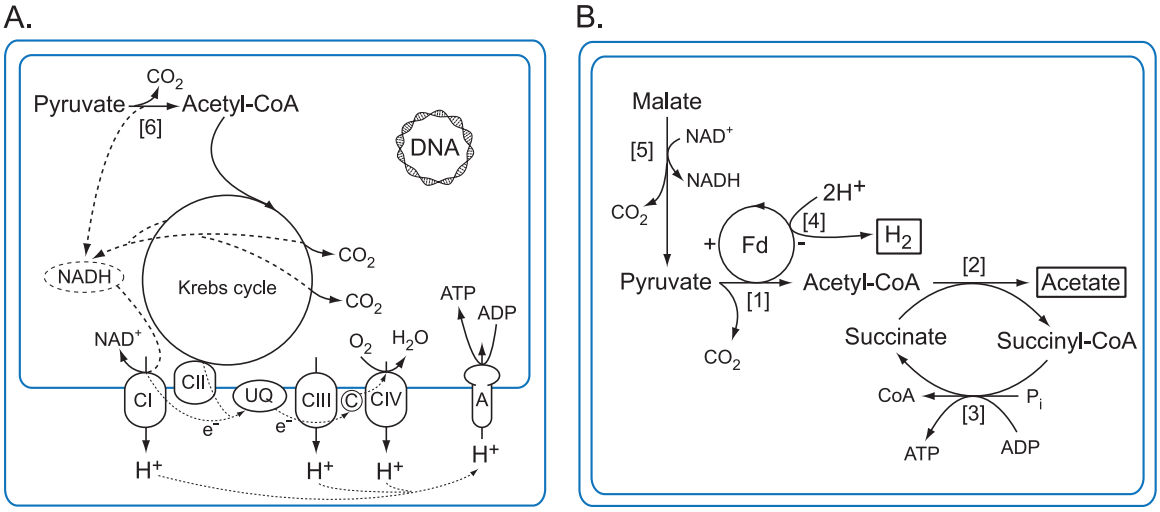


FIG 1 Two organelles in comparison. (A) Generalized metabolic scheme of pyruvate oxidation and oxidative phosphorylation in a typical oxygen-respiring mitochondrion, for example, from rat liver. (B) Generalized metabolic scheme of fermentative pyruvate oxidation in trichomonad hydrogenosomes, as proposed in the early 1970s. The presence and absence of organellar genomes are indicated. End products are boxed. Abbreviations: CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; C, cytochrome *c*; A, ATPase; Fd, ferredoxin; [1], pyruvate:ferredoxin oxidoreductase; [2], acetate:succinate CoA-transferase; [3], succinyl-CoA synthetase; [4], hydrogenase; [5], malic enzyme; [6], pyruvate dehydrogenase complex.

thought to be a major evolutionary divide between eukaryotic aerobes and their anaerobic relatives.

Data from gene, genome, and environmental sequencing projects are rapidly accumulating for eukaryotes that live in anaerobic habitats, giving clues as to what genes they possess. However, it has been repeatedly stressed—and remains true—that only for comparatively few organisms are specific biochemical data available concerning the enzymes and pathways that are actually used by the organisms and the metabolic end products that are excreted by them in their anaerobic habitats. Similarly, the biochemical role that their organelles play in ATP synthesis is known for comparatively few well-studied species. Based on those case studies, we will focus here on the enzymes, pathways, and end products of core ATP synthesis in eukaryotic anaerobes and the participation of mitochondria therein.

Even the spectrum of organelles specified by the term "mitochondria" has changed in recent years. Traditionally, the term mitochondria refers to the classical double-membrane-bounded, oxygen-respiring, crista-bearing organelles from rat liver that harbor the enzymes of the Krebs cycle (also known as the citric acid or tricarboxylic acid [TCA] cycle) and oxidative phosphorylation and that synthesize, and export to the cytosol, ATP with the help of proton-pumping electron transport chain complexes, ATP synthases, and ADP/ATP carriers (AACs) (Fig. 1A). Such would have been the description of a mitochondrion in 1973, the year when hydrogenosomes were reported for the parabasalid flagellate *Tritrichomonas foetus* (279). Hydrogenosomes are double-membrane-bounded, oxygen-sensitive, and H_2-producing organelles that occur among several groups of eukaryotic anaerobes and that synthesize ATP exclusively via substrate-level phosphorylation (Fig. 1B). For the 20 years following their discovery, the biochemical links between parabasalid hydrogenosomes and rat liver mitochondria, as shown in Fig. 1, appeared sufficiently few and sufficiently scarce that a common ancestry with mitochondria long seemed unlikely.

The first proteins characteristic of hydrogenosomes provided no links to mitochondria, for example, pyruvate:ferredoxin oxidoreductase (PFO) (also abbreviated PFOR and sometimes called pyruvate synthase) and iron-only hydrogenase ([Fe]-Hyd) (279). Although the investigation of hydrogenosomal [2Fe-2S]-ferredoxin (Fd) could have provided links to mitochondria because of the similarity of amino acid sequences and biochemical properties to mitochondrial [2Fe-2S]-Fd (164, 228, 303), those clues were not pursued at the time. Subsequent studies of hydrogenosomal succinyl coenzyme A (succinyl-CoA) synthetase (SCS) hinted more distinctly at evolutionary links between mitochondria and hydrogenosomes (57, 259, 260). The situation changed markedly, however, when four parallel reports of chaperonins common to the two organelles appeared, making it clear that hydrogenosomes are mitochondria in the evolutionary sense (63, 156, 201, 415). The discovery of more heavily reduced forms of mitochondria that do not produce ATP—mitosomes—furthermore indicated that mitochondria might indeed be ubiquitous among eukaryotes (163, 300, 507, 508, 512, 558). In the meantime, abundant evidence supporting the common ancestry of all three organelles and the ubiquity of mitochondria was amply reviewed (41, 127, 134–136, 178, 454, 520–522, 524).

Although hydrogenosomes typically lack DNA (351), some hydrogenosomes were eventually found to have preserved a genome that is homologous to mitochondrial DNA (mtDNA) (49), leaving no doubt that hydrogenosomes are anaerobic forms of mitochondria. Besides the trichomonad lineage, hydrogenosomes have been characterized for ciliates (569), chytridiomycete fungi (570), and the heterolobosean amoeboflagellate *Psalteriomonas lanterna* (56). Newly characterized organelles from the human parasite *Blastocystis hominis* share some properties with hydrogenosomes (270, 380, 468), even though evidence for H_2 production, hitherto defining for hydrogenosomes, is so far lacking for the *Blastocystis* organelles. Conversely, H_2 production has been reported for *Giardia*, a protist that lacks hydrogenosomes and synthesizes its ATP in the cytosol (287), and truncated iron-only hydrogenases are now found to be ubiquitous among eukaryotes, where they are

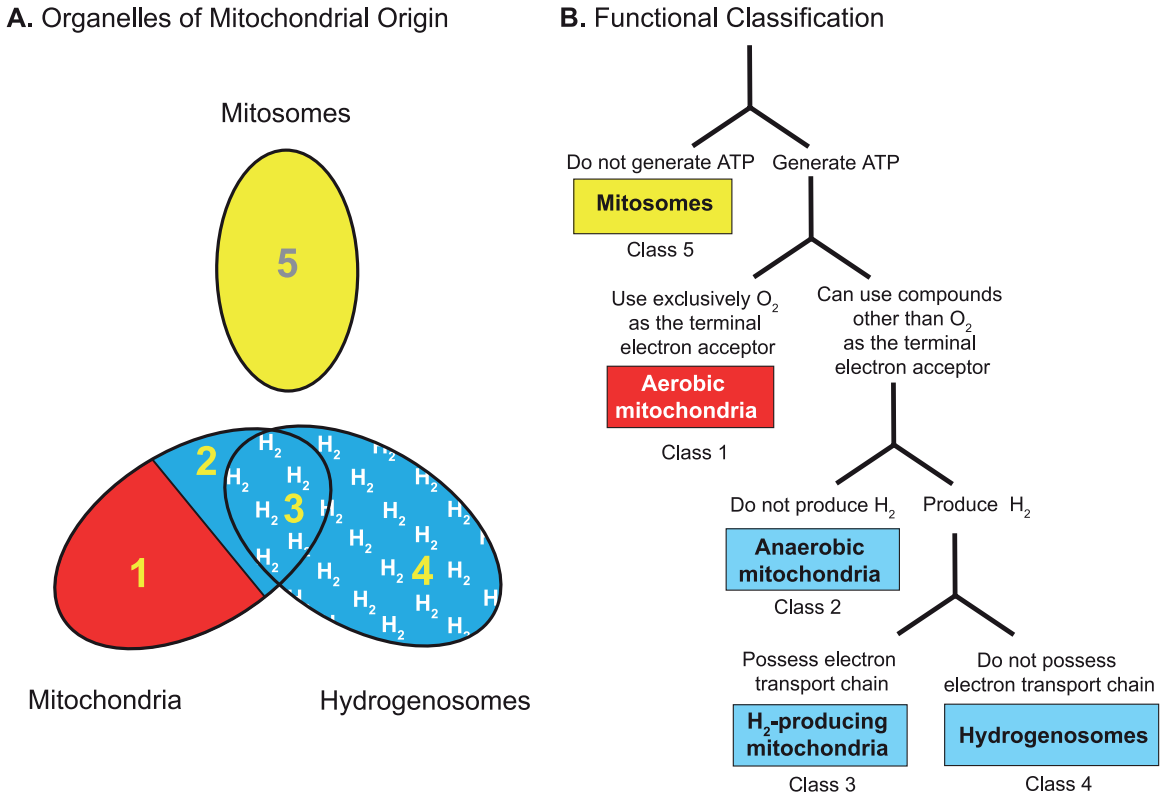


FIG 2 Organelles of mitochondrial origin. (A) The mitochondrial family of organelles, divided along functional lines (classes 1 to 5). Class 1, the canonical, rat liver-type mitochondrion (as described in most textbooks), which uses oxygen as the terminal electron acceptor; class 2, an anaerobically functioning mitochondrion, which uses an endogenously produced electron acceptor, such as fumarate, instead of oxygen; class 3, a hydrogen-producing mitochondrion, which possesses (besides a proton-pumping electron transport chain) a hydrogenase and hence can use protons as a terminal electron acceptor and is therefore *qualitate qua* also a hydrogenosome; class 4, hydrogenosomes, anaerobically functioning ATP-producing organelles of mitochondrial origin that can use protons as an electron acceptor, which results in the formation of hydrogen; class 5, mitosomes, organelles of mitochondrial origin that are not involved in ATP production. Red indicates that oxygen is consumed in the production of ATP, blue indicates the production of ATP without the use of oxygen, and yellow indicates that the organelle is not involved in the production of ATP. (B) Criteria for the functional classification of organelles of mitochondrial origin.

involved in FeS cluster biogenesis (162, 199). In addition, the chloroplasts of some eukaryotic algae have been found to produce copious amounts of H_2 under certain conditions, a development of immense biotechnological interest (184, 322, 323). Thus, the production of H_2 in eukaryotes is no longer synonymous with hydrogenosomes, while hydrogenosomes have become permanent members of the mitochondrial family of organelles.

Those developments have witnessed hundreds of original new reports over the past years dealing with mitochondria, hydrogenosomes, and mitosomes in protists. This was accompanied by the appearance of the terms "mitochondrion-like organelles" (MLOs) or "mitochondrion-related organelles" (MROs) for organelles that are mitochondria in the evolutionary sense, causing some confusion as to "who is who" among organelles of mitochondrial origin. A functional classification and a short description of the various classes among the mitochondrial family of organelles reported so far, along with a terse delineation of criteria for that classification, are given in Fig. 2.

Mitochondria produce ATP via oxidative phosphorylation. They harbor a mitochondrial genome (167), as the protein complexes of proton-pumping electron transport chains contain essential subunits that are always mitochondrially encoded (10). The canonical, textbook-type mitochondrion uses oxygen as the terminal electron acceptor.

Anaerobic mitochondria are otherwise typical mitochondria, but they function anaerobically, using compounds other than oxygen as the final electron acceptor. Most organisms with anaerobic mitochondria use an endogenously produced electron acceptor, such as fumarate, generating succinate as a major excreted end product, but environmental acceptors, such as nitrate (410), can also be used. Membrane-associated fumarate reduction is usually associated with rhodoquinone (RQ) as an electron carrier, proton pumping, and chemiosmotic ATP synthesis (501).

Hydrogen-producing mitochondria possess, in addition to their proton-pumping electron transport chain, an iron-only hydrogenase that allows them to use protons as terminal electron acceptors, resulting in hydrogen production. They can harbor cytochromes. They possess a membrane-associated, proton-pumping electron transport chain but do not use oxygen as a terminal acceptor, and they produce H_2, like hydrogenosomes. An example is found in the ciliate *Nyctotherus ovalis* (49).

Hydrogenosomes are organelles of mitochondrial origin that generate ATP via hydrogen-producing fermentations (351). They lack cytochromes, a membrane-associated electron transport chain, and a genome. They produce ATP exclusively via substrate-level phosphorylation. Hydrogenosomes have so far been found among the trichomonads, the ciliates, the chytridiomycete fungi, and excavate taxa such as *Psalteriomonas* (520).

Mitosomes are organelles of mitochondrial origin that do not produce ATP. Mitosomes of some lineages have retained components of FeS cluster assembly (163, 508), and others have retained components of sulfate activation (332).

This classification of the organelles takes into account newer findings and hence should replace the older type I and type II designations for energy metabolic types (309, 349). To signify their common origin and to underscore that these organelles are not mitochondrion-like organelles but are in fact mitochondria, in that they all evolved from the same ancestral endosymbiont as all other mitochondria, we use the term "organelle of mitochondrial origin" to designate them collectively. This is an opportune spot to cover another nomenclatural point: about 20 years ago, one of us introduced into the literature the term "amitochondriate" to designate those eukaryotes that lack typical, cytochrome-possessing mitochondria (349, 350). Although seemingly a good idea at the time, from today's perspective, it has turned out that none of the protists then designated by the term, nor any protists subsequently discovered, are indeed "amitochondriate" in the sense of ancestrally lacking mitochondria (although many do indeed lack cytochromes). Although the term "amitochondriate" has been extensively used and is still occasionally used in the literature, now is a good time to stop using it altogether.

The purpose of this review is to survey energy metabolism—with an emphasis on mitochondrial metabolism—in eukaryotes with anaerobic life-styles for part or all of their life cycles, focusing on organisms, including metazoans, where enough biochemical data are available on the main enzyme activities and excreted end products to allow the presentation of more or less realistic metabolic maps. Another nomenclatural point deserves mention. In the metazoan literature, where differing degrees of hypoxia (oxygen levels below ambient atmospheric levels) are often distinguished, it is common practice to designate environments as hypoxic or anoxic, while the term anaerobic is traditionally used to describe metabolism (169). Because we are focusing on mitochondria, here we use the term anaerobic more generally to designate life-styles, organisms, or pathways in which oxygen is not required as the terminal electron acceptor, and we will encounter cases where oxygen can be used even though it is not required.

Numerous aspects of the mitochondria of anaerobes and parasites have recently been reviewed elsewhere, including protein import (121), FeS cluster assembly (454, 520), bioenergetic aspects (266, 267), and reductive evolutionary trends (193). Monographs on the topic of hydrogenosomes and mitosomes have appeared (84, 310, 480), as have books on the evolutionary significance of mitochondria, including anaerobic forms (264, 265). Recent progress from the study of protists needs to be integrated with existing knowledge of the anaerobically functioning mitochondria of metazoans (61, 498); hence, we will cover both protists and metazoans here, but we will not address metazoans that can survive short-term hypoxia or anoxia through ethanol, lactate, or similar fermentations, aspects that have been reviewed elsewhere (194). Rather, we focus on metabolic pathways that are germane to eukaryotic anaerobes and their mitochondria. By presenting current metabolic maps for these eukaryotes in one place for comparison, and by presenting them within a current phylogenetic framework, we aim to provide a comparative picture that conveys the unity among oxygen-independent mitochondrial energy metabolic pathways among diverse eukaryotic lineages while not blurring the distinctions. We also briefly consider mitochondrial energy metabolic pathways in a more general evolutionary and earth history context.

Key reports of previous findings on this topic and related topics can be found, including reports on early progress on the topic of energy metabolism in anaerobic protists (98), general overviews (142, 349, 352, 426, 516), hydrogenosomes (32, 33, 177, 136, 351), anaerobic mitochondria (501), *Giardia intestinalis* (3, 59, 221, 222, 286, 444), *Entamoeba histolytica* (320, 406, 423), *Trichomonas vaginalis* and *Tritrichomonas foetus* (280, 381), and mitochondrial function in various medically relevant parasites (305).

A FEW DISTINCTIONS

Functional and Environmental Anaerobiosis

Particularly among animals, a distinction has to be made between environmental hypoxia (low oxygen availability) and functional hypoxia, because energy metabolism without the use of oxygen can occur either by a reduction in the availability of oxygen in the environment or as a consequence of burst-type muscular activity (61, 169). Functional hypoxia related to burst-type activity occurs usually in animals trying to escape from predators or in those in pursuit of their prey. The rate of the use of ATP by the muscles involved is too high to be met by their aerobic energy metabolism and oxygen supply. Therefore, the organism necessarily reverts to the rapid anaerobic production of ATP in its rapidly contracting muscles. In some species and tissues, phosphagens like creatine phosphate or phospho-L-arginine can be physiologically relevant "preformed" sources of ATP reserves for burst activity (169). Environmental hypoxia, on the other hand, occurs when organisms naturally inhabit niches where oxygen is scarce, such as parasitic worms living in the intestinal tract of their host (249) or protists that inhabit anoxic sediments (38, 129, 142, 291, 473). Environmental changes can also result in a decreased availability of oxygen for organisms unable to relocate to more oxic habitats.

As a consequence, environmental hypoxia is found mainly in organisms inhabiting an environment where the scarcity of oxygen is compensated for by a surplus of fermentable substrates (for example, parasites in the intestinal tract of their host) or in organisms with limited mobility that prevents them from actively escaping periods of environmental hypoxia. This occurs, for example, in intertidal marine organisms, such as the sea mussel *Mytilus edulis*, where daily interruptions of oxygen availability are forced upon the organism by the tidal cycle. During low tide, the emerged mussel closes its valves to avoid dehydration and therefore has to adapt its metabolism to hypoxic conditions and thus switches to anaerobic energy metabolism (66, 169). Freshwater snails, on the other hand, usually live at the water surface but may occasionally inhabit bottom layers low in oxygen, for instance, when they expel air from their lung cavities and sink as a response to danger.

Another strategy among various animal groups to survive prolonged periods of hypoxia is a physiological response called metabolic repression: energy metabolism is more or less turned off to reduce ATP consumption, thereby requiring little production (195). For example, marine snails that inhabit the intertidal zone can respond to severe hypoxia or anoxia with a strongly repressed metabolic rate until oxygen levels sufficient to support respiration return (181). Various marine invertebrates respond similarly, enabling them to stretch their endogenous glycogen reserves (169). Metabolic repression can be seen as a kind of metabolic resting stage; it also occurs in vertebrates, especially among diving marine

animals such as turtles and seals (109, 174, 195). However, even if the organism responds to hypoxia with a reduced metabolic rate, maintenance amounts of ATP still have to be produced without oxygen. In most free-living marine invertebrates, succinate, acetate, and propionate along with alanine and opines are the main end products of anaerobic energy metabolism (108) either during repression or during normal physiological activity. Among parasitic metazoans, the main anaerobic end products are usually lactate, succinate, acetate, and propionate, whereby, notably, the same spectrum of end products tends to be produced in the presence and in the absence of oxygen (28).

Redox Balance through Respiration and Fermentation

Since we do not consider photosynthetic ATP synthesis in this paper, when we refer to eukaryotes, we are referring to heterotrophs or heterotrophic growth. Core ATP synthesis in eukaryotes entails the oxidation of reduced carbon compounds. Glycolysis—the oxidative breakdown of glucose to pyruvate via the Embden-Meyerhof pathway—is the backbone of ATP synthesis in eukaryotes (354). Some exceptions to that rule in specialized environments entail, for example, the arginine dihydrolase pathway (155, 243, 338) in some anaerobes. The arginine dihydrolase pathway involves the conversion of arginine to ammonia and citrulline, with the phosphorolysis of the latter yielding ornithine and carbamoylphosphate, which is readily converted into NH_3, CO_2, and ATP (338). It has a function analogous to that of the urea cycle in that it is nonoxidative, and it removes nitrogen from amino acids, but in contrast to the urea cycle, it generates 1 ATP per arginine. Under physiological growth conditions, it satisfies a relevant but small fraction of the total ATP requirements of the eukaryotic organisms that possess it (59, 571) and is therefore an auxiliary rather than a core metabolic pathway. Another exception is the methylotrophic pathway in some obligately aerobic fungi, in which methanol is oxidized to CO_2, with the electrons entering the mitochondrial respiratory chain (525), or outright energy parasitism, as found for some microsporidians that appear to siphon off not only metabolites but also ATP (in exchange for ADP) from their host cells (512). Further exceptions (although they have been rare so far) are the mitochondria in gills of the lugworm *Arenicola marina*, which can use H_2S instead of glucose as a source of electrons for proton pumping and mitochondrial ATP synthesis via the electron transport chain (115, 170).

The oxidation of glucose or other organic compounds generates reduced cofactors (NADH and reduced flavin adenine dinucleotide [$FADH_2$]) that have to be reoxidized by the donation of the gained electrons to a terminal acceptor that, in its reduced form, is excreted as a metabolic end product (waste). Reduced cofactors can be reoxidized in two ways: respiration and fermentation.

Respiration is the use of a terminal electron acceptor that is obtained from the environment. Prokaryotic anaerobes display a great diversity of environmentally available terminal acceptors that they can use during ATP synthesis. Amend and Shock (13) compiled data for over 140 different core metabolic reactions involving environmentally available terminal acceptors from just 131 thermophilic anaerobic prokaryotic species alone. In eukaryotes, the entire known spectrum of core energy metabolic reactions—aerobic or anaerobic—involves closer to a dozen different main overall reactions, a paucity of biochemical diversity which, however, powers millions of known species. Perhaps the most common form of anaerobic respiration in eukaryotes, fumarate respiration, is not truly respiration at all, because fumarate (the terminal electron acceptor) is generated endogenously during metabolism, for which reason it constitutes fermentation (but fermentation involving the generation of a proton gradient). Among eukaryotes, the use of environmentally available acceptors other than oxygen is still considered to be rare, but it might be more widespread than currently thought. Nitrate is used as the terminal acceptor by denitrifying foraminiferans, which can be very widespread in marine environments (384, 410), several fungal species (248, 486, 513, 573, 574), and diatoms (232) and which was found in ciliates in one report (146). There was also one report of elemental sulfur being used as the terminal acceptor to generate H_2S as the metabolic end product (1). From today's standpoint, however, that is about all in terms of anaerobic respirations in eukaryotes.

Far more common in eukaryotic anaerobes are fermentations, the donation of electrons onto terminal acceptors that are generated by the organism during metabolism, such as pyruvate (to produce lactate and/or opines), acetaldehyde (to produce ethanol), fumarate (to produce succinate), protons (to produce hydrogen), or acetyl-CoA (to produce fatty acids and their derivatives). Fermentations entail disproportionation reactions in which the organic substrate is converted into a more reduced and a more oxidized form, for example, the conversion of glucose into ethanol and CO_2. Carbohydrates are thus suitable substrates for fermentation. Lipids, however, are too reduced to be fermented, as both oxidation and reduction of the substrate must occur. Fermentations in eukaryotes can occur entirely in the cytosol: examples include protistan parasites such as *Giardia* and *Entamoeba* (354) or specialized cells such as mature human erythrocytes, which are devoid of mitochondria (51). Fermentations can also occur partly in hydrogenosomes, as in the case of *Trichomonas* (351). Among animals, fermentation often entails malate dismutation, involving parts of the mitochondrial electron transport chain, as in the case of the anaerobic mitochondria of many marine invertebrates and parasitic worms (500, 501). Details of these fermentations are presented in later sections.

It should also be stressed that many eukaryotes live in fully oxic habitats but without using O_2 for oxidative phosphorylation. When O_2 is present, it can be used as a terminal acceptor, or it can be ignored. Oxygen is an opportunity, one that is surprisingly often declined, with yeast being the classical example. Given a sufficient amount of a fermentable substrate in the presence of oxygen, many yeasts "choose" to ferment rather than use O_2 as the terminal acceptor in mitochondria (395, 526, 535). Furthermore, many yeast species show a distinct Crabtree effect: the occurrence of alcoholic fermentation under aerobic conditions in the presence of excess sugar (395, 527). Maintenance of the baker's yeast, *Saccharomyces cerevisiae*, under aerobic cultivation conditions does not result in completely respiratory sugar metabolism, as even fully aerobic cultures exhibit a mixture of respiration and fermentation, unless they are grown with a limited sugar supply at low specific growth rates (23, 393). In *S. cerevisiae*, the biosynthesis of functional mitochondria is furthermore controlled by environmental stimuli, including the availability of oxygen and the type of carbon source (171, 344). In the laboratory, yeast can, however, be forced to use its mitochondria via growth on nonfermentable substrates such as lactate or ethanol (446).

Trypanosomes are another example of organisms that sometimes "just say no" to the use of O_2 for oxidative phosphorylation.

When they live in the mammalian bloodstream, where there is ample oxygen and glucose, they synthesize all of their ATP through substrate-level phosphorylation in glycolysis, but they excrete an oxidized end product (pyruvate) instead of a reduced end product (lactate). They maintain redox balance with the help of an alternative oxidase (AOX) in mitochondria, which uses O_2 as the terminal electron acceptor but without mitochondrial ATP synthesis. Thus, bloodstream-form trypanosomes use their mitochondria with O_2 as the terminal acceptor but without oxidative phosphorylation. This is another example of what is sometimes called aerobic fermentation (100). The bloodstream forms of the malaria parasite *Plasmodium falciparum* follow yet a different strategy. Their core energy metabolism is lactate fermentation (368a). Despite this, they have a fully functional electron transport chain in their mitochondria (505a), which is, however, used mainly to support a ubiquinone (UQ)-dependent dihydroorotate dehydrogenase of the inner mitochondrial membrane (375a). Accordingly, bloodstream malaria parasites use O_2 in their mitochondria but to reoxidize UQH_2 (ubiquinol) for pyrimidine biosynthesis and not for energy metabolism.

There is then the caveat that fermentation as a strategy to survive anoxia is not restricted to microbes; goldfish (*Carassius auratus*) are a classic example. At 10°C, goldfish can survive complete anoxia for more than a week; the end products of metabolism are ethanol and CO_2 (519), with the ethanol coming from a very standard yeast-type pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) reaction, with much higher activities of the latter, so that acetaldehyde (a cytotoxin) does not accumulate (536). The ethanol diffuses into the surrounding water. Carp (*Carassius carassius*) can also survive anaerobiosis via ethanol excretion but for periods of 140 days at 2°C (536). These ethanol-producing fish fermentations, while remarkable, do not entail the participation of mitochondria.

Anaerobes and Microaerophiles: Redox Balance with a Pinch of O_2

Several eukaryotes that lack complexes I to IV of membrane-associated electron transport in the inner mitochondrial membrane as well as the associated cytochromes and quinones can respire to a limited extent by using O_2 as the terminal acceptor, yielding H_2O as an important but very inconspicuous end product of energy metabolism. For example, oxygen-reducing NADH oxidases are present in the cytosol of *Entamoeba histolytica* (289), *Giardia lamblia* (278), *Trichomonas vaginalis* (281, 356, 489), and *Tritrichomonas foetus* (78, 345). Again, we designate eukaryotes that have no requirement for O_2 in their core energy metabolism "anaerobes," using the term anaerobic to indicate that the organisms in question do not require free O_2 for survival and multiplication. Furthermore, and importantly, the growth of anaerobes is typically inhibited by atmospheric O_2 concentrations, which are 21% (vol/vol) in air, or roughly 250 μM in water at 25°C, for which reason yeast does not qualify as an anaerobe. The basis for growth inhibition by O_2 in anaerobes is generally thought to involve the inhibition or inactivation of one or more oxygen-sensitive FeS-cluster-containing enzymes involved in core energy metabolism, such as pyruvate:ferredoxin oxidoreductase (79).

Many anaerobic protists can readily multiply in the presence of low O_2 concentrations, in the 3 to 30 μM range, corresponding to 1 to 10% of the present atmospheric levels. Some even grow slightly faster under low O_2 levels than in the complete absence of O_2 (285, 373). The basis for this slight increase is clear in some cases. Levels of O_2 in the 1 μM range elicit metabolic shifts toward the excretion of more highly oxidized end products of energy metabolism, and this in turn results in increased levels of ATP generation by the same substrate-level phosphorylation reactions that function in the absence of O_2 (286, 375). This circumstance is the basis for suggestions to designate oxygen-shunning protists "microaerophilic" instead of the more generic designation "anaerobic" (40, 285). Many protists inhabit environments where virtually no free O_2 is available, such as anaerobic sediments (38, 142, 473). In addition, numerous species, in particular among the ciliates, harbor methanogenic archaebacteria that live as endosymbionts within their hosts (132), whereby methanogens are among the strictest anaerobes known.

Thus, the terms microaerophilic and anaerobic can be equally applicable for many protists. Few eukaryotes studied in detail so far are truly strict anaerobes, in that most of them regularly encounter a bit of O_2 in their natural habitats. Accordingly, they have biochemical means for dealing with O_2 and can readily tolerate it in small amounts. For example, when a culture of *T. vaginalis* is grown in the laboratory, the culture medium does not need to be purged of O_2 at all. Rather, one simply adds a large inoculum of viable cells to the medium, and the *T. vaginalis* cells start by breaking down glucose to pyruvate but without multiplying. Trichomonads possess a cytosolic NADH oxidase, also called diaphorase, that transfers four electrons from glucose oxidation directly to O_2, yielding water (345): $2\ NADH + 2\ H^+ + O_2 \rightarrow 2\ NAD^+ + 2\ H_2O$.

The free energy available in this highly exergonic reaction is not conserved by the NADH oxidase (neither as a proton gradient nor as ATP), and the reaction proceeds until the medium is essentially free of O_2. Once the O_2 is consumed, the cells commence normal growth, channeling carbon flux into O_2-sensitive pathways, but in an environment that they have themselves made anaerobic in order for their full complement of enzymes to function.

Thus, O_2 is both a toxin and a minor alternative acceptor for achieving redox balance in many anaerobic protists, and NADH oxidases of the type possessed by *Trichomonas* (402) are very widespread. The enzyme from *Trichomonas* (281, 489) and that from *Giardia* (58, 364) have been characterized. Homologs of the NADH oxidase genes reported for *Giardia* and *Entamoeba* (364) are common among eukaryotic genomes. *Trichomonas* possesses two diaphorases in the cytosol; the NADH-dependent enzyme yields H_2O only, whereas the NADPH-dependent enzyme in addition yields H_2O_2 (81). *Giardia* possesses a cytosolic NADH oxidase and a membrane-associated NADH peroxidase (59). Based on their biochemical properties, the eukaryotic enzymes are similar to the prokaryotic NADH oxidases and NADH peroxidases, which produce H_2O_2 instead of water (157).

In addition to NADH oxidases, eukaryote anaerobes can possess flavodiiron proteins that function as O_2 scavengers, as recently characterized for *Trichomonas* hydrogenosomes (463) and for *Giardia* (112). These enzymes have close homologs encoded by several sequenced eukaryote genomes, including *Entamoeba* and several green algae. One might wonder why green algae, which are typically O_2 producers, should possess O_2-scavenging enzymes typical of anaerobes. The answer is probably one of successful generalist strategies. Some algae, such as *Chlamydomonas reinhardtii*, can switch from O_2 production to vigorous anaerobic growth in the dark within 30 min, producing large amounts of H_2 using very O_2 sensitive enzymes for fermentative ATP synthesis

(331, 357); accordingly, O_2 detoxification is an issue for *Chlamydomonas* and similar algae during anaerobic growth. Since *Chlamydomonas* is a typical soil inhabitant (326), it can regularly encounter anaerobic conditions.

METABOLIC PATHWAYS IN EUKARYOTIC ANAEROBES

In eukaryotic heterotrophs, glycolysis—the Embden-Meyerhof pathway—is the backbone of carbon and energy metabolism. During glycolysis, 1 mol glucose is oxidized to 2 mol pyruvate, with a net yield of 2 mol ATP and producing 2 mol NADH. In eukaryotes that use O_2 as the terminal electron acceptor in their mitochondria, pyruvate is further oxidized in the mitochondria through the pyruvate dehydrogenase (PDH) complex, the Krebs cycle, and O_2 respiration to yield CO_2 and water, with the synthesis of roughly an additional 25 mol ATP per mol glucose. Eukaryotes that are specialized to aerobic environments, such as land plants and land vertebrates, typically possess in addition simple cytosolic fermentations to endure short-term anaerobic functioning, resulting in end products such as lactate, via lactate dehydrogenase (LDH), or ethanol, via pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (284, 387).

Another cytosolic fermentation variant is found in several trichomonad and yeast species, which increases their glycerol production upon anoxia (469, 517). In this case, the glycolytic intermediate dihydroxyacetone phosphate (DHAP) is converted to glycerol-3-phosphate by a glycerol-3-phosphate dehydrogenase that is NADPH dependent in trichomonads (469) and NADH dependent in yeast (517) and is subsequently converted to glycerol by glycerol-3-phosphatase. Glycerol production from glucose results in a net consumption of NAD(P)H and is essential in respiratory-incompetent yeast cells (517).

In eukaryotes that do not use O_2 as the terminal acceptor, a modest diversity is known among energy metabolic pathways, which consist of components that overlap in one way or another and which are summarized in the following sections, with three main caveats mentioned here. One is core pathways. In the text and the metabolic maps, we have focused on major metabolic pathways and major end products. In many cases, there are additional minor end products, sometimes accumulated only under specific conditions. In most cases, the minor end products are not mentioned here, but in some cases they are, with H_2 production in *Giardia* being an example (287). Another caveat is strains. For the same species, there are numerous strain-specific differences with regard to the spectrum of end products detected, underscored, for example, by *Trichomonas vaginalis* (403), *Euglena gracilis* (514), and *Chlamydomonas reinhardtii* (331). A third caveat is stage-specific differences. Many of the organisms covered in this review have a life cycle in which pronounced stage-specific differences in energy metabolism exist, for example, juvenile versus adult forms of parasitic metazoans (498) or bloodstream versus insect stages of *Trypanosoma* (54, 532).

We present these pathways following a phylogenetic approach, based upon current views of eukaryote relationships, as comprising roughly six supergroups, whose exact membership and relationships to one another remain discussed (4, 180, 237). This phylogenetic framework is shown in Fig. 3, along with the kinds of mitochondria that have been found in some of the biochemically characterized anaerobes.

Animals

Animals belong to the eukaryotic supergroup currently called Opisthokonta, which includes the animals and the fungi (180). The metazoans harbor many forms that are adapted to life on land, above the soil line, where oxygen is always available, yet many invertebrates can also survive without oxygen for at least part of their life cycle, and some vertebrates, for example, carp, survive complete anoxia for months via ethanol fermentation (536). More recently, some animals were reported to complete their entire life cycle in sediments below deep-sea hypersaline haloclines (99), where there is no oxygen at all, and the mitochondria of these species of the animal phylum Loricifera appear to lack cristae, but the function of these organelles is as yet unknown. Among freshwater, marine, and parasitic invertebrates, functional hypoxia and environmental hypoxia are common. All adult parasitic worms studied so far have a fermentative metabolism and do not use oxygen to completely oxidize glucose to carbon dioxide. The anaerobic metabolism of some of these animals has been studied in detail, and representative examples are discussed in subsequent sections.

A *leitmotiv* common to anaerobic energy metabolism in animals surveyed here is that the anaerobically functioning animal mitochondria do not reveal any fundamental differences in their enzymatic repertoires relative to those of aerobically specialized lineages. Hence, in the evolutionary sense, there are no differences between aerobic and anaerobic animal lineages with respect to genes and enzymes for which to account. A caveat is that the presence of rhodoquinone seems to be restricted to organisms that can function anaerobically. In contrast, among plants, fungi, and protists, there are some notable differences among lineages with regard to the enzymes employed by anaerobes versus aerobes. However, as we discuss in the corresponding sections, these differences are minor, and so far, there are no enzymes involved in the major pathways of anaerobic energy metabolism that are truly specific to any one eukaryotic lineage; that is, enzymes used for anaerobic energy metabolism by one eukaryotic supergroup are also found in at least one other, and they are virtually all present in the green alga *Chlamydomonas*, indicating that—just as for oxidative phosphorylation—the differences reflect a presence in the eukaryote common ancestor and differential loss. This observation indicates that the evolutionary process of specialization to an anaerobic life-style in metazoans is distinct from the process in protists and leads to a less diverse spectrum of end products. In other words, among animals, the anaerobic species divert their core carbon flux through enzymes that are also possessed by the aerobes but in such a manner as to generate a different spectrum of end products, shunting and obviating the need for O_2-dependent terminal oxidation reactions.

***Fasciola hepatica* (liver fluke).** The adult liver fluke, *Fasciola hepatica*, is a parasitic flatworm that lives in the bile ducts of its host, mainly cattle and sheep, but humans can also become infected. In the free-living and early larval stages of the animal's life cycle, mitochondria of *F. hepatica* use oxygen in a conventional way, and these stages degrade glucose completely to CO_2 via the Krebs cycle. Upon the penetration of the host and the development of the parasite in the liver and, subsequently, the bile ducts, this fully aerobic metabolism is gradually replaced by an anaerobic partial oxidation of glucose, which nevertheless still involves mitochondria (502). During this metabolic development, first the

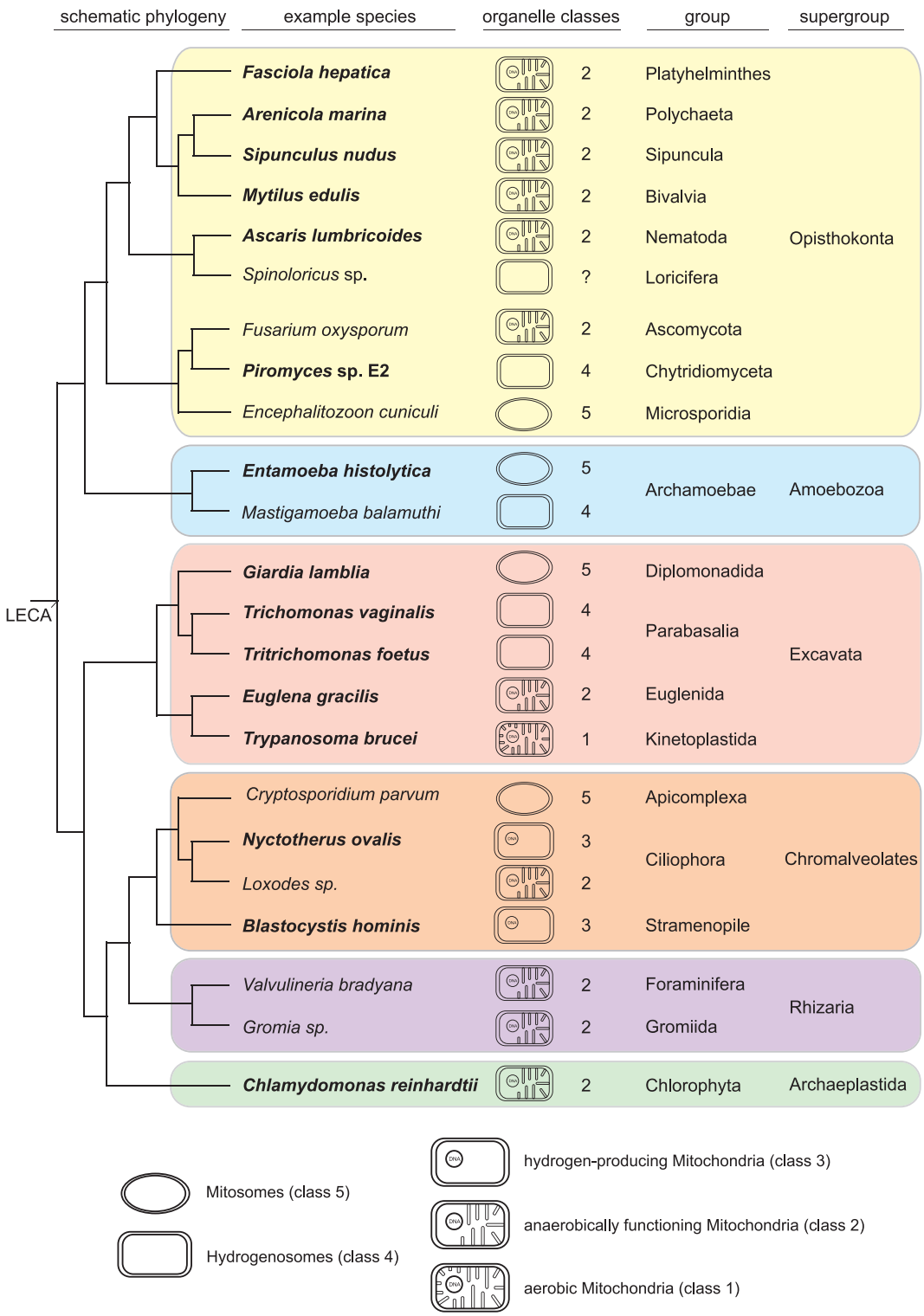


FIG 3 Phylogenetic distribution of organelles of mitochondrial origin. The distribution is plotted across a schematic phylogeny for the six currently recognized major clades or supergroups of eukaryotes (4, 180, 237). Species for which metabolic maps are presented in this review are shown in boldface type. The mitochondrion class for each example species is indicated, and numbers in the column "organelle classes" correspond to the scheme described in the legend of Fig. 2. The presence of a genome is indicated by encircled DNA. Sizes of individual mitochondrial types are not drawn to scale. LECA, last eukaryotic common ancestor. Note that functional information is absent for the loriciferan organelle. Note the absence of lineages lacking organelles of mitochondrial origin among eukaryotes.

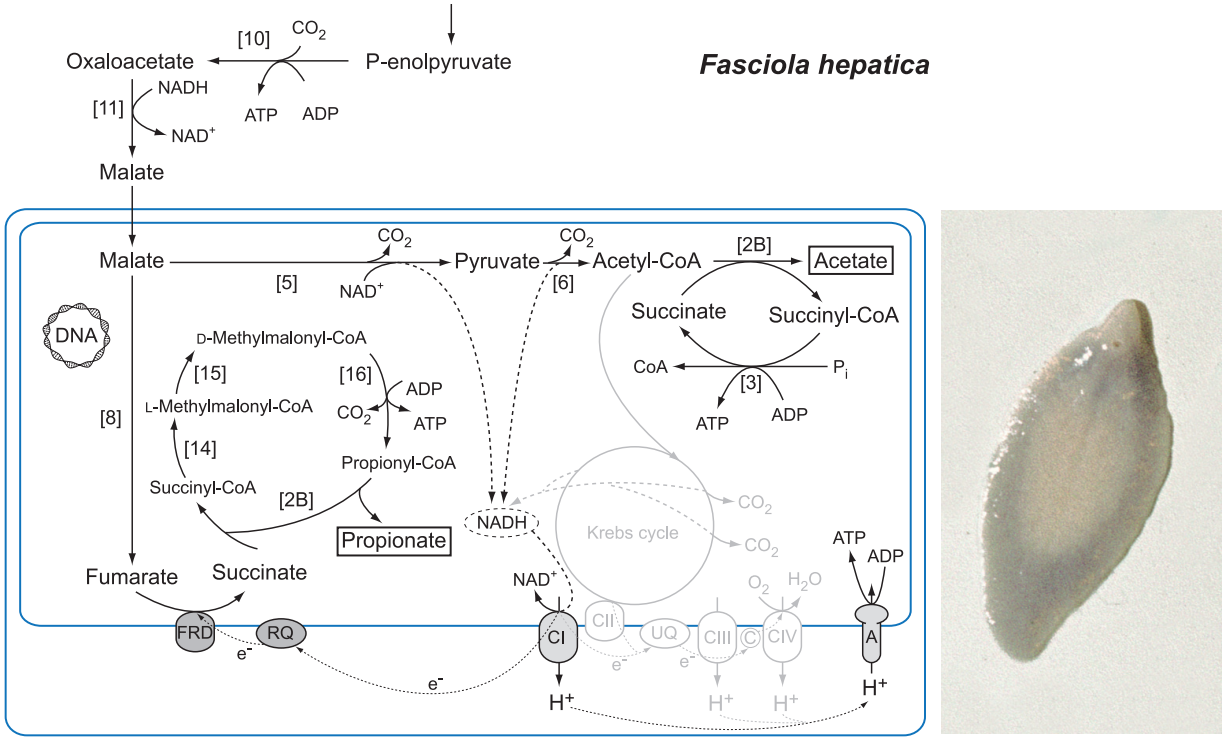


FIG 4 Major pathways of energy metabolism in anaerobic mitochondria of the adult parasitic platyhelminth *Fasciola hepatica* (common liver fluke). The map is redrawn based on data described previously (500). The main end products of the adult parasites are acetate and propionate, with minor amounts of lactate and succinate. In the presence of oxygen, this metabolism of the adult helminth remains unchanged. Aerobic mitochondrial metabolism, occurring in free-living and juvenile parasitic stages of *F. hepatica* in the mammalian host and leading to the reduction of oxygen and water production, is shaded in gray (and in all following metabolic figures as well) to distinguish it from anaerobic respiration and fermentation pathways, drawn in black. Abbreviations: CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; RQ, rhodoquinone; C, cytochrome *c*; A, ATPase; FRD, fumarate reductase; [2B], acetate:succinate CoA-transferase (subfamily 1B); [3], succinyl-CoA synthetase; [5], malic enzyme; [6], pyruvate dehydrogenase complex; [8], fumarase; [10], phosphoenolpyruvate carboxykinase (ATP dependent); [11], malate dehydrogenase; [14], methylmalonyl-CoA mutase; [15], methylmalonyl-CoA epimerase; [16], propionyl-CoA carboxylase. The photograph shows *Fasciola hepatica* at the adult stage, with a length of ca. 2.5 cm. (Photograph by Louk Herber, Utrecht University, Netherlands.)

aerobic production of acetate becomes the major pathway for the production of ATP by *F. hepatica* during its migration period in the liver parenchyma. Later on, in the bile ducts, the anaerobic production of acetate and propionate becomes the most important source of ATP (499). These two end products are formed in the anaerobically functioning mitochondria via a split pathway called malate dismutation, a fermentative route for the degradation of carbohydrates that is common to most parasitic helminths. In all life cycle stages of this parasite, the classical metazoan fermentation product, lactate, is only a minor end product.

In the anaerobic energy metabolism of *Fasciola* adults (Fig. 4), phosphoenolpyruvate (PEP) from glycolysis is converted to oxaloacetate via an ATP-linked PEP carboxykinase (ATP-PEPCK), which is subsequently reduced by cytosolic malate dehydrogenase (MDH), reoxidizing the glycolytic NADH. This malate is imported into the mitochondrion, where it is degraded via malate dismutation. A portion of the malate is oxidized to acetate, and another portion is reduced to succinate, which is then further metabolized to propionate. This way, malate provides a source of electrons (NADH) for the electron transport chain as well as a sink for these electrons, and when acetate and propionate are produced in a ratio of 1:2, the process is in redox balance.

In the oxidative, NADH-generating branch (Fig. 4), a portion of the L-malate is oxidized by an NAD-linked malic enzyme (NAD-ME) to pyruvate, which is further oxidized by the pyruvate dehydrogenase (PDH) complex to acetyl-CoA. An acetate:succinate CoA-transferase (ASCT) transfers the CoA moiety of acetyl-CoA to succinate, generating succinyl-CoA and acetate, a major end product of *Fasciola* adults. ASCTs were only recently characterized for eukaryotes, and it was shown that these enzymes are family I-type CoA-transferases, where ASCT is only one member of a multitude of different acetate-producing enzymes in eukaryotes (reviewed in reference 503). The ASCT in anaerobically functioning *Fasciola* mitochondria is closely related to the CoA-transferases of bacteria such as *Roseburia* and to that of *Artemia franciscana*, a brine shrimp, and these enzymes all belong to subfamily 1B of the CoA-transferases (503, 528). Succinyl-CoA synthetase (SCS) (also called succinate thiokinase and abbreviated STK in many papers), a canonical Krebs cycle enzyme (93), conserves the energy in the thioester bond of succinyl-CoA by ATP synthesis from succinyl-CoA, ADP, and P_i via substrate-level phosphorylation involving succinyl phosphate as a reaction mechanism intermediate (197), regenerating CoA for the PDH reaction and succinate for the ASCT reaction. One mole acetate, 2 mol CO_2, and 2 mol NADH are produced per mol malate oxidized via this route.

The above-described production of acetate by the oxidation of part of the malate, which results in the formation of NADH, is balanced by the reduction of another part of the malate to succinate, via two reactions that reverse part of the Krebs cycle. First,

malate is converted to fumarate via the enzyme fumarase running in reverse, and the fumarate produced then serves as the terminal electron acceptor for the electrons from the oxidative branch of malate dismutation and is reduced to succinate. This fumarate reduction is coupled to an anaerobically functioning electron transport chain in which electrons are transferred from NADH to fumarate via complex I, rhodoquinone (RQ), and a membrane-associated fumarate reductase (FRD) (Fig. 4). Complex I is similar to complex I in aerobic mitochondria, but RQ and FRD are specific for anaerobic mitochondria. The use of RQ instead of ubiquinone (UQ), the quinone used by aerobically functioning mitochondria, is essential. RQ is important because it has a lower standard redox potential than UQ, which is the reason why reduced RQ can be used by FRD to donate electrons to fumarate, producing succinate, a reaction where UQ would not readily work (530).

The membrane-associated FRD found in *Fasciola* resembles structurally, and is related to, complex II (succinate dehydrogenase [SDH]) (501) but is distinct from the soluble NADH-dependent FRD found in several protists, including trichomonads and trypanosomes, that catalyzes the NADH-dependent reduction of fumarate to succinate (90, 354).

The succinate produced by FRD is a major excreted end product in many organisms that perform malate dismutation. In *Fasciola*, however, this succinate is decarboxylated to propionate, and one additional ATP is gained (383) (Fig. 4). The CoA moiety from propionyl-CoA is transferred to succinate by virtue of a dual substrate specificity of ASCT (528), generating propionate as a metabolic end product and succinyl-CoA. A cycle regenerates propionyl-CoA, involving the vitamin B_{12}-dependent enzyme methylmalonyl-CoA mutase, methylmalonyl-CoA epimerase, and propionyl-CoA carboxylase. The propionyl-CoA carboxylase reaction generates propionyl-CoA and produces ATP via substrate-level phosphorylation (Fig. 4).

During this malate dismutation, protons are pumped by complex I, and the resulting proton gradient is harnessed by the mitochondrial ATP synthase, i.e., oxidative phosphorylation via pumping at complex I alone and without O_2 as the terminal acceptor (67a, 248a, 435a). Overall, this anaerobic energy metabolism in *Fasciola* generates propionate and acetate (in addition to CO_2) as major end products, in a ratio 2:1, respectively. The process yields roughly 5 ATPs per glucose: ATP is formed via substrate-level phosphorylation during glycolysis in the cytosol (2 ATPs per glucose) and inside the mitochondria by the formation of acetate (1 ATP) as well as propionate (1 ATP), and next to that, ATP is formed via proton pumping at complex I and the mitochondrial ATP synthase (~1 ATP per glucose) but in the absence of oxygen.

***Ascaris* (giant roundworm).** The parasitic nematodes *Ascaris lumbricoides* and *Ascaris suum* inhabit the small intestine of their respective hosts, humans and pig, and can grow up to 25 cm in length (males) or 40 cm (females). They feed on the food present in the gut and synthesize glycogen during periods of host feeding. The resulting glycogen storage serves as an energy reserve during host fasting. The adult parasite exhibits anaerobic energy metabolism, and like most other parasitic helminths, *Ascaris* performs malate dismutation, including fumarate reduction by RQ, to maintain redox balance [see "*Fasciola hepatica* (liver fluke)"], producing acetate, succinate, and propionate (Fig. 5). However, *Ascaris* is unusual, as acetyl-CoA and propionyl-CoA are further condensed and reduced to the branched-chain fatty acids 2-methylbutanoate and 2-methylpentanoate through a pathway similar to the reversal of β-oxidation.

The result is that *Ascaris* excretes a complex mixture of acetate, succinate, propionate, 2-methylbutanoate, and 2-methylpentanoate as end products (251). Methylbutanoate is formed by the condensation of acetyl-CoA with propionyl-CoA, and methylpentanoate is formed by the condensation of two propionyl-CoA moieties, followed by the subsequent reduction of the condensation products (Fig. 5). The pathway corresponds in some respects to the reversal of β-oxidation found in mammalian mitochondria, yet the *Ascaris* enzymes function physiologically in the direction of acyl-CoA synthesis and not oxidation. The final reaction in this pathway, the NADH-dependent reduction of 2-methyl branched-chain enoyl-CoA, requires complex I, RQ, electron transport flavoprotein (ETF) oxidoreductase, and two soluble components, ETF and 2-methyl branched-chain enoyl-CoA reductase. ETF:ubiquinone oxidoreductase (ETF-UO) is a membrane-bound, iron-sulfur flavoprotein that plays an important role in the β-oxidation of fatty acids in mammalian mitochondria by shuttling reducing equivalents from a soluble ETF to ubiquinone. In adult *A. suum* mitochondria, ETF:rhodoquinone oxidoreductase (ETF-RO) functions in the opposite direction: rotenone-sensitive, proton-pumping NADH oxidation at complex I reduces soluble ETF, which is subsequently reoxidized by a 2-methyl branched-chain enoyl-CoA reductase, which generates acyl-CoA, and a CoA transferase releases the fatty acid (249, 250, 298). This branched-chain fatty acid formation provides an additional route for the oxidation of NADH in *Ascaris* and thus functions as an electron sink when fumarate reduction is limited. It was shown that UQ cannot replace RQ in this NADH-dependent ETF-RO (or in the FRD reaction) (298). Ultimately, the terminal electron acceptor of glucose oxidation is acetyl-CoA, a principle that is revisited in the section on *Euglena* below.

***Mytilus edulis* (common mussel).** The common or blue mussel, *Mytilus edulis*, inhabits the intertidal zone and undergoes environmentally induced hypoxia during low tide, when the animal closes its valves to avoid desiccation and thereby switches to an anaerobic energy metabolism (Fig. 6). It was one of the first animal species whose anaerobic energy metabolism was investigated in detail (109). Although it is known that different tissues in *M. edulis* respond differently to hypoxia, some generalizations can be made (107). The response to hypoxia involves the accumulation of opines. Opine formation is a cytosolic fermentation pathway in which pyruvate is condensed with an amino acid by a specific dehydrogenase that reduces the Schiff base with glycolytic NADH, resulting in an iminoacid derivative, an opine (176), as the end product of energy metabolism. The condensation of pyruvate and arginine by octopine dehydrogenase results in the formation of octopine as the reduced end product, whereas condensation with alanine or glycine results in the formation of alanopine and strombine, respectively (143, 169).

These opine pathways are present in diverse marine phyla, such as the Cnidaria, Mollusca, and Annelida (107, 145, 169, 283, 445). ATP generation by these pathways is equivalent to that of lactate formation, with 2 mol ATP per mol glucose degraded. The suggested advantage of the opine pathways is that the anaerobic opine end products are less acidic than lactate, and, because 1 amino acid is consumed for each opine synthesized, these end products are produced in an osmotically neutral process that reduces the os-

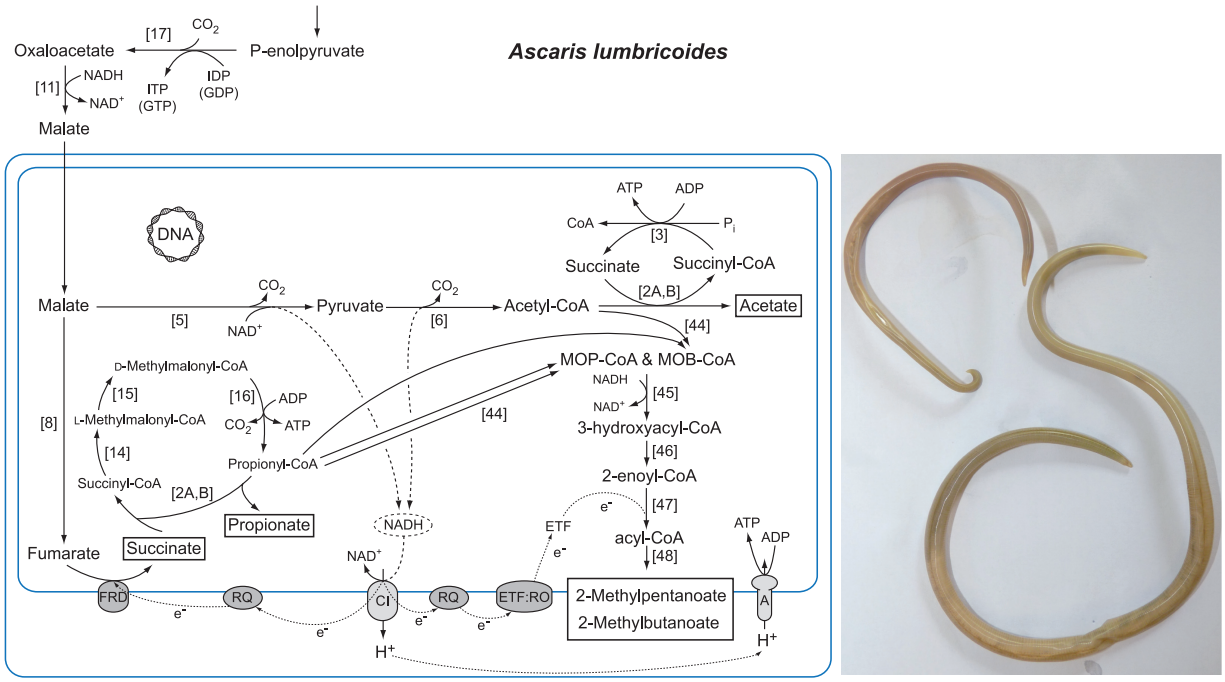


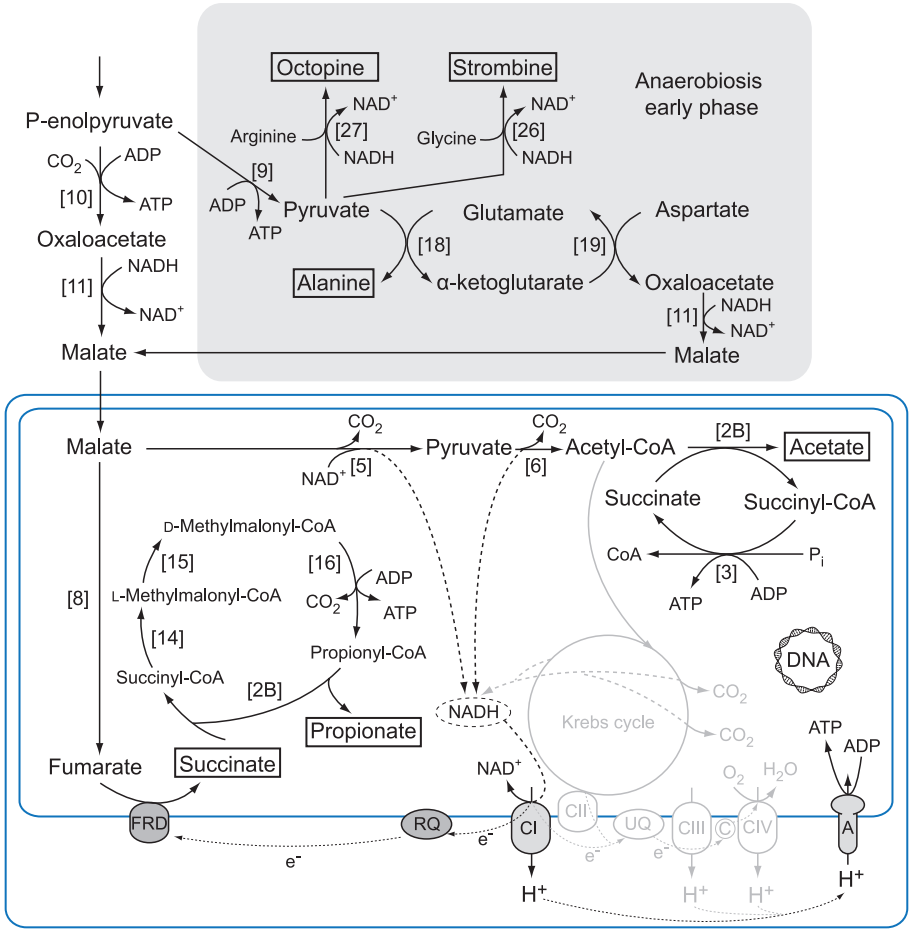
FIG 5 Major metabolic pathways and electron transport chain in mitochondria of the adult nematode *Ascaris lumbricoides* (giant roundworm). The map is redrawn based on data reported previously (249). Mature forms of this animal, a large and very common parasitic worm, inhabit the small intestine of their hosts. Like most parasitic worms, *Ascaris* performs malate dismutation to obtain redox balance and uses rhodoquinone to donate electrons for fumarate reduction (434). However, *Ascaris* is unusual, as acetyl-CoA and propionyl-CoA are used for the production of two branched-chain fatty acids, 2-methylbutanoate and 2-methylpentanoate, which are formed by the condensation of an acetyl-CoA and a propionyl-CoA or two propionyl-CoAs, respectively, with the subsequent reduction of the condensation products (436, 437). These short branched-chain fatty acids are typical end products for *Ascaris* (249, 251, 298). Abbreviations: MOP-CoA, 2-methyl-3-oxo-pentanoyl-CoA; MOB-CoA, 2-methyl-3-oxo-butanoyl-CoA; CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; RQ, rhodoquinone; C, cytochrome *c*; A, ATPase; FRD, fumarate reductase; [2A, B], acetate:succinate CoA-transferase (subfamilies A and B); [3], succinyl-CoA synthetase; [5], malic enzyme; [6], pyruvate dehydrogenase complex; [8], fumarase; [11], malate dehydrogenase; [14], methylmalonyl-CoA mutase; [15], methylmalonyl-CoA epimerase; [16], propionyl-CoA carboxylase; [17], phosphoenolpyruvate carboxykinase (ITP/GTP dependent); [44], condensing enzyme; [45], 2-methyl acetoacyl-CoA reductase; [46], hydratase; [47], 2-methyl branched-chain enoyl-CoA reductase; [48], acyl-CoA transferase. The photograph shows *Ascaris lumbricoides* worms at the adult stage, with lengths of ca. 15 cm (top, male) and 30 cm (bottom, female). (Photograph by Rob Koelewijn, Harbor Hospital, Rotterdam, Netherlands.)

motic stress associated with the accumulation of lactate (24). The enormous amino acid pools maintained by mollusks for osmotic balance are the source of amino acids for this process.

During the early phase of hypoxia in *Mytilus*, redox balance is maintained through this production of opines from pyruvate. However, the opines are not excreted as end products. They remain in tissues of the animals, for example, in the body wall tissue, and upon a return to aerobic conditions, the opines are reoxidized (107, 169). This is regarded as a general strategy employed by animals that accumulate opines (107, 169). The temporary accumulation and reoxidation, rather than the excretion, of end products are also encountered during lactate fermentations in humans (194) and during wax ester fermentation in *Euglena* (62, 514). In addition to opines, large amounts of alanine and succinate are produced. These products are the result of the coupled formation of alanine from glycogen and succinate from aspartate (Fig. 6) (107). This succinate is produced via the part of the Krebs cycle between oxaloacetate and succinate running in reverse, which, in fact, is the reductive branch of malate dismutation, as discussed above in the section on *Fasciola*. In this way, aspartate transamination and its further conversion to succinate are coupled to glycogen fermentation and the production of alanine. This degradation of aspartate and the concomitant production of alanine are characteristic features of many anoxia-tolerant marine invertebrates (169). These marine invertebrates have a large pool of free amino acids and possess large glycogen reserves that can constitute up to 10 to 35% of the animal's dry weight.

Under conditions of prolonged anaerobiosis, a transition to complete malate dismutation sets in, and the typical end products (acetate, propionate, and succinate) accumulate in *M. edulis* (107). The role of fumarate reductase in *Mytilus* has been studied (107), and the presence of rhodoquinone, essential for the reductive branch of malate dismutation, has been shown for *Mytilus* (531).

***Arenicola marina* (lugworm).** Another very well studied marine invertebrate with tidal cycles of anaerobiosis is the polychaete lugworm *Arenicola marina* (572). It burrows into the sand of intertidal zones; at low tide, it is exposed to extreme hypoxia and to high concentrations of sulfide (170). The overall pattern of anaerobic energy metabolism characterized for *Arenicola* is the same as that found for *Mytilus*: opine production in early anaerobiosis, with the degradation of aspartate and the concomitant production of alanine, and malate dismutation during prolonged anaerobiosis, with the typical spectrum of end products—acetate, propionate, and succinate—accumulating (Fig. 7), whereby the presence of RQ has been shown (531). During the early anaerobic phase, the pyruvate formed via the Embden-Meyerhof pathway



Mytilus edulis

FIG 6 Major pathways of facultative anaerobic energy metabolism in mitochondria of the free-living mollusk *Mytilus edulis* (blue mussel). The map is redrawn based on data reported previously (107). Living attached to hard substrates, for example, rocks in intertidal habitats, the bivalve has to face anaerobiosis periodically. Oxygen-independent cytosolic energy metabolism produces ATP via substrate-level phosphorylation accompanied by the formation of various end products, including alanopine, strombine, and alanine (11). Under conditions of prolonged anaerobiosis, propionate is preferentially formed instead of succinate in mitochondria. Fumarate reduction is electron transfer chain coupled, and rhodoquinone serves as an electron donor to fumarate reductase, as in other anaerobic mitochondria (107, 531). The light gray rectangle highlights metabolic pathways preferentially employed during the early phase of anaerobiosis. The gray shading of lines is described in the legend of Fig. 4. Abbreviations: CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; RQ, rhodoquinone; C, cytochrome *c*; A, ATPase; FRD, fumarate reductase; [2B], acetate:succinate CoA-transferase (subfamily 1B); [3], succinyl-CoA synthetase; [5], malic enzyme; [6], pyruvate dehydrogenase complex; [8], fumarase; [9], pyruvate kinase; [10], phosphoenolpyruvate carboxykinase (ATP dependent); [11], malate dehydrogenase; [14], methylmalonyl-CoA mutase; [15], methylmalonyl-CoA epimerase; [16], propionyl-CoA carboxylase; [18], alanine aminotransferase; [19], aspartate aminotransferase; [26], strombine dehydrogenase; [27], octopine dehydrogenase. The photograph shows *Mytilus edulis* at the adult stage, with a length of ca. 6 cm. (Photograph by Louk Herber, Utrecht University, Netherlands.)

can be converted to strombine and to alanine. In the case of *Arenicola*, however, the L-alanine formed can be converted to D-alanine by alanine racemase (445).

In contrast to *Mytilus*, *Arenicola* burrows into the sulfidic sediment (solfatara) and is therefore regularly exposed to high sulfide concentrations. Sulfide inhibits cytochrome *c* oxidase (170), for which reason sulfide is toxic during aerobic growth. *Arenicola* deals with this sulfide with the help of sulfide:quinone oxidoreductase (SQR), which oxidizes sulfide and donates the electrons to quinones in the mitochondrial electron transport chain (496). Mitochondrial SQR oxidizes sulfide to an enzyme-bound persulfide (495). The final product of mitochondrial sulfide oxidation in *Arenicola* is thiosulfate, the production of which appears to require oxygen via a sulfur dioxygenase and the sulfur-transferase activity of mitochondrial rhodanese (189). The sulfur dioxygenase homolog in humans was recently characterized and is directly involved in mitochondrial sulfur metabolism, and null mutants induce severe disease phenotypes (505). Long considered a toxin, sulfide has recently been recognized as a signaling molecule and a mediator of cardiovascular function in mammals (45, 231).

Although homologous SQR genes are very widespread in animals and fungi (496), still comparatively little is known about mitochondrial sulfide metabolism. In the ribbed mussel *Geukensia*, sulfide oxidation can drive ATP synthesis (116). Recent work on another inhabitant of marine sediments, the annelid *Urechis unicinctus*, revealed that it possesses the same SQR enzyme and that its mitochondria, especially mitochondria from the hindgut, can also synthesize ATP with the help of protons pumped by using electrons stemming from sulfide (299).

***Sipunculus nudus* (peanut worm).** The free-living peanut marine worm, *Sipunculus nudus*, is an annelid (358) that lives in

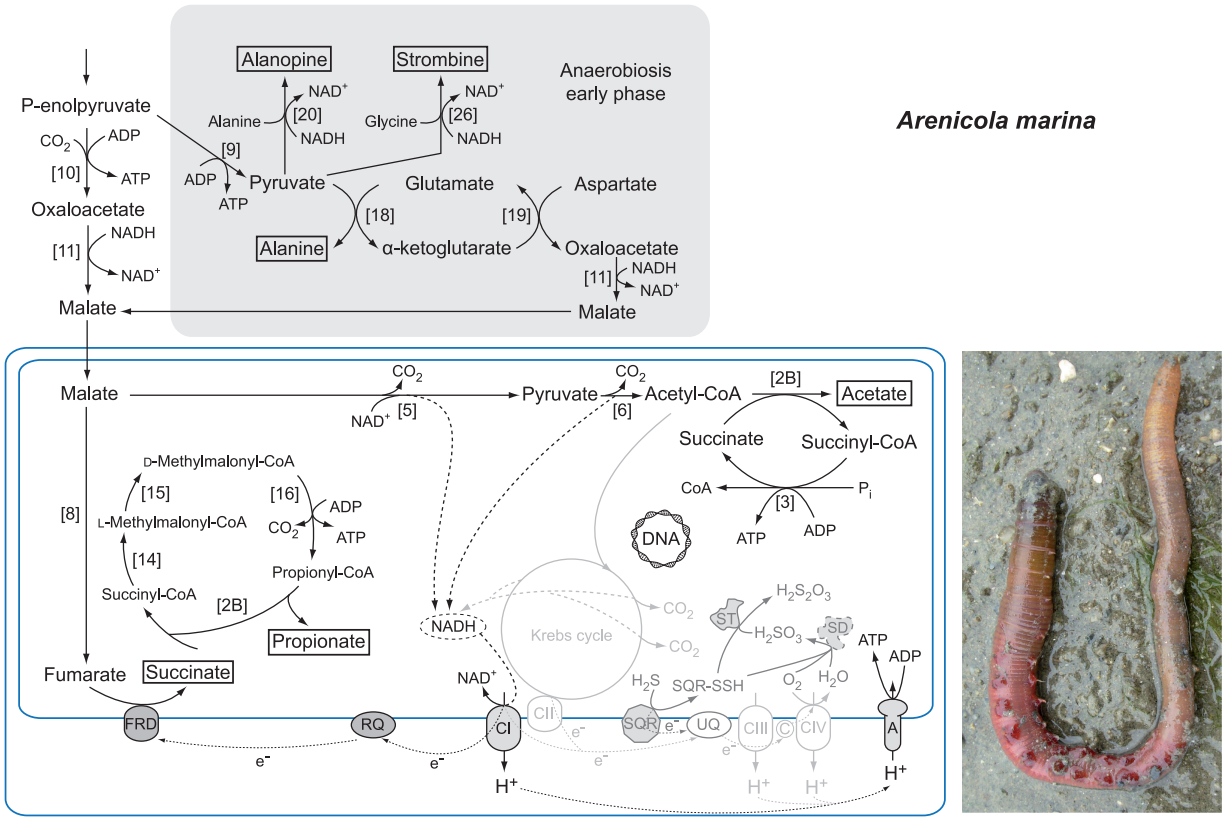


FIG 7 Major pathways of energy metabolism in mitochondria of the free-living annelid polychaete *Arenicola marina* (lugworm or sandworm). The worm inhabits intertidal sediments and produces ATP predominantly by an aerobic respiratory chain during high tide. At low tide, it is exposed for several hours to hypoxia (445) and also has to deal with high concentrations of H_2S (up to 2 mM), which is a potent inhibitor of respiratory complex IV (495, 496). *A. marina* protects itself intrinsically by means of membrane-bound flavoprotein sulfide:quinone oxidoreductase, which oxidizes hydrogen sulfide and transfers the electrons to the quinone pool. The generation of the final oxidized sulfur species, thiosulfate, can occur in an oxygen-dependent manner in cooperation with a sulfur dioxygenase and a sulfur transferase (189). The animal can also reduce fumarate in anaerobic respiration by utilizing rhodoquinone (531) and can produce acetate and ATP via substrate-level phosphorylation. The gray shading of lines is described in the legend of Fig. 4; the light gray rectangle highlights metabolic pathways preferentially employed during the early phase, as described in the legend of Fig. 6. Abbreviations: CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; RQ, rhodoquinone; C, cytochrome *c*; A, ATPase; FRD, fumarate reductase; SQR, sulfide:quinone oxidoreductase; ST, sulfur transferase; SD, sulfur dioxygenase; [2B], acetate:succinate CoA-transferase (subfamily 1B); [3], succinyl-CoA synthetase; [5], malic enzyme; [6], pyruvate dehydrogenase complex; [8], fumarase; [9], pyruvate kinase; [10], phosphoenolpyruvate carboxykinase (ATP dependent); [11], malate dehydrogenase; [14], methylmalonyl-CoA mutase; [15], methylmalonyl-CoA epimerase; [16], propionyl-CoA carboxylase; [18], alanine aminotransferase; [19], aspartate aminotransferase; [20], alanopine dehydrogenase; [26], strombine dehydrogenase. The photograph shows *Arenicola marina* at the adult stage, with a length of ca. 20 cm. (Photograph by Auguste Le Roux [http://commons.wikimedia.org/wiki/File:Arenicola_marina.JPG] [Wikimedia Commons].)

sandy sediments ranging from intertidal to subtidal zones down to a 900-m depth. It often experiences hypoxia and encounters environmental sulfide, although *Sipunculus* typically encounters lower sulfide concentrations (~13 μM) than those encountered by *Arenicola* (~340 μM) (543). *Sipunculus* deals with the scarcity of oxygen by means of the same pathways discussed for other marine invertebrates when facing hypoxia and anoxia: the production of opines and alanine in the cytosol and the production of succinate, acetate, and propionate in the mitochondria upon encountering sustained anoxic conditions (169), as shown in Fig. 8. Under a wide range of hypoxic conditions (oxygen levels below the ambient atmospheric level of 159 torr, corresponding to roughly 250 μM in solution), the metabolic rate (oxygen consumption) of *Sipunculus* declines linearly with decreasing oxygen partial pressure (391). Below levels corresponding to about 40 μM, severe hypoxia sets in, the metabolic rate declines more rapidly, and the animals begin to accumulate succinate as evidence of the onset of anaerobic energy metabolism. The point at which anaerobic energy metabolism sets in corresponds to the oxygen tension at which the respiratory oxygen demand exceeds the environmental oxygen supply; this was defined as the critical oxygen partial pressure (391).

Like most facultatively anaerobic metazoans, *Sipunculus* does not show a Pasteur effect (440); that is, glycolytic flux in *Sipunculus* is not increased under hypoxic conditions (181). Instead, hypoxia reduces glycolytic flux and alters the spectrum of end products produced. The switch from the production of primarily opines and alanine under conditions of transient hypoxia to primarily succinate, acetate, and propionate under conditions of sustained hypoxia appears to involve enzymatic regulation at the level of phosphoenolpyruvate carboxykinase (PEPCK) (169). The role of intracellular pH changes as a possible regulatory mechanism for propionyl-CoA carboxylase (185) and for PEPCK (169) has been discussed. In *Sipunculus*, exposure to cold temperatures (0°C to 4°C) can also induce the accumulation of acetate, succinate, and propionate along with opines (576). Hypoxia and high CO_2 levels can furthermore induce acidosis in the animals, with an associated downregulation of metabolism (390).

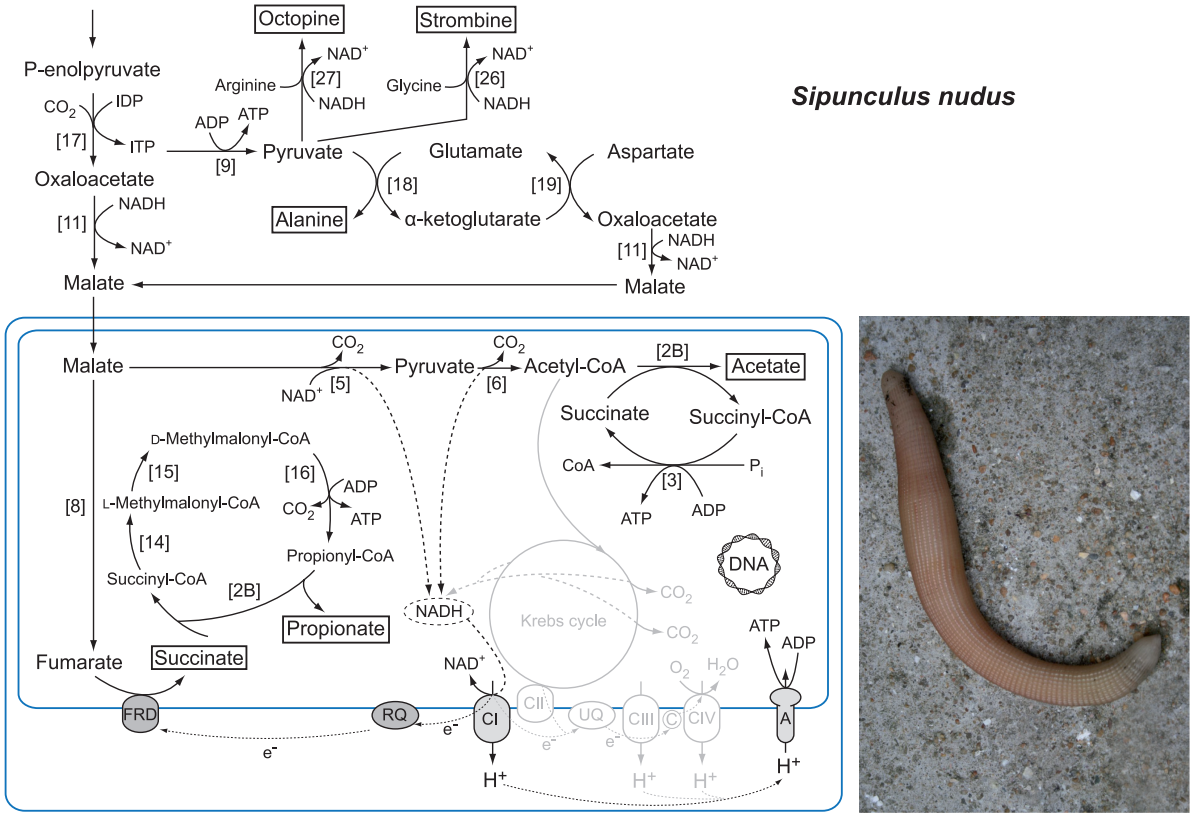


FIG 8 Major pathways of energy metabolism in mitochondria of the peanut worm, *Sipunculus nudus*. The map is redrawn based on data reported previously (169). This free-living worm inhabits sandy marine sediments ranging from intertidal to subtidal zones down to a 900-m depth, where it often encounters hypoxia. *S. nudus* deals with low oxygen by using the same pathways as those found in other marine invertebrates facing hypoxic or anoxic conditions. Fumarate serves as the terminal electron acceptor in the anaerobic branch of the respiratory chain and enables the synthesis of ATP not only via oxidative phosphorylation but also through propionate production from succinate. Additional ATP can be generated from succinyl-CoA in the pyruvate degradation pathway, leading to acetate as an end product (169). The gray shading of lines is described in the legend of Fig. 4. Abbreviations: CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; RQ, rhodoquinone; C, cytochrome *c*; A, ATPase; FRD, fumarate reductase; [2B], acetate:succinate CoA-transferase (subfamily 1B); [3], succinyl-CoA thiokinase; [5], malic enzyme; [6], pyruvate dehydrogenase complex; [8], fumarase; [9], pyruvate kinase; [11], malate dehydrogenase; [14], methylmalonyl-CoA mutase; [15], methylmalonyl-CoA epimerase; [16], propionyl-CoA carboxylase; [17], phosphoenolpyruvate carboxykinase (ITP/GTP dependent); [18], glutamate:pyruvate transaminase; [19], glutamate:oxaloacetate transaminase; [26], strombine dehydrogenase; [27], octopine dehydrogenase. The photograph shows *Sipunculus nudus* at the adult stage, with a length of ca. 15 cm. (Photograph courtesy of Matt du Fort.)

A strictly anoxic animal among the loriciferans. All animals with anaerobic metabolism discussed above do encounter oxygen permanently or from time to time. Explorations of permanently anoxic environments revealed recently that these habitats are not entirely prohibitive to life of multicellular animals. Three so-far-unnamed species (*Spinoloricus* sp., *Rugiloricus* sp., and *Pliciloricus* sp.) of the phylum Loricata, a group of small marine worms, were recently recovered from a deep basin at the bottom of the Mediterranean Sea (99), where they inhabit a nearly salt-saturated brine that, because of its density (>1.2 g/cm^3), does not mix with the waters above. As a consequence, this environment is completely anoxic and has been so for 50,000 years (99). Due to the activity of sulfate reducers, the brine contains sulfide at a concentration of 2.9 mM. That paper provided biological evidence that these organisms indeed live in this environment. While no information on their biochemistry is as yet available, electron microscopy revealed that their cells contain organelles of a mitochondrial size that have no cristae. It will be of enormous significance to learn about their energy metabolism and the nature of their mitochondria. It should be noted that environmental sequencing studies indicated that despite such harsh conditions, this anoxic and sulfidic environment is teeming with other eukaryotic microbes (9, 129). An understanding of the so-far-unexplored biology of these organisms might provide a glimpse of what much of Earth's ecology might have been like in the far more distant past (324a) and might disclose novel combinations of known biochemical mechanisms or even unexpected metabolic pathways.

Fungi

The other major clade of the opisthokonts encompasses the fungi. In terms of energy metabolism, the fungi are a very diverse group, with many lineages possessing oxygen-respiring mitochondria, some possessing hydrogenosomes (142), some possessing mitosomes (558), some using elemental sulfur as a terminal acceptor (1), and some performing ammonia fermentation or nitrate respiration (573).

***Piromyces* sp. strain E2 and *Neocallimastix*.** *Piromyces* sp. strain E2 and *Neocallimastix frontalis* are chytridiomycetes that inhabit the rumen of mammalian herbivores (30). They are obligate anaerobes and possess hydrogenosomes (343, 568) and [Fe]-Hyd (544). Typical end products of carbohydrate breakdown in *Piromyces* are lactate, ethanol, formate, acetate, hydrogen, and

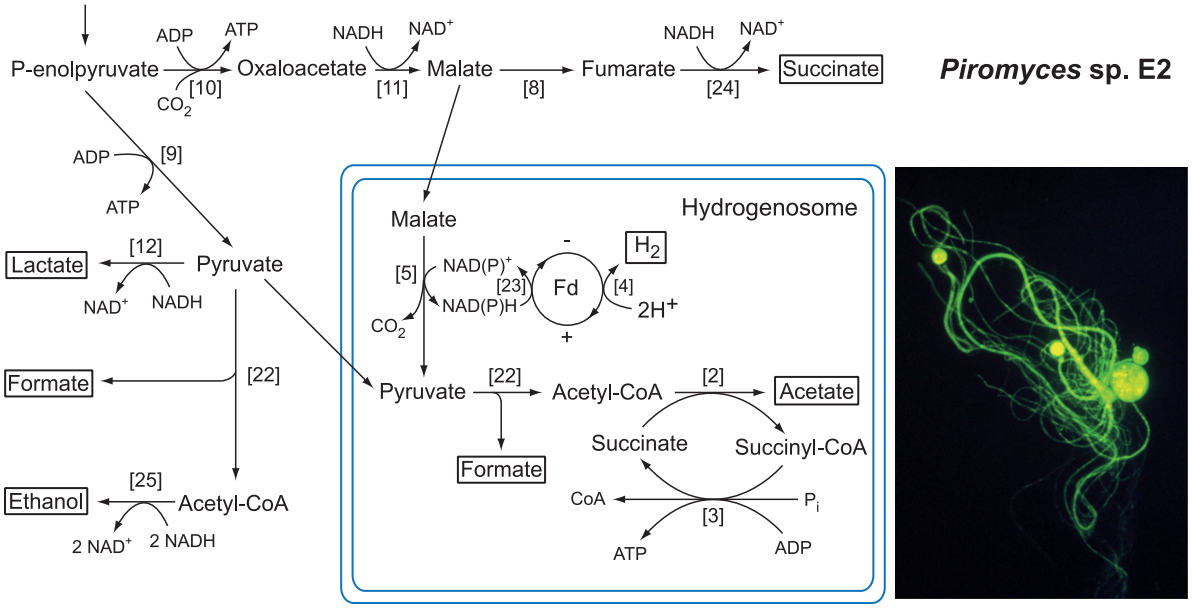


FIG 9 Mixed-acid fermentative metabolism of the hydrogenosome-bearing anaerobic chytridiomycete fungus *Piromyces* sp. E2, a rumen inhabitant. The map is redrawn based on data reported previously (47). Contrary to anaerobic parabasalian parasites, which use pyruvate:ferredoxin oxidoreductase, these common rumen inhabitants of many herbivorous mammals use pyruvate:formate lyase for pyruvate catabolism in their hydrogenosomes (47). Carbohydrate can also be metabolized to the end products succinate, lactate, formate, and ethanol in the cytosol. Bifunctional alcohol dehydrogenase E (ADHE), having both alcohol dehydrogenase and acetaldehyde dehydrogenase activities, mediates the cytosolic formation of ethanol. Note that metabolic maps reported previously by Yarlett et al. (570) and Boxma et al. (47) differ with respect to the involvement of ferredoxin in the H_2-producing reactions. With regard to the problematic circumstance that H_2 production from NAD(P)H is sketched, see the text. Abbreviations: Fd, ferredoxin; [2], acetate:succinate CoA-transferase; [3], succinyl-CoA synthetase; [4], hydrogenase; [5], malic enzyme; [8], fumarase; [9], pyruvate kinase; [10], phosphoenolpyruvate carboxykinase (ATP dependent); [11], malate dehydrogenase; [12], lactate dehydrogenase; [22], pyruvate:formate lyase; [23], NAD(P)H:ferredoxin oxidoreductase; [24], fumarate reductase (soluble); [25], alcohol dehydrogenase E. The photograph shows *Piromyces* sp. E2; the fluorescing region (sporangia and hyphae) is ca. 250 μm across. (Reprinted from reference 178a with permission from Elsevier.)

succinate, the relative amounts of which can vary with culture conditions (494). Boxma et al. (47) measured fluxes for various end products in *Piromyces* sp. E2, including formate and H_2, upon which the map shown in Fig. 9 is based.

Formate production in *Piromyces* stems from pyruvate:formate lyase (PFL) activity (47), an enzyme also present in *Neocallimastix* (154) but also active in chlorophyte algae (21). PFL is present both in *Piromyces* hydrogenosomes and in the cytosol and generates acetyl-CoA in both compartments. In the cytosol, acetyl-CoA is reduced via a bifunctional aldehyde/alcohol dehydrogenase (ADHE) to yield CoASH and ethanol; in hydrogenosomes, the CoA moiety of acetyl-CoA is transferred to succinate via an ASCT, generating acetate as an end product (47) and succinyl-CoA, which serves as the substrate for succinyl-CoA synthetase (SCS) for substrate-level phosphorylation and the regeneration of CoASH (93). ADHE is also present in chlorophyte algae (22) and in several heterotrophic protists, such as *Giardia* and *Entamoeba*.

In labeling experiments using [6-^{14}C]glucose and [U-^{14}C]glucose, Boxma et al. (47) showed that succinate was formed from oxaloacetate via a malate intermediate and involving a soluble fumarate reductase. The lack of labeled CO_2 and the formation of formate and acetate plus ethanol in 1:1 molar ratios indicated that PFL, rather than pyruvate:ferredoxin oxidoreductase (PFO) or pyruvate dehydrogenase (PDH), which would have generated 1 mol $^{14}CO_2$ per mol pyruvate, was the main route of pyruvate breakdown in the hydrogenosomal metabolism of *Piromyces*. PFL has a glycyl radical mechanism and requires the PFL-activating enzyme (PFL-AE), which is present in chytrids (154), as well as the PFL-deactivating enzyme, which is provided as a side activity of ADHE (154). There have been conflicting reports regarding the presence and/or activity of PFO in anaerobic chytrids. Early studies found PFO activity instead of PFL activity in two *Neocallimastix* species (316, 317, 570), while in a third species, *N. frontalis*, no PFO activity was found (368). Genome sequence information will clarify the matter further. Recombined homologs of PFO are present in the yeast genome (and many other fungal genomes) as sulfite reductase (200, 419). ATP is exported from chytrid hydrogenosomes to the cytosol via an ATP-ADP translocase belonging to the mitochondrial carrier family (MCF) (523, 545).

We note that the flow of electrons from NAD(P)H to H_2 sketched in Fig. 9 and described previously (47) is energetically quite unfavorable and hence unlikely to be fully correct (see the passage regarding the trimeric *Thermotoga* hydrogenase in the section on *Trichomonas vaginalis* for a more in-depth discussion). Since PFO appears to be lacking as a source of low-potential ferredoxin in *Piromyces* (47) but has been detected in other chytrids (317, 570), the mechanism of limited hydrogen production in *Piromyces*—roughly 50% to 20% of formate production (47)—remains poorly understood.

Fusarium oxysporum. Extensive work has been done on the anaerobic energy metabolism of the soil-dwelling fungal pathogen of plants *Fusarium oxysporum*; related ascomycete species such as *Cylindrocarpon tonkinense* and *Aspergillus nidulans* have also been studied in depth. Details of the denitrification pathway of *Fusarium* have largely been worked out (248, 341, 486, 513, 573). Deni-

trification among various ascomycetes has been described (574). In *Fusarium*, denitrification occurs under low-oxygen conditions, involves mitochondria, and entails the oxidation of reduced carbon sources, such as ethanol, to acetate with a deposition of the electrons onto nitrate to generate N_2O, which is excreted as an end product (487, 573). Dissimilatory nitrate reductase (dNar) and dissimilatory nitrite reductase (dNir) in the mitochondrion reduce NO_3^- to NO, which is further reduced by a soluble nitric oxide reductase, a cytochrome P450 enzyme called p450nor, to produce N_2O (150, 487, 573). *Fusarium* dNar has not been characterized at the gene level but has properties in common with bacterial NarGHI proteins (515, 573). The nitrite reductases, NirK, from *Fusarium* and *Aspergillus* are homologous to copper-containing proteobacterial nitrite reductases, have conserved homologs in *Acanthamoeba castellanii* and *Chlamydomonas reinhardtii*, and likely have a mitochondrial origin (241, 242, 359). The *Fusarium* NO reductase, NoR, is not homologous to prokaryotic nitrate reductases but is a recruited P450 enzyme (488). *Fusarium* denitrification takes place in mitochondria and is coupled to the mitochondrial electron transport chain and ATP synthesis (487, 573).

Under more strictly anoxic conditions, the reduction of nitrate proceeds to the level of NH_4^+ as the excreted end product, a process called ammonia fermentation (485, 574). This involves a different set of nitrate-metabolizing enzymes altogether, assimilatory nitrate reductase (aNar or niaD) and assimilatory nitrite reductase (aNir or niiA), which are localized in the cytosol (573). Also involved in the ammonia fermentation pathway of *Fusarium* is acetate kinase (ACK) (485), an enzyme that is also found in *Chlamydomonas* (21, 357). Formate dehydrogenase was also reported to be involved in *Fusarium* denitrification (515), and there has been one report of the participation of PFL activity in the generation of endogenous formate (258).

Abe et al. (1) showed that *Fusarium* will grow under anaerobic conditions on a variety of reduced carbon sources using elemental sulfur, S_8, as the terminal electron acceptor, generating H_2S as the reduced end product in a 2:1 molar ratio relative to acetate. Among eukaryotes studied so far, the use of elemental sulfur as a terminal electron acceptor is restricted to *Fusarium*. Hypoxic *A. nidulans* can also excrete large amounts of branched-chain amino acids as an alternative pathway to achieve redox balance (455).

Denitrification has been reported for foraminifera that inhabit aquatic sediments (410) and for diatoms (232), but none of the enzymes in these systems have been characterized so far, in contrast to *Fusarium*. Nitrite reduction coupled to ATP synthesis was also measured for mitochondria of higher-plant roots, where NO was the end product (474), but the quantitative contribution of this route to the core ATP supply is as yet unknown.

Microsporidia. Microsporidia are obligate intracellular parasites that can infect a broad spectrum of hosts (110). The group harbors important human pathogens, including *Encephalitozoon cuniculi*, which causes digestive and nervous system syndromes (234). The microsporidia are now recognized as highly derived fungi (192, 555), although their exact placement within the fungi is not clear (220). Their metabolic organization is the result of reductive evolution due to their specialized obligate parasitic lifestyle. The microsporidians *Trachipleistophora hominis* (558) and *E. cuniculi* (234, 512) are energy parasites; they have little in the way of energy metabolism at all. They can steal the ATP that they need from their host via ADP/ATP translocases localized in their plasma membranes. This type of reduction can go to an extreme. The microsporidian *Enterocytozoon bieneusi*, for example, has lost all but 2 genes for the 21 enzymes of glycolysis, all genes for the pentose phosphate pathway, and all genes for trehalose metabolism (238). This not only makes the parasite strictly dependent on the host for energy but also poses a problem with regard to the recycling of NADH. A possibility might be a glycerol-3-phosphate shuttle that moves reducing equivalents across the mitochondrial membrane, as glycerol-3-phosphate dehydrogenase has been identified on microsporidian genomes. The reducing equivalents could subsequently feed into the microsporidian alternative oxidase (556). In the microsporidian *Antonospora locustae*, both glycerol-3-phosphate dehydrogenase and the alternative oxidase, in addition to Hsp70, localize to the mitosome (122).

As another example, the energy parasite *E. cuniculi* has lost all of the mitochondrial carrier family (MCF) members, even including the ADP/ATP carrier (AAC); instead, it now uses the bacterial type of nucleotide transporter, which is not related to the AAC but is found in plastids and the intracellular pathogenic bacteria *Rickettsia* and *Chlamydia*, to import ATP from the cytosol of their eukaryotic host cells (512). The *E. cuniculi* genome harbors four genes for this nucleotide transporter, and all four gene products are expressed: three are localized to the *E. cuniculi* plasma membrane, while the fourth is localized to mitosomes, where it is thought to provide cytosolic ATP to the mitosome (512). Contrary to *E. cuniculi*, the microsporidian *A. locustae* possesses the MCF-type AAC, which was suggested to provide energy for Hsp70-dependent protein import into mitosomes and the export of iron-sulfur clusters to the cytosol (557). Transport processes (including ADP/ATP transport) on the surface of microsporidia were recently reviewed (554). Microsporidia appear to recruit host mitochondria around their surface, apparently in line with their life-style as ATP-stealing energy parasites (439, 554).

The mitochondrial-type protein import system in microsporidian mitosomes has been reviewed (549), as have protein import and tRNA import into organelles of mitochondrial origin in general (282). The characterization of mitochondrial-type iron-sulfur assembly proteins in microsporidia suggests that iron-sulfur protein assembly is the key function of their mitosomes (163), which do not appear to play a direct role in energy metabolism.

Amoebozoa: *Entamoeba histolytica*

The eukaryotic group called Amoebozoa (4) includes members that lack ATP-producing organelles, such as *Entamoeba histolytica*, an intestinal pathogen of humans, and members that have oxygen-respiring mitochondria, such as *Dictyostelium discoideum*, a free-living amoeba that inhabits soil and compost (25). *E. histolytica* is an anaerobe, and the main end products of its energy metabolism are alanine, CO_2, ethanol, and acetate (337, 349), whereby the relative amounts of ethanol and acetate produced depend upon trace oxygen levels. The enzymes of its energy metabolism are localized exclusively in the cytosol, as reviewed elsewhere (354), from which the map shown in Fig. 10 is adapted.

In *Entamoeba*, glycolytic phosphoenolpyruvate (PEP) is converted to pyruvate via high levels of activity of pyruvate:orthophosphate dikinase (PPDK) (405) rather than the classical pyruvate kinase (PYK). However, a homolog of PYK encoded by the *Entamoeba* genome (290) was subsequently found to be expressed at levels of activity approaching that of PPDK (422) and thus appears to represent an alternative route to pyruvate under some conditions. Another alternative shunt pathway to pyruvate, by-

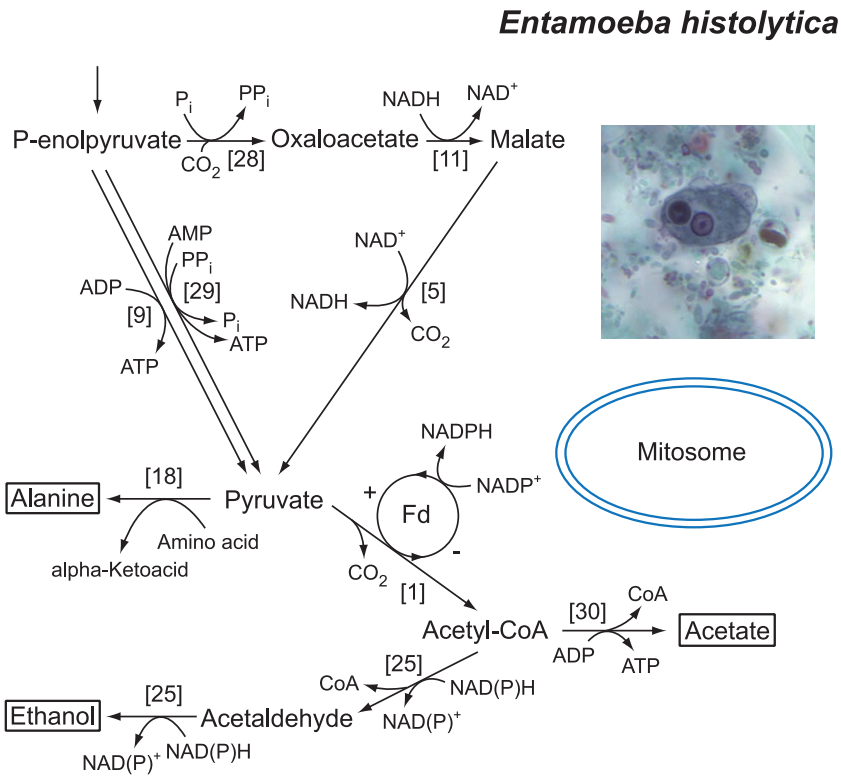


FIG 10 Major metabolic pathways in the anaerobic intestinal parasite *Entamoeba histolytica*, a parasitic amoeboid protist and the causative agent of amoebiasis. The map is redrawn based on data reported previously (354). The amoebozoan infects humans and other primates; its energy metabolic pathways are localized in the cytosol, which also harbors mitosomes (5, 507). Pyruvate:ferredoxin oxidoreductase is used for pyruvate decarboxylation/oxidation, as in parabasalian parasites; ATP is synthesized through substrate-level phosphorylation via acetyl-CoA synthetase (ADP forming) (431). Although not involved in energy metabolism, mitosomes in *E. histolytica* harbor enzymes of sulfate activation as a major function (332). Abbreviations: Fd, ferredoxin; [1], pyruvate:ferredoxin oxidoreductase; [5], malic enzyme; [9], pyruvate kinase; [11], malate dehydrogenase; [18], alanine aminotransferase; [25], alcohol dehydrogenase E; [28], phosphoenolpyruvate carboxytransferase (PP_i dependent); [29], pyruvate:orthophosphate dikinase; [30], acetyl-CoA synthetase (ADP forming). The photograph shows an *Entamoeba histolytica* trophozoite with an ingested erythrocyte, with a length of ca. 20 μm. (Photograph from the Centers for Disease Control and Prevention [CDC], Atlanta, GA.)

passing both the PYK and PPDK reactions, is PEP carboxylation to oxaloacetate by PEP carboxytransferase (PP_i dependent), followed by malate dehydrogenase (MDH) and malic enzyme (ME) reactions (Fig. 10) (406). Pyruvate is oxidized via pyruvate:ferredoxin oxidoreductase (PFO) (407, 414). The resulting acetyl-CoA is then converted into a mixture of acetate, which allows the synthesis of one additional ATP per pyruvate, and ethanol, which allows the regeneration of NAD^+, depending upon the redox state of the cell. The acetate-generating enzyme of *Entamoeba*, acetyl-CoA synthase (ADP forming), is a comparatively rare enzyme among eukaryotes (503), but the same enzyme is used in the core energy metabolism of *Giardia intestinalis* (428), and homologs occur in the genome of the diatom *Thalassiosira pseudonana*, in apicomplexan genomes (several *Plasmodium* species and *Cryptosporidium muris*), in the stramenopile *Blastocystis hominis*, and in the excavate taxon *Naegleria gruberi* (371). The ethanol-generating enzyme is a bifunctional aldehyde/alcohol dehydrogenase (ADHE), a fusion protein consisting of an N-terminal aldehyde dehydrogenase (ALDH) (acetylating) domain and a C-terminal alcohol dehydrogenase (ADH) domain (138). *Entamoeba* ADHE converts 1 mol acetyl-CoA and 2 mol NADH to 1 mol ethanol and 2 mol NAD^+ (60). The enzyme is typically a monomer of 97 kDa and is expressed at high levels in green algae (21, 22, 357), *G. intestinalis* (97), and chytrid fungi (47), with homologs occurring in the genomes of several other diverse eukaryotes, including the apicomplexan *Cryptosporidium hominis*. The ability to excrete amino acids (alanine) is also manifest in *Entamoeba* (87). The treatment of *Entamoeba* infections typically involves nitroimidazole compounds (metronidazole) (354), which are activated to highly cytotoxic derivatives via metabolism with low-potential electrons stemming from reactions such as those catalyzed by PFO (208, 256, 340) or thioredoxin reductase (271, 272).

Investigations of *Entamoeba* led to the discovery of the mitosome (300, 507), by virtue of its ability to import the chaperonin Cpn60. The *Entamoeba* mitosome possesses a mitochondrial-type ADP/ATP translocator (80); its function is still under investigation (520). Recently, the enzymes of sulfate activation were localized to the mitosomes of *Entamoeba* (332, 333), providing a new perspective on mitosomal functions. Other possible functions might have to do with FeS cluster maturation and hydroperoxide detoxification via the rubrerythrin system (302). Because carbon flux and energy metabolism in *Entamoeba* are known to be cytosolic processes (354), the role of mitosomes in core energy metabolism, if any, can be peripheral at best. The *Entamoeba* genome encodes several [Fe]-hydrogenase homologs (290). The presence of mRNA for a short-form [Fe]-hydrogenase in *Entamoeba* was shown by reverse transcription (RT)-PCR (365), and the activity of recombinant *Entamoeba* hydrogenase expressed in *Escherichia*

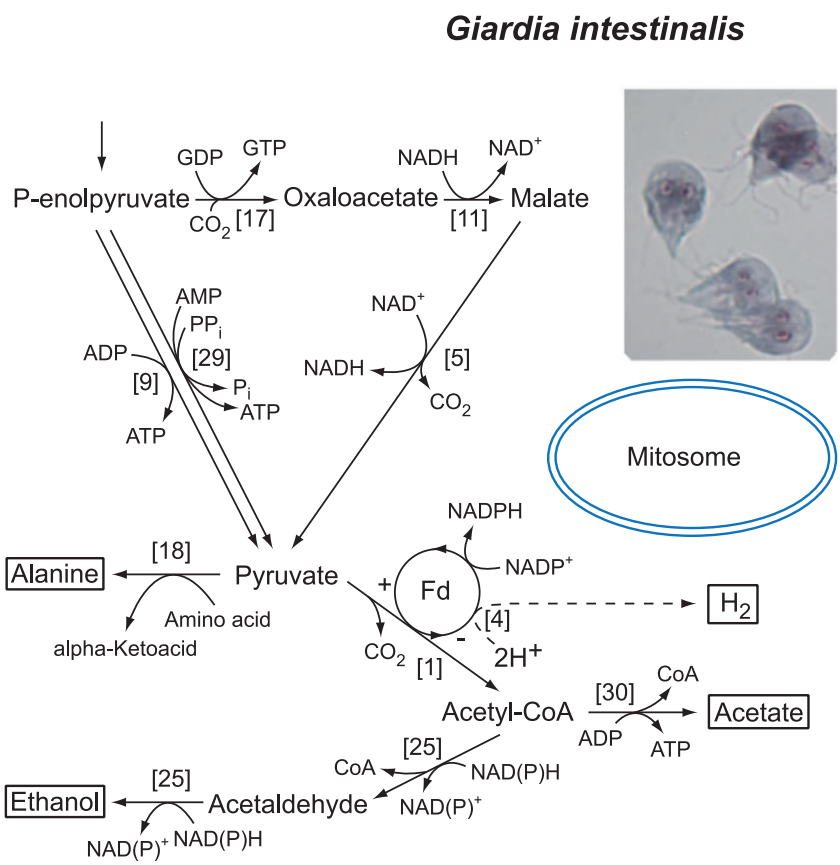


FIG 11 Major pathways of energy metabolism in a parasite of the small intestine, *Giardia intestinalis* (also called *Giardia lamblia*), a flagellated protist parasite and the causative agent of giardiasis, a diarrheal infection of humans. The map is redrawn based on data reported previously (354), taking hydrogenase activity into account (287). *Giardia* mitosomes are not directly involved in energy metabolism but are involved in FeS cluster biogenesis (508). In *Giardia*, as in *Entamoeba*, the typically hydrogenosomal (and sometimes mitochondrial) enzymes PFO and [Fe]-Hyd have been recompartmentalized to the cytosol during evolution. *G. lamblia* produces molecular hydrogen under strictly anoxic conditions (287), as indicated by the dashed line. Abbreviations: Fd, ferredoxin; [1], pyruvate:ferredoxin oxidoreductase; [4], hydrogenase; [5], malic enzyme; [9], pyruvate kinase; [11], malate dehydrogenase; [17], phosphoenolpyruvate carboxykinase (GTP dependent); [18], alanine aminotransferase; [25], alcohol dehydrogenase E; [29], pyruvate:orthophosphate dikinase; [30], acetyl-CoA synthetase (ADP forming). The photograph shows *Giardia intestinalis* at the trophozoite stage, with a length of 15 to 20 μm. (Photograph from the CDC, Atlanta, GA.)

coli was reported, but endogenous hydrogenase enzymatic activity in *Entamoeba* has not yet been reported.

Because of the potential of the mitosome to harbor drug targets, protein import into *Entamoeba* mitosomes has been intensely studied as of late (118). A free-living relative of *Entamoeba*, *Mastigamoeba balmuthii*, has several enzymes of core energy metabolism in common with *Entamoeba* (183, 564) and also possesses mitochondria that import Cpn60 (159).

Excavate Taxa

Like the Amoebozoa, the eukaryotic supergroup Excavata (4, 237) includes members with oxygen-respiring mitochondria, such as many euglenids, and members that lack organelles involved in core ATP synthesis, such as *Giardia intestinalis*. Like the opisthokont supergroup, members with hydrogenosomes, such as *Trichomonas vaginalis*, also occur. Our focus here is on organisms whose metabolisms have been investigated. Genome sequencing and expressed sequence tag (EST) data are becoming available for many excavate taxa, which allow inferences about biochemical functions in organelles, for example, the hydrogenosome-like organelles of *Sawyeria marylandensis* (27), the hydrogenosomes of *Psalteriomonas lanterna* (102), or the organelle of mitochondrial origin in *Naegleria gruberi* (149, 161, 371), but there are insufficient direct experimental data available concerning enzyme activities and excreted end products to generate metabolic maps for these organisms.

Giardia intestinalis. *Giardia intestinalis* (syn., *Giardia lamblia*) is an intestinal unicellular parasite that causes acute and chronic diarrhea in humans (17). The vegetative trophozoites of this flagellated protist propagate rapidly in the anaerobic environment of mammalian intestines but are susceptible to molecular oxygen. They cease growth under aerobic conditions. Its energy metabolic map (Fig. 11) very closely parallels that of *Entamoeba* (354). The main differences are the presence of a GTP-dependent phosphoenolpyruvate carboxykinase (PEPCK) in the pyruvate kinase (PYK)/pyruvate:orthophosphate dikinase (PPDK) shunt (278) and the presence of an [Fe]-hydrogenase (287), homologous to the enzyme found in chytrids or trichomonads. As with *Entamoeba*, both PPDK and PYK activities are present at comparable levels (377), although the latter activity was long thought to be lacking (207, 327). Also as with *Entamoeba*, the main end products are acetate and ethanol, usually a mixture of both, depending upon redox conditions: in the presence of small amounts of oxygen, the parasite utilizes diaph-

orase (H_2O-producing NADH oxidase) activity (58) to maintain redox balance, and more acetate than ethanol is produced; in the absence of O_2, redox balance is maintained via the excretion of ethanol as the main end product (278, 354, 375). However, when *Giardia* is grown under fastidiously anoxic conditions, a substantial amount of H_2 is produced (287). The [Fe]-hydrogenase encoded by the *Giardia* genome is implicated in its H_2 production. Another diplomonad parasite, *Spironucleus vortens*, also produces H_2 (334). The level of production of alanine as an end product (130) is also increased in the strict absence of oxygen (374).

ADP-forming acetyl-CoA synthetase (ACS) from *Giardia* has been purified to homogeneity (428), and the gene has been characterized (430, 431). *Giardia* pyruvate:ferredoxin oxidoreductase (PFO) was detected in cell homogenates (278), has been biochemically studied and purified to homogeneity (510), and was investigated at the molecular level (417). Paget et al. (375) studied the effects of oxygen levels on fermentation end products in *Giardia*. The *Giardia* diaphorase activity plays an important role in the oxygen metabolism of the anaerobe (429). Diaphorase is a flavoprotein that can mediate one-electron transfers from two-electron donors (NADH), thereby generating toxic reactive oxygen species (ROS). Li and Wang (275) showed that the overexpression of the diaphorase in *Giardia* significantly enhanced the susceptibility of the cells to oxygen, while a knockdown of this enzyme resulted in more oxygen-tolerant *Giardia* cells that grew equally well under anaerobic and aerobic conditions. In addition to the diaphorase, *Giardia* also possesses a flavodiiron protein that can reduce O_2 to water (112).

The involvement of mitosomes in mitochondrial-type iron-sulfur cluster assembly was demonstrated for *Giardia* (508), following hints from the phylogeny of the IscS enzymes from *Giardia* and *Trichomonas* (483). The mitochondrial-type monothiol glutaredoxin involved in the mitosomal iron-sulfur cluster biogenesis pathway was recently identified in *Giardia* (397). Recent proteomic studies of *Giardia* mitosomes revealed the presence of all key components of the iron-sulfur cluster assembly machinery, including the cysteine desulfurase, IscS; the scaffold proteins IscU, Nfu, and IscA; and monothiol glutaredoxin (224, 397). Mitosomes also contain [2Fe-2S]-ferredoxin, which might provide the reducing equivalents required for the formation of iron-sulfur clusters, and a complete set of chaperones that are involved in the transfer of preassembled FeS clusters into apoproteins.

Protein import across the outer mitosomal membrane of *Giardia* might involve *G. intestinalis* Tom40 (*Gi*Tom40), which is a beta barrel protein homologous to the yeast outer mitochondrial membrane translocase Tom40 (96). Neither genomic nor proteomic analyses have uncovered *Giardia* homologs for components of the Tim17/22/23 family, which are essential for the formation of the inner membrane translocation pore of the TIM complex in yeast. However, in yeast, the TIM complex interacts with the PAM (presequence translocase-associated motor) complex, three components of which, Pam18, Pam16, and HSP70, are present in *Giardia* mitosomes. Thus, while several proteins involved in protein translocation across the inner membrane of *Giardia* mitosomes and *Trichomonas* hydrogenosomes are clearly homologous to their counterparts in mitochondria (96, 119), their specific functions are not yet known in detail; hence, the mechanisms of protein import into mitosomes remain to be elucidated. The mitochondrial processing peptidase (MPP) from *Giardia* is another example: it functions as a monomeric beta-MPP coevolving in structure and charge distribution with mitosomal targeting presequences, which are short and, hence, not as positively charged as mitochondrial presequences (459). Ethanol production from acetyl-CoA entails a bifunctional alcohol/aldehyde dehydrogenase (ADHE), which has been purified and characterized (427, 428). The *Giardia* genome sequence was reported (342), and the cell biology, cell cycle stages, and pathogenicity of *G. intestinalis* were recently reviewed (17).

Trichomonas vaginalis. *Trichomonas vaginalis* is a parasitic protist that inhabits the human urogenital tract; it is the causative agent of a widespread sexually transmitted disease in humans, trichomoniasis (381, 449). The *T. vaginalis* genome is marked by a high frequency of duplicate genes (71). Trichomonads possess hydrogenosomes, which produce molecular hydrogen as an end product of fermentative energy metabolism (349, 351, 480). Hydrogenosomes were discovered in trichomonads (279). The most extensively characterized hydrogenosomes are those of the parabasalid flagellate *T. vaginalis* (354, 355). The main end products of energy metabolism in *T. vaginalis* are glycerol, lactate, and ethanol (produced in the cytosol) as well as H_2, CO_2, and acetate (produced in hydrogenosomes) (Fig. 12). Glycerol is produced from dihydroxyacetone phosphate via glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase (354, 469). Ethanol is generated from pyruvate, as in yeast, via pyruvate decarboxylase and alcohol dehydrogenase (256, 477). Lactate is produced in the cytosol by a lactate dehydrogenase (LDH) that is derived phylogenetically from the duplication and modification of a preexisting malate dehydrogenase (MDH) (563). Alanine is accumulated as a minor end product of *T. vaginalis* glucose metabolism (82) and can represent up to 30 to 34% of the amino acid pool in *Trichomonas* cells (420). Alanine is thus an end product (87), but it is apparently not excreted, as it is not detected among the excreted end products in cell-free medium (493), thus paralleling to some extent the situation with opines in metazoans (see "Animals" above) or wax esters in *Euglena* (see below), end products which are sequestered by the organism and not excreted. Glycerol is detected both within cells (82) and in medium (493).

In trichomonad hydrogenosomes, pyruvate and malate from glycogen or glucose degradation can be imported from the cytosol, and malate is then converted in the organelle into pyruvate via malic enzyme (124, 205). A cytosolic isoenzyme of malic enzyme also exists (120). Isolated hydrogenosomes produce 1 mol each H_2, CO_2, and acetate along with 1 mol ATP per mol pyruvate (470). Pyruvate is decarboxylated by pyruvate:ferredoxin oxidoreductase (PFO) in hydrogenosomes (205, 209, 351), generating CO_2, acetyl-CoA, and reduced ferredoxin (Fd). PFO has several FeS clusters, and its mechanism involves a radical intermediate (114, 400a). Fd carries electrons to ferredoxin-dependent [Fe]-hydrogenase (64, 378, 578), which donates them to protons to generate molecular hydrogen (209, 351). PFO from *Trichomonas* has been biochemically studied and purified to homogeneity (204). The CoA moiety of acetyl-CoA (from the PFO reaction) is transferred to succinate by acetate:succinate CoA-transferase (ASCT), yielding acetate as an end product, and succinyl-CoA, which serves as a substrate for succinyl-CoA synthetase (SCS) (259, 260), also called succinate thiokinase (STK), as in the case of mitochondria, yielding ATP (or GTP) through substrate-level phosphorylation. The acetate-generating enzyme of hydrogenosomes, ASCT, which was originally described for *Trichomonas* (277), was only recently characterized at the molec-

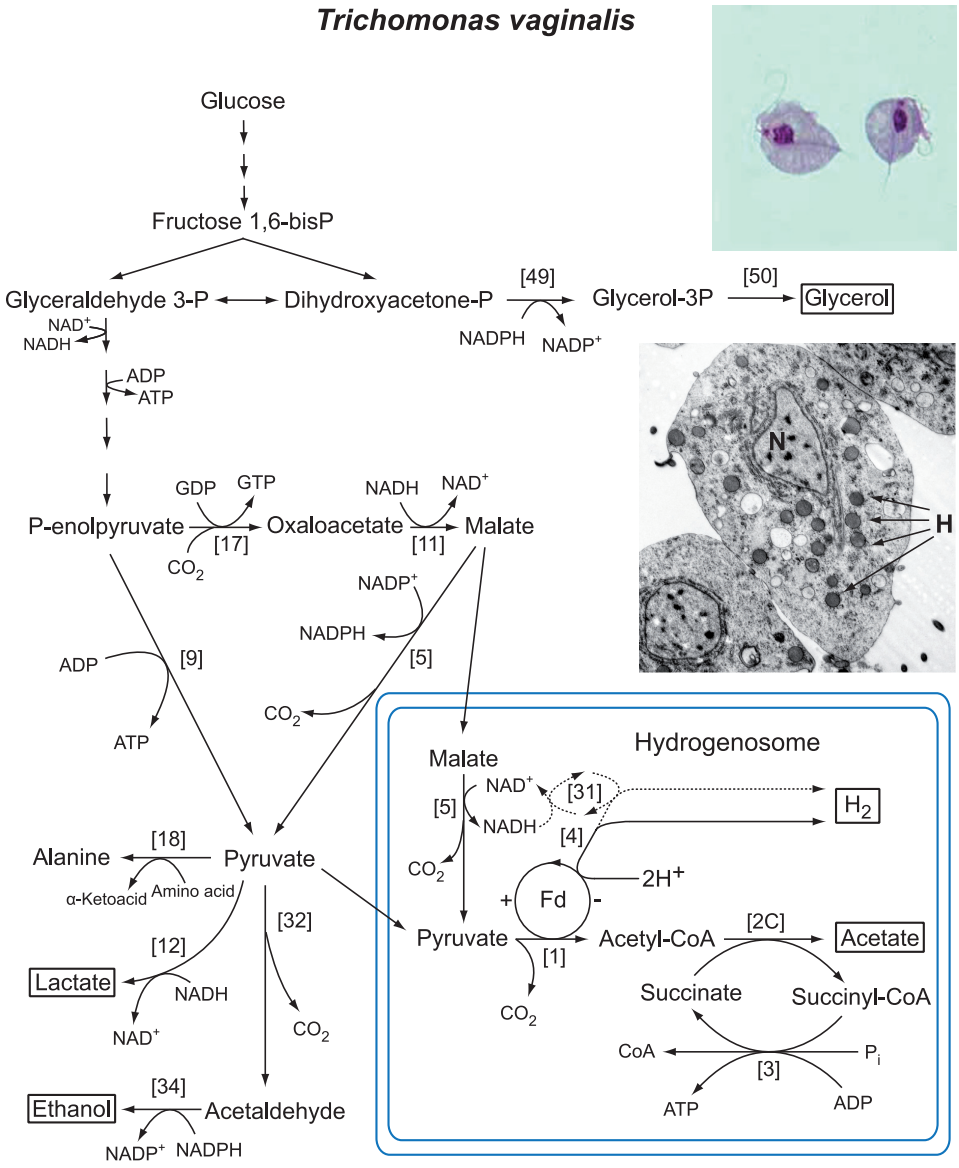


FIG 12 Major pathways of anaerobic, molecular hydrogen-producing, fermentative metabolism in hydrogenosomes of the flagellated protist parasite *Trichomonas vaginalis*, a sexually transmitted pathogen of the human urogenital tract. The map is redrawn based on data reported previously (209, 349). Hydrogenosomal pyruvate breakdown involves pyruvate:ferredoxin oxidoreductase and functional 51-kDa and 24-kDa subunits of the NADH dehydrogenase module in complex I, which reoxidize NADH stemming from malate oxidation (71, 209, 349). The 51-kDa and 24-kDa subunits of mitochondrial complex I function in association with [Fe]-Hyd in *Trichomonas* (209), possibly in a manner similar to that of the trimeric [Fe]-Hyd of *Thermotoga* (448) (see the text). Additional major end products of cytosolic fermentations in *T. vaginalis* include alanine, lactate, ethanol, and glycerol (469). Abbreviations: Fd, ferredoxin; [1], pyruvate:ferredoxin oxidoreductase; [2C], acetate:succinate CoA-transferase subfamily 1C (503); [3], succinyl-CoA synthetase; [4], hydrogenase; [5], malic enzyme; [9], pyruvate kinase; [11], malate dehydrogenase; [12], lactate dehydrogenase; [17], phosphoenolpyruvate carboxykinase (GTP dependent); [18], alanine aminotransferase; [31], 51-kDa and 24-kDa subunits of the NADH dehydrogenase module of complex I; [32], pyruvate decarboxylase; [34], alcohol dehydrogenase (NADPH dependent); [49], glycerol-3-phosphate dehydrogenase; [50], glycerol-3-phosphatase. The light micrograph shows *Trichomonas vaginalis* at the trophozoite stage, with a length of 7 to 30 μm (Photograph from the CDC, Atlanta, GA.) The transmission electron micrograph shows hydrogenosomes (H) and the nucleus (N). (Photograph courtesy of Kathrin Bolte, University of Marburg, Germany.)

ular level (529). The *Trichomonas* ASCT enzyme is distinct from the ASCT enzyme that generates acetate as an end product in trypanosome mitochondria (503, 528, 533).

In addition to cytosolic diaphorase activities, there is a hydrogenosomal flavodiiron protein that transfers electrons to O_2 to produce water (463). The hydrogenosomal [2Fe-2S]-ferredoxin of *Trichomonas* has been characterized (164, 228, 540); it is one of the few *Trichomonas* proteins that has been studied at the crystal structure level (92). Trichomonad hydrogenosomes contain components derived from complex I of the mitochondrial respiratory chain that help to maintain redox balance by reoxidizing NADH from the malic enzyme reaction (209). The 51-kDa and 24-kDa proteins from mitochondrial complex I (128, 209) copurify as a protein complex, and they can reduce the *Trichomonas* [2Fe-2S]-ferredoxin using NADH as the electron donor (209).

The potential involvement of NADH in H_2 production, as de-

scribed by Hrdy et al. (209) and as shown in Fig. 12, might appear to be problematic at first sight, because the midpoint potential of NADH is not sufficiently negative to generate H_2. Although not yet shown for eukaryotes, the likely solution to this situation comes from a process recently discovered for prokaryotes called electron bifurcation (274): Schut and Adams (448) showed that the trimeric [Fe]-hydrogenase of *Thermotoga maritima* operates in such a way as to accept 1 electron from NADH and 1 electron from the low-potential reduced Fd generated by PFO per molecule of H_2 produced. Despite the involvement of NADH, the overall reaction is energetically favorable because of the participation of the low-potential ferredoxin, which drives the reaction forward. The trimeric *Thermotoga* [Fe]-hydrogenase consists of three subunits, α, β, and γ. The α-subunit is homologous to the [Fe]-hydrogenase of *Trichomonas*, while the β- and γ- subunits are homologous to the 51- and 24-kDa subunits of complex I (209), respectively, as sequence comparisons have readily shown. This finding suggests that eukaryote [Fe]-hydrogenase might be trimeric and function similarly to the *Thermotoga* enzyme in some species, such as *Trichomonas*. Consistent with this view, in the anaerobic chytrids, the 51- and 24-kDa subunits of complex I are present, and in the ciliate *Nyctotherus*, they are C-terminally fused to the [Fe]-hydrogenase catalytic subunit (48). However, not all eukaryotic hydrogenases will be trimeric, because the 51- and 24-kDa subunits of complex I are lacking in some eukaryotic anaerobes (*Giardia*, for example), but they are present in others.

Protein import into *Trichomonas* hydrogenosomes can involve the mitochondrial-type processing peptidase (459), but some proteins are imported into hydrogenosomes without processing and without the help of N-terminal transit peptides (66a, 325). Several of the enzymes underpinning *Trichomonas* core carbon and energy metabolism are abundant proteins possessing FeS clusters (518), and iron metabolism in *Trichomonas* and related parasites has been studied in depth (476, 481). *Trichomonas* hydrogenosomes have a mitochondrial-type FeS cluster assembly machinery (478), and frataxin, a mitochondrial protein of iron homeostasis, from *Trichomonas* hydrogenosomes has been studied (117). [Fe]-hydrogenase of the type found in *Trichomonas* hydrogenosomes requires specific maturases, HydEFG, for the assembly of the FeS clusters and the attachment of ligands to the active-site H-cluster (396). One of the [Fe]-hydrogenases in *Trichomonas* is a fusion protein carrying at its C terminus the same NAD-, flavin mononucleotide (FMN)-, and flavin adenine dinucleotide (FAD)-binding domain as that found at the C terminus of the pyruvate: $NADP^+$ oxidoreductase (PNO) of *Euglena* (419).

The maturation of eukaryotic Fe-Hyd was first studied for the green alga *Chlamydomonas*, which requires at least three additional proteins, HydE, HydF, and HydG (392), that are also present in trichomonad hydrogenosomes (396) and in other eukaryotes that express Fe-Hyd (211), but their presence in chytrids has not yet been established. The function of these hydrogenase maturases involves the synthesis and insertion of small ligands (319, 453), including CN^- and CO, that are attached to Fe and S atoms of the H-cluster, the active site of the enzyme.

Several *Trichomonas* Fe-hydrogenases are found as fusion proteins possessing an FAD-NAD one-electron- to two-electron-converting module (209), which also occurs in *Euglena*, *Cryptosporidium*, and *Blastocystis* PNOs (270, 419). However, this functional module is distinct from the 51-kDa and 24-kDa complex I subunits that are associated with the trimeric *Thermotoga* hydrogenase (the β- and γ-subunits thereof) that are involved in low-potential ferredoxin-dependent NADH oxidation (448) and that are also present in *Trichomonas* hydrogenosomes (209).

The ATP generated in trichomonad hydrogenosomes is thought to reach the cytosol via a mitochondrial-type ADP/ATP carrier homolog, Hmp31 (126, 545). The spectrum of mitochondrial carrier family (MCF) proteins is remarkably small, in addition to the ATP/ADP carrier. Rada et al. (398) identified five other MCF members in *Trichomonas*. There are 44 distinct MCF proteins in humans, for comparison. In addition to the pathways shown in Fig. 12, there is also a contribution of the arginine dihydrolase pathway to energy metabolism, but it is a quantitatively minor contribution (571). The first enzyme of the arginine dihydrolase pathway, arginine deiminase, is present in the hydrogenosome, while other components are cytosolic (338).

Tritrichomonas foetus. *Tritrichomonas foetus* is an important parasite of livestock that can infect the placenta, lung, and lymph nodes of fetal cattle, causing abortion (449). Hydrogenosomes were first discovered in this anaerobic flagellate (279). When grown under conditions of iron starvation, *T. foetus* has reduced PFO levels (518).

The spectrum of end products of *T. foetus* (Fig. 13) parallels that of *Trichomonas vaginalis* (Fig. 12), with the exceptions that lactate is missing (256) and that in *T. foetus*, up to 50% of the carbon end product is excreted as succinate (421). In *T. foetus*, succinate is produced from phosphoenolpyruvate (PEP) via PEP carboxykinase (PEPCK), entailing a cytosolic fumarase and a soluble NADH-dependent fumarate reductase (354), by a route paralleling those also found for trypanosomes (39) and anaerobic fungi (47). This mode of succinate formation is thus distinctly different from that found for anaerobic metazoans, where a membrane-bound fumarate reductase and rhodoquinone are involved [see "*Fasciola hepatica* (liver fluke)" for details].

Like *T. vaginalis*, *T. foetus* possesses a hydrogenosomal adenylate kinase (113). The cytosolic malate dehydrogenase (202), the hydrogenosomal malic enzyme (ME) (203, 205), and the ME isoenzymes (206) have been characterized, as has the ATP-conserving hydrogenosomal succinyl-CoA synthetase (SCS) (227). Succinate is produced via a soluble, cytosolic fumarate reductase (346). The enzymes of the *T. foetus* glycolytic route leading to PEP have been studied (328, 329).

Differences between *T. vaginalis* and *T. foetus* metabolisms are more pronounced in metronidazole-resistant lines. Both parasites shunt hydrogenosomal metabolism during resistance, but metronidazole-resistant *T. vaginalis* cells excrete mainly lactate, whereas resistant *T. foetus* cells excrete mainly ethanol (256). The mode of action of metronidazole (321) involves the activation of the compound through low-potential electron donors derived from metabolism (273, 340). The *Tritrichomonas* ferredoxin has been characterized (303). ME, [Fe]-hydrogenase, SCS, and adenylate kinase were purified from *T. foetus* hydrogenosomes (280).

Trypanosoma brucei. *Trypanosoma brucei* is a parasitic protist of the order Kinetoplastida that causes African sleeping sickness in humans and Nagana in livestock. Trypanosomatids exhibit several unusual cell biological features, such as (i) the presence of only a single mitochondrion containing a special structure, the kinetoplast, that comprises a giant network of thousands of catenated circular DNAs (106, 292); (ii) that most mitochondrial mRNAs of trypanosomatids are subject to elaborate and precise posttranscriptional RNA processing that inserts hundreds and de-

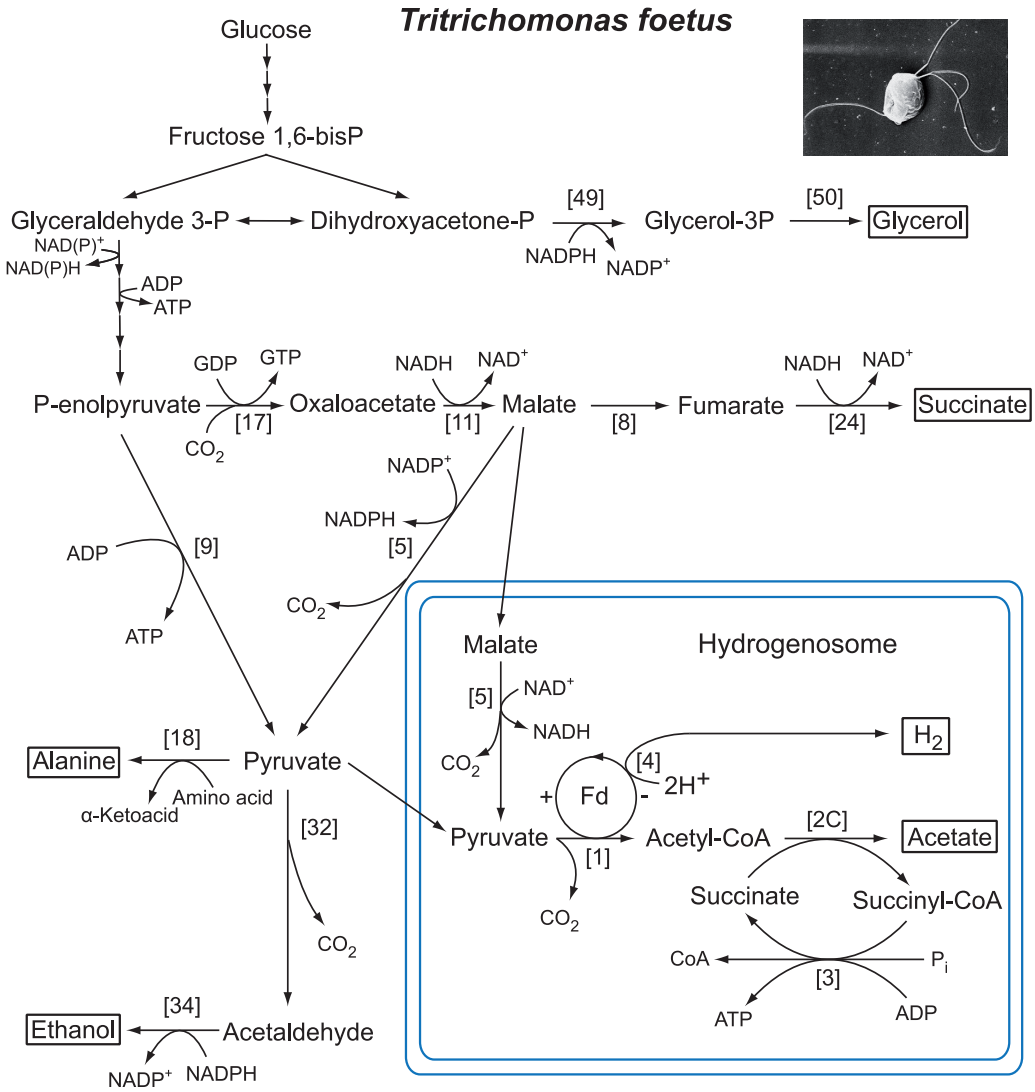
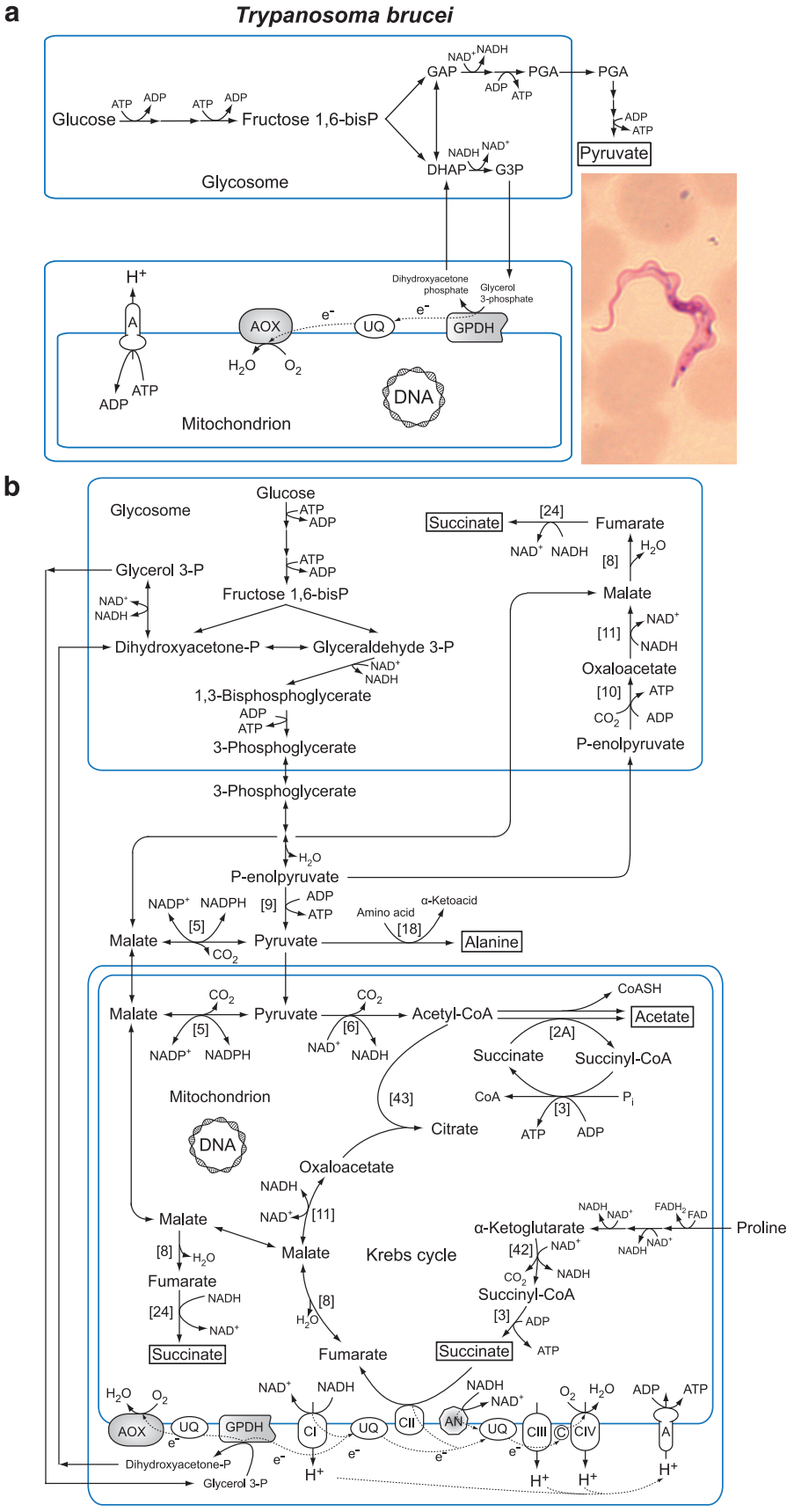


FIG 13 Major pathways of energy metabolism leading to acetate end product formation in hydrogenosomes of the flagellated protist parasite *Tritrichomonas foetus*, a pathogen of the bovine urogenital tract. The map was redrawn based on data reported previously (354, 469). The hydrogenosomal fermentation of *T. foetus* is very similar to that of *Trichomonas vaginalis*. Pyruvate:ferredoxin oxidoreductase mediates the generation of acetyl-CoA, and protons serve as the terminal electron acceptor; NADPH can be reoxidized through alcohol dehydrogenase (469). The 51- and 24-kDa subunits of complex I (209) have so far not been identified in *T. foetus*. Cytosolic succinate production through soluble fumarate reductase is lacking in metronidazole-resistant *Tritrichomonas foetus* strains (256). Abbreviations: Fd, ferredoxin; [1], pyruvate:ferredoxin oxidoreductase; [2C], acetate:succinate CoA-transferase subfamily 1C (503); [3], succinyl-CoA synthetase; [4], hydrogenase; [5], malic enzyme; [8], fumarase; [9], pyruvate kinase; [11], malate dehydrogenase; [17], phosphoenolpyruvate carboxykinase (GTP dependent); [18], alanine aminotransferase; [24], fumarate reductase (soluble); [32], pyruvate decarboxylase; [34], alcohol dehydrogenase (NADPH dependent); [49], glycerol-3-phosphate dehydrogenase; [50], glycerol-3-phosphatase. The photograph shows *Tritrichomonas foetus* at the trophozoite stage, with a length of ca. 15 μm. (Reprinted from reference 365a with permission from Elsevier.)

letes tens of uridylates (475); and (iii) sequestering the major part of the glycolytic pathway within peroxisome-like organelles called glycosomes (182). The energy metabolism of all trypanosomatids studied so far is dependent upon O_2 for redox balance, but their energy metabolism is fermentative and thus encompasses many of the enzymes common to anaerobic eukaryotes (500a). Thus, it is meaningful to include trypanosomes in this review even though they do not display anaerobic energy metabolism. The life cycle of *T. brucei* comprises distinct developmental stages in the mammalian host and in the insect vector, the tsetse fly. The energy metabolism of *T. brucei* differs drastically between these life cycle stages (54, 532).

In all investigated life cycle stages, the mitochondrion of *T. brucei* is aerobically functioning (i.e., oxygen is the terminal electron acceptor); however, pyruvate is not oxidized to carbon dioxide, and instead, fermentation-like end products are produced. Therefore, the mitochondrial energy metabolism of trypanosomatids can be considered an intermediate between classical aerobically functioning mitochondria, such as those of mammals, and true anaerobically functioning mitochondria, such as those of most parasitic helminths and several marine organisms (500a, 501).

In the long slender bloodstream form of *T. brucei*, glycolysis is the only source of ATP production. Glucose is converted to pyru-



vate as the sole end product (Fig. 14a), and no ATP is generated by oxidative phosphorylation (182). To maintain glycosomal redox balance under aerobic conditions, NADH is reoxidized by a mitochondrial glycerol-3-phosphate shuttle (369). This shuttle is coupled to a plant-like alternative oxidase that does not translocate protons over the mitochondrial inner membrane and thus does not generate any extra ATP (85). In bloodstream forms, the mitochondrial ATP synthase even operates in reverse and consumes ATP to generate $\Delta\Psi$ by proton pumping (365b, 442, 539a).

The transformation of bloodstream form *T. brucei* into the procyclic insect stage is accompanied by striking changes in energy metabolism (Fig. 14b). Glycosomal metabolism is then extended, such that part of the PEP is imported from the cytosol and is subsequently metabolized to succinate via PEPCK, malate dehydrogenase, fumarase, and a soluble glycosomal NADH:fumarate reductase (39). This pathway consumes 2 mol NADH per mol succinate formed, and therefore, glycosomal succinate production is used to maintain redox balance (538). In contrast to the bloodstream form, in the procyclic insect stage, pyruvate, the end product of glycolysis, is not excreted but is further metabolized inside the mitochondrion. However, although all enzymes are present in this procyclic insect stage, the Krebs cycle does not function as a cycle; instead, parts of it are used for purposes other than the complete degradation of mitochondrial substrates (537, 538). Consequently, pyruvate is not completely oxidized to carbon dioxide by Krebs cycle activity but instead is converted mainly into acetate. Acetate production from acetyl-CoA occurs mainly by a two-enzyme cycle, in which ASCT transfers the CoA moiety of acetyl-CoA to succinate, yielding acetate and succinyl-CoA, which is subsequently converted by SCS with the concomitant production of ATP (53, 503, 413, 533). The *T. brucei* ASCT enzyme is not homologous to the ASCT enzymes used by *Trichomonas vaginalis* hydrogenosomes and *Fasciola hepatica* mitochondria (503). Although the ASCT gene is not an essential gene, because an alternative acetate-producing pathway exists, the importance of ASCT for the normal physiological phenotype of the parasite has been clearly shown (413).

Procyclic *T. brucei* cells contain a completely developed mitochondrion, which also uses amino acids, such as proline and threonine, as substrates for ATP production. Mitochondria of procyclic *T. brucei* contain a more classical respiratory chain, which produces ATP by oxidative phosphorylation (370). Next to its function in energy metabolism, the *T. brucei* mitochondrion is essential for iron-sulfur cluster assembly (460), Ca^{2+} transport (114a, 539a), and fatty acid biosynthesis (86, 173).

Euglena gracilis. Euglenids are a broad and diverse group containing many typical flagellate inhabitants of shallow freshwater environments, but relatives can also be found in anoxic marine (38, 129a) and anoxic freshwater (457a) ecosystems, whereby some members possess organelles ultrastructurally similar to hydrogenosomes (129a, 457a). Only one member of the euglenids has been extensively studied from a biochemical standpoint: *Euglena gracilis* (62). Grown aerobically, *Euglena gracilis* expresses pyruvate dehydrogenase (PDH) in mitochondria (196) and respires O_2 but by using a slightly modified Krebs cycle that is also found among some alphaproteobacteria (168). The shunt involves the replacement of α-ketoglutarate dehydrogenase by α-ketoglutarate decarboxylase and succinate semialdehyde dehydrogenase (62). When grown under anaerobic conditions (Fig. 15), *Euglena* uses acetyl-CoA as the terminal electron acceptor and produces wax esters as end products of metabolism (62, 213–215, 514).

Some *E. gracilis* strains accumulate wax esters at levels up to 57% of their dry weight (514). Wax ester fermentation by *Euglena* is similar in general principle to the synthesis of branched short-chain fatty acids in *Ascaris* or butyrate in *Dasytricha*: fatty acids are synthesized from acetyl-CoA condensation with an acyl-CoA (starting with acetyl-CoA or propionyl-CoA), a reduction of the resulting 3-oxoacid to 3-hydroxy acid, dehydration thereof, and a reduction of the resulting *trans*-enoyl-CoA to the elongated acyl-CoA. In contrast to malonyl-CoA-dependent fatty acid synthesis, the acetyl-CoA-dependent *Euglena* route allows net fermentative ATP synthesis from glucose, because acetyl-CoA is condensed without prior ATP-dependent carboxylation to malonyl-CoA (213, 215, 443). The step catalyzed by *trans*-2-enoyl-CoA reductase (NADPH dependent) circumvents the reversal of an O_2-dependent step in β-oxidation (197). A portion of the fatty acids is reduced to alcohols, esterified with another fatty acid, and deposited into the cytosol as wax (wax ester fermentation). The fatty acyl-CoA reductase and the wax synthase involved in the synthesis of the medium-chain wax aliphatic side chains of *Euglena* were recently characterized (490); the gene is ubiquitous among eukaryotes. Upon a return to an oxic environment, the stored waxes are degraded via aerobic dissimilation in the mitochondrion (213).

Similar to the situation for anaerobic mitochondria of meta-

FIG 14 Major pathways of compartmentalized energy metabolism in bloodstream (a) and procyclic (b) life cycle stages of the flagellated and fully oxygen-dependent protist parasite *Trypanosoma brucei*. The maps are redrawn based on data reported previously (54, 500a, 538). (a) In bloodstream forms, *Trypanosoma brucei* mitochondria help to maintain cell redox homeostasis via glycerol-3-phosphate dehydrogenase and alternative oxidase, which, together with ubiquinone, constitute the whole O_2-consuming respiratory chain, which does not, however, produce ATP. The inner mitochondrial membrane ATPase works in reverse; that is, it pumps protons to the intermembrane space while hydrolyzing ATP to ADP. The major metabolic end product pyruvate is generated in the cytosol. The photograph shows a long slender bloodstream form of *Trypanosoma brucei* with a length of ca. 25 μm (circles in the background are erythrocytes). (Photograph by Rob Koelewijn, Harbor Hospital, Rotterdam, Netherlands.) (b) Energy metabolism is more complex in procyclic *T. brucei* stages. Succinate is generated both in glycosomes by a soluble fumarate reductase and in mitochondria via a recently functionally characterized soluble mitochondrial fumarate reductase (90). Acetate is produced in mitochondria mainly by means of the acetate:succinate CoA-transferase/succinyl-CoA synthetase cycle, but an alternative pathway is also present (413). In *T. brucei*, no lactate is formed from pyruvate (538); instead, another end product commonly found in facultative anaerobic animals, alanine, is produced by alanine aminotransferase. The respiratory chain contains not only an alternative oxidase and glycerol-3-phosphate dehydrogenase but also an alternative rotenone-insensitive NADH dehydrogenase (90). Abbreviations: CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; C, cytochrome *c*; A, ATPase; AOX, alternative oxidase; AN, alternative, rotenone-insensitive NADH dehydrogenase; GPDH, glycerol-3-phosphate dehydrogenase; G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; PGA, 3-phosphoglycerate; [2A], acetate:succinate CoA-transferase (subfamily 1A); [3], succinyl-CoA synthetase; [5], malic enzyme; [6], pyruvate dehydrogenase complex; [8], fumarase; [9], pyruvate kinase; [10], phosphoenolpyruvate carboxykinase (ATP dependent); [11], malate dehydrogenase; [18], alanine aminotransferase; [24], fumarate reductase (soluble, NADH dependent); [42], α-ketoglutarate dehydrogenase; [43], citrate synthase.

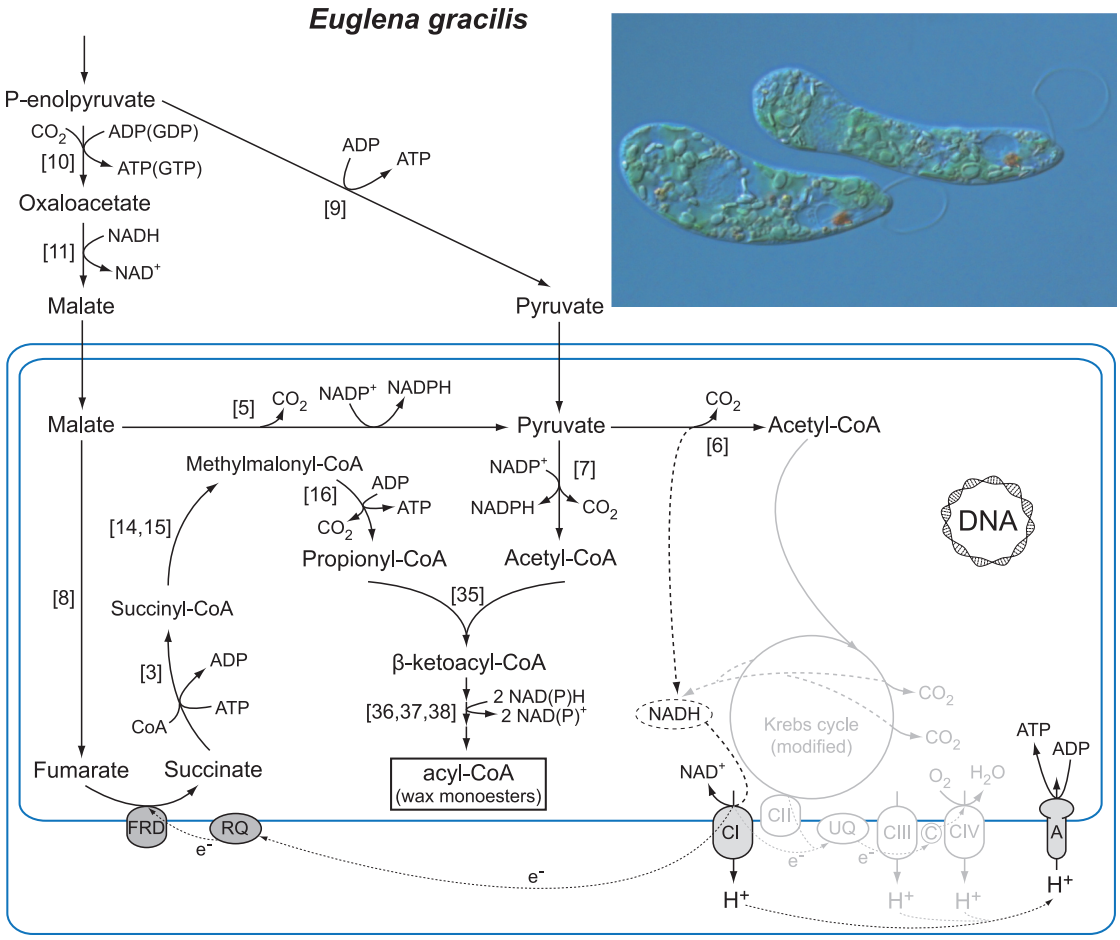


FIG 15 Wax ester fermentation in mitochondria of the facultatively anaerobic photosynthetic flagellate *Euglena gracilis*. The map is redrawn based on data reported previously (514). Under anaerobic conditions, this photosynthetic euglenid, which acquired its plastids through secondary endosymbiosis, uses acetyl-CoA produced by pyruvate:$NADP^+$ oxidoreductase (419) as the terminal electron acceptor, leading to the formation of an unusual end product among eukaryotes: wax esters (213, 443, 514). Mitochondrial wax ester fermentation includes anaerobic fumarate respiration and the same propionyl-CoA formation pathway as the one found in mitochondria of facultative anaerobic animals excreting and/or accumulating propionate. Abbreviations: CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; RQ, rhodoquinone; C, cytochrome *c*; A, ATPase; FRD, fumarate reductase; [3], succinyl-CoA synthetase; [5], malic enzyme; [6], pyruvate dehydrogenase complex; [7], pyruvate:$NADP^+$ oxidoreductase; [8], fumarase; [9], pyruvate kinase; [10], phosphoenolpyruvate carboxykinase (ATP dependent); [11], malate dehydrogenase; [14], methylmalonyl-CoA mutase; [15], methylmalonyl-CoA epimerase/racemase; [16], propionyl-CoA carboxylase; [35], α-ketoacyl synthase; [36], β-ketoacyl reductase; [37], β-hydroxyacyl dehydrogenase; [38], *trans*-2-enoyl-CoA reductase. (Photograph of *Euglena gracilis* [American Type Culture Collection] by D. J. Patterson, L. Amaral-Zettler, M. Peglar, and T. Nerad, reprinted from the Encyclopedia of Life [http://eol.org/pages/918864/overview], which was published under a Creative Commons license.)

zoa, wax ester fermentation of *Euglena* involves mitochondrial fumarate reduction and thus utilizes rhodoquinone (RQ) (196) for the synthesis of propionyl-CoA, although reduced RQ can also donate electrons to other components of the *Euglena* mitochondrial respiratory chain (73), including the cytochrome bc_1 complex and the alternative oxidase (72). Propionyl-CoA is used as the starter for the synthesis of fatty acids with odd-number chain lengths, which comprise about 50% (by weight) of hydrocarbon chains in accumulated wax esters under various conditions (235) and in different *Euglena* strains (514). Propionyl-CoA is produced via the same short methylmalonyl-CoA route as that found for animal mitochondria (443), and homologs of the underlying enzymes, methylmalonyl-CoA mutase and propionyl-CoA carboxylase (which provides ATP via substrate-level phosphorylation), are abundantly expressed in euglenid EST data (6). Methylmalonyl-CoA mutase from *Euglena* was recently characterized (335); the enzyme is also present in humans, where it is one of our only two vitamin B_{12} (cobalamin)-dependent enzymes (418), whereby *Euglena* was once the standard assay for serum B_{12} levels, because of its B_{12}-dependent ribonucleotide reductase (506).

When grown under anaerobic conditions, *Euglena* expresses PNO (216–218), which performs the oxidative decarboxylation of pyruvate and is a fusion protein, with an N-terminal PFO domain fused to a C-terminal flavoprotein domain with NAD-, FMN-, and FAD-binding modules (360, 419). The flavoprotein domain is found in many other proteins, sometimes alone as NADPH:cytochrome P450 reductase and sometimes fused to other domains, such as in nitric oxide synthases of metazoans. The flavoprotein domain is best understood as a transducer of one-electron transport (from the FeS clusters of the PFO domain) to two-electron transport (NADPH). PNO supplies acetyl-CoA and NADPH for the production of wax esters. PNO was first described for *Euglena*

and was long considered an enzyme unique to the *Euglena* lineage, but the same PNO (PFO fusion) is found in the apicomplexan *Cryptosporidium* (419) as well as in *Blastocystis* (270). More recently, PNO homologs from many disparate eukaryotic lineages have been turning up abundantly in EST sequencing projects (211), indicating that the enzyme is far more widespread than previously thought. Sequences clearly homologous to PFO occur in "typical" eukaryotes as well but in the guise of sulfite oxidases among fungi (200, 419), which exhibit fusions similar to those of the *Euglena* mitochondrial PNO.

Excavate pathogens and metronidazole. Many excavate protists, including trichomonads, *Giardia*, and *Entamoeba*, are important pathogens of humans and livestock. The most widely used selective drugs against amitochondriate parasitic protists are nitroimidazole derivatives (208, 271, 288, 541). Metronidazole (1-hydroxyethyl 2-methyl 5-nitroimidazole) was the first member of this group to be introduced and is still widely used today. The mechanism of action involves the reduction of the nitro moiety, thereby converting the nitroimidazole precursor into highly cytotoxic agents with a short half-life (201, 348, 516). The reduction can be driven by electrons from low-redox-potential donors, such as from pyruvate oxidation through pyruvate:ferredoxin oxidoreductase (114, 403) or through thioredoxin reductase (271). The products of the reduction are short-lived cytotoxic products, and their exact identity remains uncertain despite extensive research. The one-electron reduction product, a nitro free radical, and the two-electron reduction product, a hydroxylamine derivative, have been proposed to be active compounds.

Clinical resistance to 5-nitroimidazoles has been observed for *Giardia*, *Entamoeba*, and *Trichomonas* (256, 257, 262, 271, 516). Resistance can also be developed in cultured organisms exposed to increasing concentrations of the drugs. The levels of resistance seen in clinical cases and those obtainable under laboratory conditions vary across species. Moderate resistance can be obtained easily for *Trichomonas vaginalis* (482), less easily for *Giardia intestinalis* (516), and only with great difficulty for *Entamoeba histolytica* (551).

The mechanism of resistance can often be attributed to decreased levels of enzymes that activate the drug (261, 263, 403, 516, 551). With low, clinically relevant, levels of resistance in *T. vaginalis*, the organisms are less oxygen tolerant (402), indicating that modifications of an oxygen-detoxifying system affect toxicity. In laboratory strains of *E. histolytica* with decreased drug susceptibility, decreases in levels of reducing enzymes are accompanied by increased levels of enzymes involved in oxygen detoxification (551).

Alveolates and Stramenopiles

Alveolates and stramenopiles are sometimes grouped together as chromalveolates for convenience (4), but it is increasingly clear that they are not a monophyletic group (141, 432). In particular, the relationship of the cryptophytes, the haptophytes, and the other groups that were once called "chromalveolates" is presently being debated (18, 65).

Nyctotherus ovalis. *Nyctotherus ovalis* is an anaerobic ciliate that lives in the hindgut of cockroaches. This protist harbors organelles of a mitochondrial origin (class 3 in Fig. 2), considered to be a link between mitochondria and hydrogenosomes, since this organelle unites the hallmark features of mitochondria (a DNA genome and an electron transport chain) and those of hydrogenosomes (hydrogen production) (49). Electron microscopy revealed the presence of methanogenic bacteria in close association with these organelles, which demonstrates the *in vivo* production of hydrogen in these *Nyctotherus* organelles (158). The presence of a gene encoding an [Fe]-hydrogenase in the genome of *Nyctotherus* further indicated hydrogen production (48). On the other hand, the *Nyctotherus* organelle was shown to contain DNA (7, 534). Characterizations of respiratory chain components of this hydrogen-producing organelle uncovered biochemical features characteristic of anaerobic mitochondria (49). Furthermore, the organelle was shown to possess a typical ciliate mitochondrial genome (48, 49, 101); this finding established once and for all the evolutionary identity of mitochondria and hydrogenosomes as different manifestations of one and the same organelle.

Metabolic studies (49) together with the annotation of the nearly completely sequenced mitochondrial genome of *Nyctotherus* allowed a reconstruction of the organellar metabolism (101) (Fig. 16). *Nyctotherus* was shown to consume glucose as a substrate and to excrete acetate, succinate, lactate, and ethanol as end products (49). Acetate and succinate are probably produced within the hydrogen-producing mitochondrion, in which succinate production occurs via a part of the Krebs cycle (malate-fumarate-succinate), used in a reductive direction (101). Acetate is produced from pyruvate, which is converted to acetyl-CoA by pyruvate dehydrogenase (PDH) and subsequently to acetate by an acetate:succinate CoA-transferase (ASCT) belonging to subfamily 1A, with sequence similarity to the ASCT from *Trypanosoma brucei* (503). ASCT transfers the CoA moiety of acetyl-CoA to succinate, yielding acetate and succinyl-CoA, which is subsequently converted by succinyl-CoA synthetase (SCS) (also called succinate thiokinase [STK] in many papers) with the concomitant production of ATP. The oxidative decarboxylation of pyruvate to acetyl-CoA by PDH results in the reduction of NAD^+ to NADH. The oxidation of NADH in *Nyctotherus* is thought to occur in part by a truncated electron transport chain in which complex I passes the electrons from NADH through rhodoquinone to complex II, which then uses fumarate as an electron acceptor to produce succinate. Another part of NADH is possibly reoxidized by the [Fe]-hydrogenase, involving the 51- and 24-kDa subunits of complex I that are C-terminally fused to the [Fe]-Hyd catalytic subunit (48, 448), thereby releasing molecular hydrogen. The hydrogen-producing mitochondrion of *Nyctotherus* appears to generate a proton gradient (49), possibly involving complex I of the electron transport chain. This proton gradient is probably not used for ATP synthesis, as a gene encoding an ATP synthase seems to be lacking (101). The inferred metabolism of *Nyctotherus* is remarkably similar to the (also inferred) organellar metabolism of the phylogenetically distant anaerobic stramenopile *Blastocystis* (101).

Blastocystis. *Blastocystis hominis* is a common inhabitant of the human gastrointestinal tract. It has been reported to be a strict anaerobe, but it nonetheless possesses cristate mitochondria that can accumulate dyes such as rhodamine 123, MitoLight, and MitoTracker (363, 468), suggesting that it actively maintains an electrochemical proton gradient across its mitochondrial membrane. Similar to *Euglena gracilis* (419), the conversion of pyruvate to acetyl-CoA in *Blastocystis* involves an active PNO that is localized in the organelle (270), and although a PDH is encoded by the genome (468), no PDH activity was detected in the organelle (270). In addition, a PFO gene has been identified as well (103). However, enzyme assays failed to detect any PFO activity, while high levels of activity have been found for PNO (270). The trans-

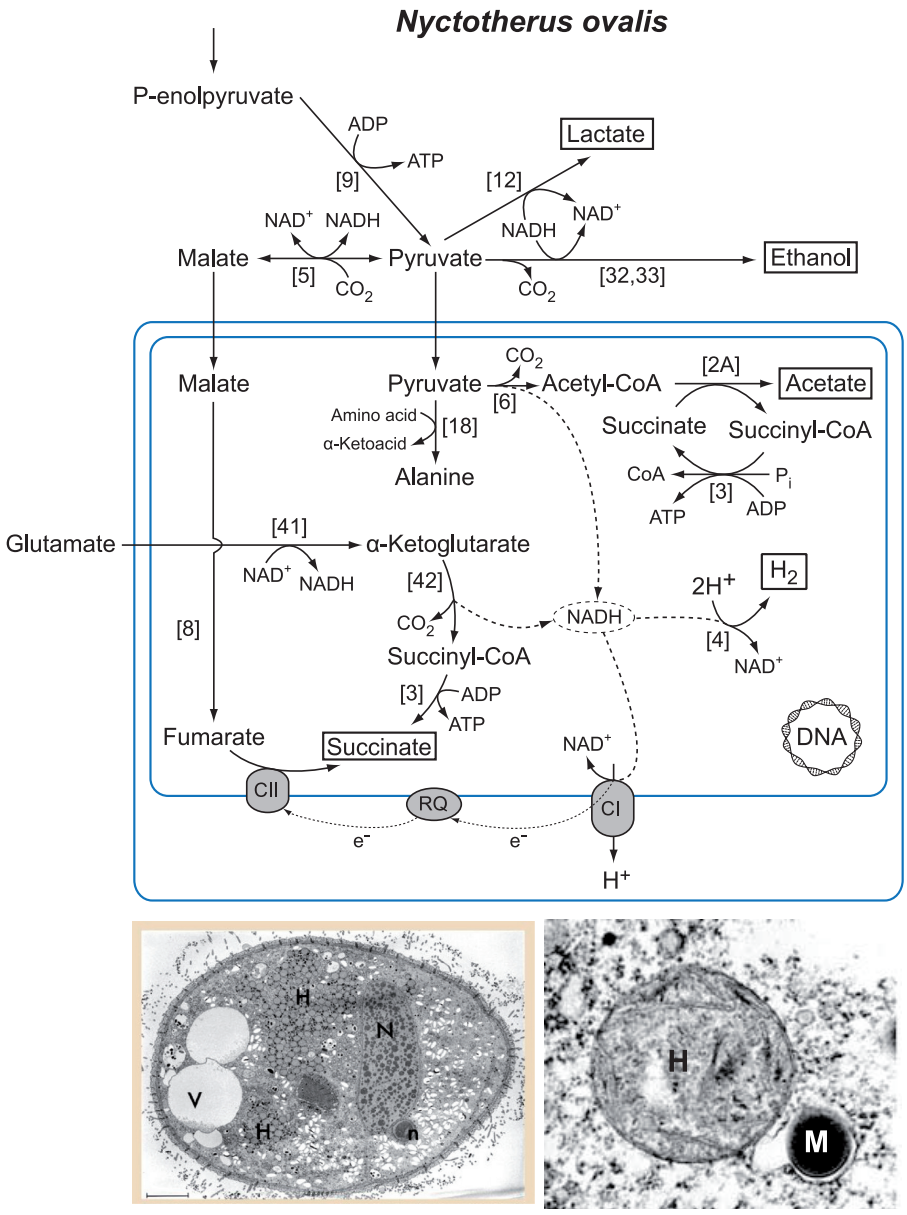


FIG 16 Tentative map of major pathways of energy metabolism in hydrogen-producing mitochondria of the anaerobic ciliate *Nyctotherus ovalis*. The map is redrawn based on data reported previously (49). The ciliate lives in the hindgut of cockroaches and harbors organelles that provided a link between mitochondria and hydrogenosomes (49). The Krebs cycle is incomplete and is likely used in the reductive direction (177). A proton gradient is generated, probably by a functional respiratory complex I, which passes the electrons from the NADH pool through rhodoquinone to complex II, acting as fumarate reductase synthesizing succinate (101). Redox balance is also achieved with the help of hydrogenase, releasing molecular hydrogen, hence the term hydrogen-producing anaerobic mitochondria (49). ATP can be synthesized by substrate-level phosphorylation, producing acetate. Abbreviations: CI, respiratory complex I; RQ, rhodoquinone; CII, fumarate reductase/succinate dehydrogenase; [2A], acetate:succinate CoA-transferase subfamily 1A (503); [3], succinyl-CoA synthetase; [4], hydrogenase; [5], malic enzyme; [6], pyruvate dehydrogenase complex; [8], fumarase (predicted); [9], pyruvate kinase; [12], lactate dehydrogenase; [18], alanine aminotransferase; [32], pyruvate decarboxylase; [33], alcohol dehydrogenase (NADH dependent); [42], α-ketoglutarate dehydrogenase (predicted); [41], glutamate dehydrogenase. The photograph on the left shows *Nyctotherus ovalis*, with a length of ca. 80 μm. The hydrogenosomes (H) are surrounded by endosymbiotic methane-producing archaebacteria (dark spots). N, macronucleus; n, micronucleus; V, vacuole. (Reprinted from reference 7 with permission of Macmillan Publishers Ltd.) The right photograph shows a closeup view of a *Nyctotherus* hydrogenosome (H) and an associated methanogen (M). (Reprinted from reference 49 with permission of Macmillan Publishers Ltd.)

formation of acetyl-CoA to acetate leads to the synthesis of ATP via substrate-level phosphorylation using an acetate:succinate CoA-transferase, succinyl-CoA synthetase cycle (197, 468), as is found for *T. vaginalis*, and high levels of activity of both enzymes in the organelle were measured (270). *Blastocystis* has an active cytosolic lactate dehydrogenase (270). From these biochemical data, it can be reasonably predicted that acetate and lactate could be major metabolic end products, but no information on accumulated *Blastocystis* end products is currently available. The genome data suggest the presence of many more mitochondrial enzymes (103, 468); accordingly, the map shown in Fig. 17, suggesting the nature of major metabolic pathways, could be incomplete.

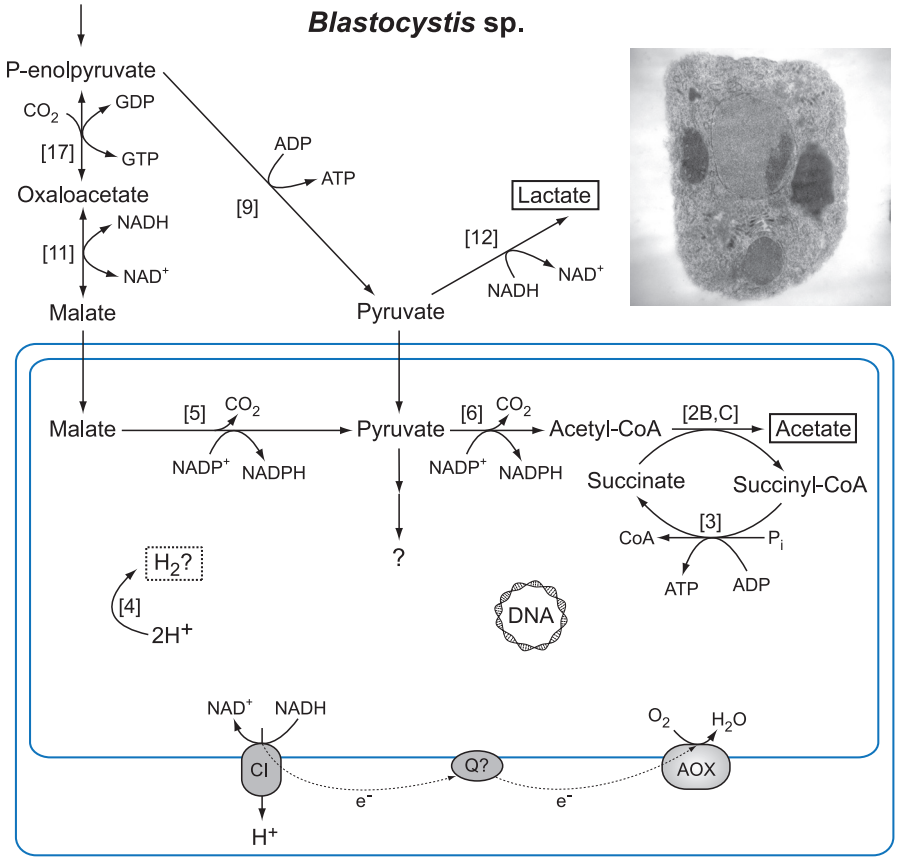


FIG 17 Putative major pathways of anaerobic energy metabolism in the organelle of mitochondrial origin in *Blastocystis hominis*, a protozoan parasite and a common inhabitant of the human gastrointestinal tract. The map is redrawn based on data reported previously (270). The conversion of pyruvate to acetyl-CoA is carried out by pyruvate:NADP$^+$ oxidoreductase (PNO) (270), the same fusion protein found in *Euglena gracilis*. The transformation of acetyl-CoA to acetate leads to the synthesis of ATP via substrate-level phosphorylation. The alternative oxidase might help *Blastocystis* cope with oxygen stress conditions in the host intestine and prevent the formation of reactive oxygen species. Hydrogen production has not yet been shown for *Blastocystis* (as indicated by a question mark), although [Fe]-Hyd localizes to the organelle (468). In this sense, the mitochondria of *Blastocystis* resemble the hydrogen-producing mitochondria found in the ciliate *Nyctotherus ovalis* and represent another example demonstrating that mitochondria and hydrogenosomes utilize a common enzyme toolkit. The question mark indicates another possible pathway (103, 270, 468). Abbreviations: CI, respiratory complex I; Q?, quinone (of an uncertain nature); AOX, alternative oxidase; [2B, C], acetate:succinate CoA-transferase (members of subfamilies 1B and 1C [503] are present in the genome); [3], succinyl-CoA synthetase; [4], hydrogenase; [5], malic enzyme; [7], pyruvate:NADP$^+$ oxidoreductase; [9], pyruvate kinase; [11], malate dehydrogenase; [12], lactate dehydrogenase; [17], phosphoenolpyruvate carboxykinase (GTP dependent). The photograph shows *Blastocystis* sp. at the trophozoite stage, with a length of ca. 35 μm. (Photograph courtesy of C. G. Clark, London School of Hygiene and Tropical Medicine, London, United Kingdom.)

The situation regarding the Krebs cycle in *Blastocystis* is confusing, as different studies have suggested different results. A large-scale EST study suggested that the enzymes for the second half of the Krebs cycle from succinyl-CoA to oxaloacetate are present (468), while no EST encoding enzymes capable of converting acetyl-CoA into succinyl-CoA was identified (i.e., no citrate synthase, aconitase, isocitrate dehydrogenase, or α-ketoglutarate dehydrogenase). On the other hand, in cell fractionation studies, the *Blastocystis* organelle was shown to contain malic enzyme, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and succinyl-CoA synthetase activities (270). Mitochondrial targeting sequences were predicted to be present on a putative fumarase (103, 468) and malate dehydrogenase (103), although the former seems more likely to represent an aspartate ammonia-lyase based on sequence comparisons. *Blastocystis* might represent a species complex of up to nine subtypes (471). A genome project (103) and a biochemical study (270) were conducted on a subtype 7 species, while an EST study (468) was conducted with subtype 1; since these subtypes belong to two different clades, this might explain many of the differences among those three studies.

Similar to *Nyctotherus ovalis*, a partial complex I seems to be present, suggesting that the organelle might have some proton-pumping potential (103, 468), in agreement with the ability of the organelle to accumulate dyes such as rhodamine 123 (363). Genes encoding complex I components were also identified in the *Blastocystis* mitochondrial genome (380, 552). In addition, all four complex II subunits were identified (103, 468), although whether this complex functions as a succinate dehydrogenase (SDH) or a fumarate reductase (FRD) is unknown, and it is not known which quinones are present. No evidence for complexes III and IV or an ATP synthase was found, in agreement with earlier biochemical studies (577). Genomic studies identified an alternative oxidase (AOX) and an [Fe]-hydrogenase. The latter protein was localized to the organelle by using antibodies (468), but hydrogenase activity was not detected (270).

Rhizaria and Denitrification

The biochemical details of anaerobic energy metabolism have not yet been characterized for any members of the eukaryotic supergroup Rhizaria. However, it has long been known that foraminiferans, members of the Rhizaria (4), are prominently represented in the protist communities of anoxic and sulfidic marine sediments (36–38). Hence, there can be little doubt that there exist broadly based anaerobic eukaryote communities in such environments. Foraminiferans have attracted considerable interest as of late with a report by Risgaard-Petersen et al. (410) that sediment-inhabiting foraminiferans are capable of performing complete denitrification, that is, the conversion of NO_3^- to N_2. In contrast to the situation described for the fungus *Fusarium oxysporum*, where the enzyme activities involved in nitrate respiration to the level of N_2 or to NH_4^+ have been characterized (574), the enzymes behind the foraminiferan pathway are not at all clear, although it was documented by 4′,6-diamidino-2-phenylindole (DAPI) staining and microscopy that the foraminiferans themselves, not associated prokaryotes, are performing the denitrification reaction (410). In a more recent report demonstrating the broader occurrence of nitrate respiration among many facultative anaerobic members of the Rhizaria (384), it is less certain that the denitrification is not due to prokaryotic partners, although the sequestration of nitrate by the rhizarians is highly suggestive that the protists themselves, and not associated symbionts, are performing nitrate respiration.

Archaeplastida

Higher plants produce oxygen but can encounter anaerobic conditions in roots or at the seedling stage due to submersion. Archaeplastida (4) is the name currently used to designate plants and algae with plastids derived from primary endosymbiosis and surrounded by two membranes (18, 472). The typical higher-plant response to anaerobiosis is the cytosolic fermentation of carbohydrate reserves, involving pyruvate decarboxylase and either alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), or both (539). In anaerobic maize seedlings, about 20 glycolytic and fermentation enzymes are induced, accounting for 70% of the soluble protein (424). However, these anaerobic responses do not involve mitochondria, except perhaps in a signaling role (336, 424). Under anaerobic conditions, some higher-plant mitochondria can produce small amounts of NO from nitrite, and this might involve ATP synthesis, but the NO respiratory rate is only about 0.1% of that measured for oxygen (175), such that the quantitative contribution to energy metabolism is minor. For unicellular green algae, the situation is very different: under conditions of anaerobiosis, *Chlamydomonas* (see below) behaves like *Trichomonas*.

Chlamydomonas. *Chlamydomonas reinhardtii* is a green alga that possesses the generalist, facultative anaerobic physiology of a typical soil microbe (326). Interest in the anaerobic metabolism of *C. reinhardtii* has risen dramatically in recent years because of its ability to produce H_2 as a possible biofuel under certain growth conditions (187, 322, 399), with the biology of hydrogenases playing a central role in that development (198, 542). When grown aerobically, the alga respires oxygen with a normal manifestation of oxidative decarboxylation via pyruvate dehydrogenase (PDH) and oxidative phosphorylation in mitochondria (70). When grown anaerobically, it produces acetate, formate, ethanol, and hydrogen as major end products (357) (Fig. 18). Different *C. reinhardtii* strains differ substantially with respect to the relative amounts of different end products accumulated, with strain-specific variations in formate and glycerol production being particularly pronounced (331).

The enzymes involved in anaerobic energy metabolism in *Chlamydomonas* are to a large extent the same as those of other anaerobic protists: [Fe]-Hyd (184), bifunctional alcohol dehydrogenase E (ADHE) (22); pyruvate:formate lyase (PFL) (21), and, characterized only at the gene and transcript levels so far, pyruvate:ferredoxin oxidoreductase (PFO) (21, 357). There are duplicate gene copies for several of the enzymes involved, and the localization of the enzymes is not yet fully resolved. The map shown in Fig. 18 is based on data presented previously (331, 357, 491, 492). One [Fe]-Hyd isozyme, HydA1, is localized in the plastid, where it can accept electrons from ferredoxin (PetF) of the photosynthetic electron transfer chain (560). Acetate production in *Chlamydomonas* appears to differ from that in most eukaryotes studied so far (503), in that a phosphotransacetylase (PTA) and acetate kinase (ACK) system is used, as earlier biochemical studies (255) and more recent mitochondrial proteomic studies (21) as well as transcriptomic studies (357) have revealed. Based on the current genome sample, ACK has numerous well-conserved homologs in other green algae, many fungi, and *Entamoeba*, while the occurrence of PTA is more restricted among green algae, the oomycete (stramenopile) *Phytophthora*, and the alveolate *Perkinsus*. The ACK activity of *Fusarium* is involved in the fungal ammonia fermentation pathway (485, 486, 574).

The catalytic mechanism of PFL involves a glycyl radical. This radical is generated by the PFL-activating enzyme (PFL-AE), which is present in *Chlamydomonas* and other eukaryotes that possess PFL (154, 467). The deactivating enzyme for PFL is ADHE, which is also present in *Chlamydomonas* and generally in eukaryotes that possess PFL (21).

Many eukaryotes that do not produce hydrogen, such as mammals, lack [Fe]-Hyd but nonetheless express homologs of [Fe]-Hyd, which are called either Nar1, Narf (29), or IOP (iron-only hydrogenase-like protein). In humans, IOP1 is involved in cytosolic FeS cluster assembly (465). In *Chlamydomonas*, the IOP homolog is named Hyd3 and also appears to be involved in cytosolic FeS cluster biogenesis (162). Several maturase proteins are required for FeS cluster formation specific for [Fe]-Hyd; these proteins are called HydG and HydEF, the eukaryotic versions of which were first characterized for *Chlamydomonas* (392). HydEFG are radical SAM (*S*-adenosyl methionine) proteins and are conserved via a common ancestry in *Trichomonas vaginalis* (396), which expresses the homologous [Fe]-Hyd.

The prospect of the use of green algae as a source of H_2 in biofuel production has sparked interest in the anaerobic metabolism of chlorophytes in general, and this is turning up some new insights. For example, a *Chlorella vulgaris* strain isolated from anaerobic sediment not only produces H_2 in anaerobic fermentative growth but also accumulates butyrate in medium (14). The finding that the land plants (derived chlorophytes) apparently have lost many of their anaerobic capabilities, or have specialized them to ethanolic fermentations involving pyruvate decarboxylase and ADH, is not surprising: like mammals, they are specialized to life above the soil line, and today's atmosphere is not prone to going anoxic. The anaerobic capabilities of *Chlamydomonas* and of the chlorophytes in general strikingly underscore the conserved na-

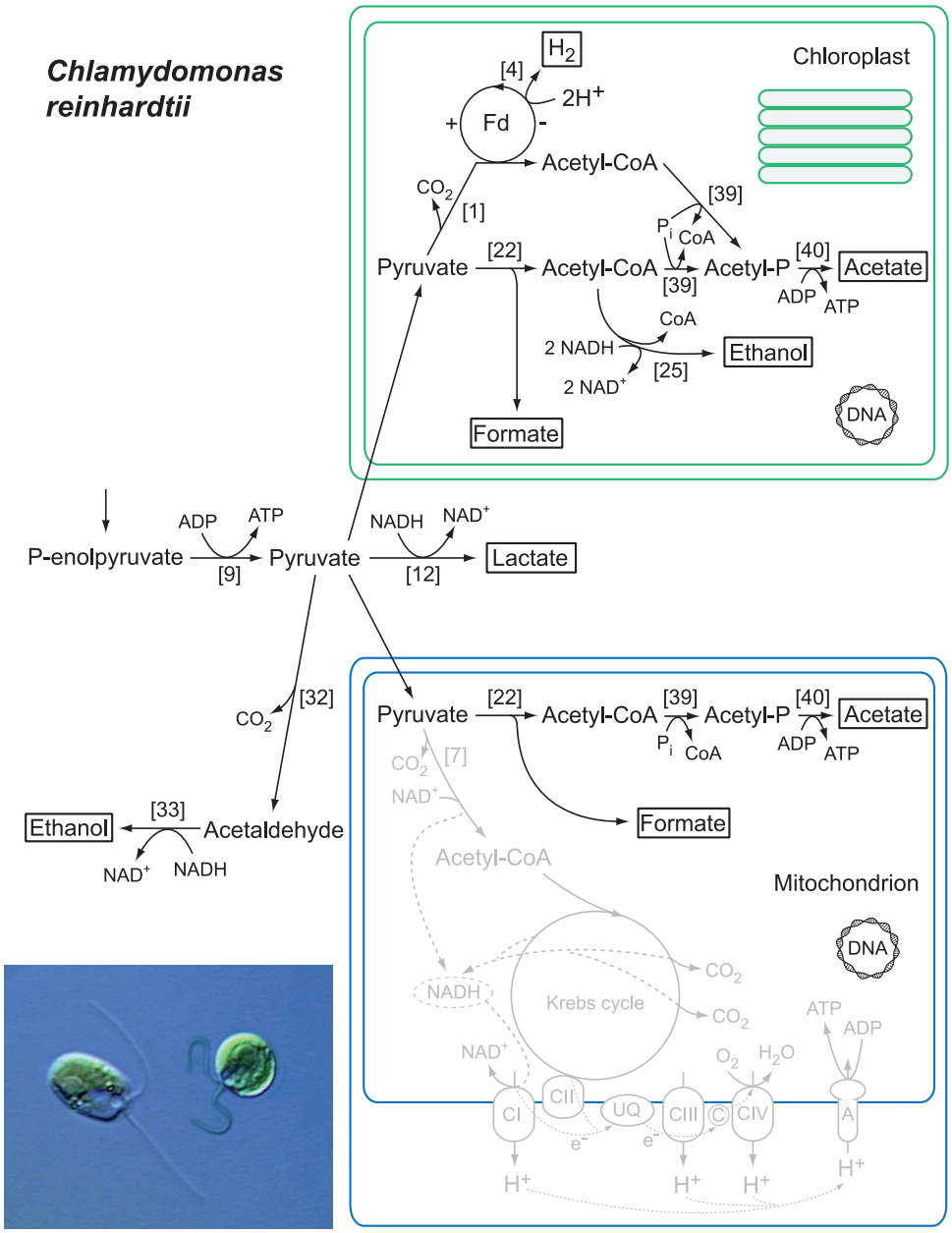


FIG 18 Major pathways of anaerobic energy metabolism in the green alga *Chlamydomonas reinhardtii*, whereby the suggestions for their compartmentation are still putative. The map was redrawn based on data reported previously (331). This typical soil inhabitant can produce oxygen but is often faced with anoxic conditions in nature (331). When grown anaerobically, *C. reinhardtii* generates virtually the same end products as those generated by *Trichomonas* but utilizing pyruvate:formate lyase, pyruvate:ferredoxin oxidoreductase, lactate dehydrogenase, and pyruvate decarboxylase (357). The localization of fermentative pathways leading to the formation of acetate, ethanol, and formate end products has yet to be fully clarified. Abbreviations: CI to CIV, respiratory complexes I to IV; UQ, ubiquinone; C, cytochrome *c*; A, ATPase; Fd, ferredoxin; [1], pyruvate:ferredoxin oxidoreductase; [4], hydrogenase; [6], pyruvate dehydrogenase complex; [9], pyruvate kinase; [12], lactate dehydrogenase; [22], pyruvate:formate lyase; [25], alcohol dehydrogenase E; [32], pyruvate decarboxylase; [33], alcohol dehydrogenase (NADH dependent); [39], phosphotransacetylase; [40], acetate kinase. The photograph shows *Chlamydomonas reinhardtii*, with a length of ca. 10 μm. (Photograph courtesy of Cytographics Inc., Victoria, Australia.)

ture of anaerobic energy metabolism across diverse eukaryotic groups.

EVOLUTIONARY CONSIDERATIONS

Evolving Concepts

It has taken several decades to reach the conclusion that in both biochemical and evolutionary terms, mitochondria are central to anaerobic energy metabolism in eukaryotes. This is quite the opposite of what everyone thought when hydrogenosomes were discovered in the early 1970s, a time when even the endosymbiotic origin of mitochondria and chloroplasts was not obvious (43, 74). This also contrasts with the consensus of the 1980s, when the endosymbiotic origin of mitochondria was undisputed. For various reasons, anaerobic protists, including trichomonads, diplo-

monads, and microsporidians, came to be viewed as primitive lineages of eukaryote evolution that branched off before the origin of mitochondria (75, 349, 464, 546, 562).

There was also a window of time when the notion was entertained that hydrogenosomes were descended from a *Clostridium*-like endosymbiont that was distinct from the mitochondrion (347, 553), but in the 1990s, it became evident that the "early-branching" status of anaerobic protists was an artifact of molecular phylogenetics and that the hydrogenosomes of various lineages are mitochondria in the evolutionary sense (133, 382). The subsequent discovery of mitosomes and their characterizations in several disparate lineages made a strong case for the ubiquity of mitochondria (300, 507, 508, 558). In the past 10 years, it became clear that all eukaryotes possess an organelle of mitochondrial origin (Fig. 2) (134, 454, 458). New order came to the eukaryotic tree of life, in which anaerobic protists such as trichomonads, diplomonads, and microsporidians found homes next to mitochondrion-bearing lineages within the six or so eukaryotic supergroups currently recognized (4, 25, 180, 192, 237, 457) (Fig. 3). Mitochondria are thus ubiquitous among eukaryotes, and they are also germane to energy metabolism in eukaryotic anaerobes. In energetic terms, mitochondria themselves, whether aerobic or anaerobic (not using oxygen in the role of the terminal electron acceptor), now appear causal to the origin of eukaryote complexity in that they removed the tight constraints that prokaryotic cell organization places on the prokaryotic genome and cell complexity (267); that is, the foundation of the eukaryote cell complexity rests on the origin of >1,000 novel gene families that arose in the eukaryote common ancestor, those supporting the eukaryotic cell cycle, endomembrane traffic, flagella, and the like. That phase of gene invention required amounts of energy per gene (or per genome) that only the internalized configuration of bioenergetic membranes afforded by mitochondria can supply (267).

Anaerobic Energy Metabolism: Present in the Eukaryote Common Ancestor

Throughout those developments, the central evolutionary question has been, What is the origin of anaerobic energy metabolism in eukaryotes? From the previous sections of this review, it has hopefully become evident that anaerobic protists possess a few enzymes, about 10, that mammals do not. In contrast, anaerobic metazoans survive by using the same enzymes as humans do in their mitochondria albeit with parts of the Krebs cycle running backwards—including a modified succinate dehydrogenase (SDH) functioning as a fumarate reductase (FRD) (416, 433, 500)—and with complex I reducing rhodoquinone (RQ) instead of ubiquinone (UQ). Thus, for the origin of anaerobic energy metabolism in metazoans, there is no differential gene content whatsoever to account for, only the origin of the cofactor RQ, whose biosynthesis is so far unresolved, whereby we recall that RQ also occurs in metazoans such as *Caenorhabditis* (484) and in protists such as *Euglena* (196). For the origin of anaerobic energy metabolism in protists, the question is, Were the genes present in the single eukaryote common ancestor, or do they clearly reflect multiple origins, and if the former is true, does their single origin coincide with the origin of mitochondria? (131, 309, 501). This has in turn given rise to two main competing alternative hypotheses for the origin of anaerobic energy metabolism in protists: (i) the enzymes were present in the eukaryote ancestor and were inherited vertically by modern groups, or (ii) they were lacking in the eukaryote ancestor (which would then implicitly have been a strict aerobe) and were acquired in different eukaryotes groups independently via lateral gene transfers (LGTs). Those views generated very different predictions with regard to the evolutionary patterns of the underlying genes.

If the enzymes of anaerobic energy metabolism were present in the unicellular eukaryote common ancestor, different eukaryotic groups should tend to have different subsets of the same ancestral collection of genes, allowing for differential loss, and some eukaryotes should have preserved nearly the entire set.

However, if anaerobic energy metabolism was lacking in the eukaryote common ancestor and had been acquired independently by different eukaryotic lineages, eukaryotic aerobes should lack such genes altogether, and the anaerobes of different eukaryotic lineages should tend to possess fundamentally different collections of genes reflecting acquisitions from the multitude of ways in which prokaryotic anaerobes can conserve energy in anaerobic habitats.

The distribution of the enzymes for the pathways underlying anaerobic energy metabolism presented here reveals that there is no fundamental difference between the major eukaryotic groups surveyed with respect to either an aerobic or anaerobic life-style or the enzymes involved. All major groups of eukaryotes currently recognized harbor aerobes and anaerobes (Fig. 3), and if we compare the anaerobes, the following is observed.

For the metazoans, the core anaerobic pathway typified by *Fasciola* consists of (i) malate dismutation involving malic enzyme (ME), pyruvate dehydrogenase (PDH), and conventional Krebs cycle enzymes and (ii) the propionyl-CoA cycle, involving four enzymes that are also expressed in humans, methylmalonyl-CoA mutase, methylmalonyl-CoA epimerase, propionyl-CoA carboxylase, and propionate:succinate CoA-transferase. The metazoan FRD enzymes are currently indistinguishable from SDH at the level of the primary structure (501), acetate:succinate CoA-transferase (ASCT) homologs function in aerobes (503), and complex I accepts both UQ and RQ as substrates, such that the only truly "special" component of the metazoan anaerobic mitochondrion core pathway is RQ.

For the protists, the enzymes of *Trichomonas* hydrogenosomes, pyruvate:ferredoxin oxidoreductase (PFO), [Fe]-Hyd, ASCT, and succinyl-CoA synthetase (SCS) (a Krebs cycle enzyme), occur in combinations with various other enzymes which themselves are not lineage specific (as genome database searching quickly reveals). Examples include soluble FRD (present in *Tritrichomonas* and in *Trypanosoma*), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (*Tritrichomonas* and plants), a bifunctional aldehyde/alcohol dehydrogenase (ADHE) and acetyl-CoA synthetase (ACS) (ADP forming) (*Giardia* and *Entamoeba*), the propionyl-CoA cycle (*Blastocystis*), Krebs cycle enzymes (*Nyctotherus* and *Euglena*), pyruvate:formate lyase (PFL) (*Piromyces* and *Chlamydomonas*), acetate kinase (ACK) and phosphotransacetylase (PTA) (also called phosphate acetyltransferase) (*Chlamydomonas*; both are also present in the oomycete *Phytophthora*, in the apicomplexan relative *Perkinsus*, and in the moss *Physcomitrella*), or PFO fused to an $NADP^+$-reducing domain as pyruvate:$NADP^+$ oxidoreductase (PNO) (*Euglena*, *Blastocystis*, *Perkinsus*, and the apicomplexan *Cryptosporidium*) or fragmented across proteins as sulfite reductase in fungi (200, 419).

The differences in anaerobic energy metabolism observed

among the protist lineages are minor and clearly reflect, in our view, differential loss from a facultatively anaerobic ancestral state of eukaryotic energy metabolism. The different lineages possess merely different subsets of the same ancestral collection of genes. Furthermore, almost the entire collection is preserved within *Chlamydomonas reinhardtii* (21). We consider this to be very strong evidence in favor of the enzymes being present in the eukaryote common ancestor. Ginger et al. (161) surveyed the phylogenetic distributions of some of the same enzymes and found evidence for the presence of genes for anaerobic energy metabolism in the eukaryote common ancestor, followed by differential loss.

Had the origin of the anaerobic life-style in the different eukaryotic lineages surveyed here entailed independent lateral gene acquisitions, as has been suggested (16, 211), one would expect different eukaryotic lineages to have acquired different collections of genes as a result of their independent solutions to the anaerobic survival problem. However, this has not been observed. For example, there are no known eukaryotic anaerobes that perform sulfate reduction, anaerobic ammonium oxidation, anaerobic methane oxidation, uranium oxide reduction, or dozens of other anaerobic energy metabolic pathways (13), even though eukaryotes inhabit environments where such prokaryote physiologies are abundant and successful strategies (291, 473).

Instead, eukaryotic anaerobes of different lineages possess different subsets of a common stock of only about 50 genes and enzymes (Table 1). This is a very modest number of basic tools in the anaerobic toolbox, similar to the number of genes required for subunits of mitochondrial complex I. These 50 enzymes are used in various combinations in different lineages—minor variations on a theme, as the maps presented in this paper show. If we examine the distribution of the underlying genes across different eukaryotic groups using a standard database search approach (Fig. 19), it is clear that there are no patterns to suggest lineage-specific acquisitions. Rather, the genes in question were present in the eukaryote common ancestor and have undergone differential loss in various lineages.

It follows that the ancestral eukaryote stem was able to respire oxygen when present but in the absence of oxygen was also able to perform anaerobic respiration and fermentations of the type found for the green alga *C. reinhardtii*. This free-living soil inhabitant can gladly respire oxygen like humans, but after 30 min of anaerobic growth in the dark, it uses the same enzymes as those used by *Trichomonas* or *Piromyces* to produce the same spectrum of metabolic end products as those heterotrophs (331, 357). Clearly, *Chlamydomonas* has preserved the largest subset of ancestral eukaryote gene diversity for an anaerobic life-style (21) characterized so far (Fig. 19), while some highly reduced lineages, such as the microsporidians, have lost almost all energy metabolic functions (Fig. 19), in accordance with their life-style as energy parasites (238, 512, 554, 558).

Further evidence for the presence of anaerobic energy metabolism in the eukaryote common ancestor comes from the presence of derived versions of [Fe]-Hyd and PFO in the genomes of eukaryotic aerobes. Narf, also called Nar1 or Hyd3, is a eukaryote-specific derivative of [Fe]-Hyd that has conserved all but one (the H-cluster) of the FeS clusters of [Fe]-Hyd (199) and is present in all eukaryotes sampled so far. In *Chlamydomonas*, the Hyd3 protein, like its homologs in yeast and humans, is involved in FeS cluster biogenesis (162). This indicates that [Fe]-Hyd was present in the eukaryote common ancestor, where it duplicated to give rise to Narf (Hyd3), which then acquired an essential role in FeS biogenesis that has remained conserved across eukaryotes, even after the loss of the ancestral [Fe]-hydrogenase gene in many oxygen-adapted lineages. Another such example is the sulfite oxidase of many oxygen-adapted fungi, an enzyme consisting of two catalytic subunits corresponding to the yeast Met5 and Met10 genes (88), which are derived from eukaryotic PNO via domain rearrangement (200, 419). The presence of functionally specialized forms of PNO [a fusion of PFO and a one-electron-accepting, $NAD(P)^+$-reducing domain] and [Fe]-Hyd in diverse eukaryotic aerobes suggests that the genes were present in a facultatively anaerobic eukaryote common ancestor.

A notable aspect of Fig. 19 is that virtually all of the enzymes of eukaryotic energy metabolism are more similar to proteobacterial homologs than to archaebacterial homologs, with enolase being the possible exception (183). This pattern of similarity is not at all surprising for proteins germane to mitochondrial energy conservation, because of the eubacterial origin of mitochondria. The eubacterial nature of cytosolic proteins involved in eukaryote energy metabolism was initially more puzzling, however. It became evident through studies of individual genes (183, 204, 304, 311, 313) and stood in contrast to the findings, emerging at the same time, that eukaryotic transcription (269) and translation (219, 563) proteins are of an archaebacterial nature. This functional and phylogenetic dichotomy of eukaryotic genes into operational (for biochemical functions, mainly eubacterial) and informational (for gene expression, mainly archaebacterial) classes (412) is now well documented (89, 139) and most easily attributed to endosymbiosis and gene transfer at the origin of eukaryotes, in the simplest scenario, gene transfer from the common ancestor of mitochondria and hydrogenosomes to an archaebacterial host (91, 309). It could be argued that increased evolutionary rates in archaebacterial proteins might be able to explain patterns such as those shown in Fig. 19 (76), but specific investigations of ancient duplicate gene pairs showed that the evolutionary rate of archaebacterial genomes is not increased at all; if anything, it is lower than that of eubacteria (95).

Hence, the similarity between enzymes of eukaryotic and eubacterial energy metabolisms, in particular the glycolytic pathway (309), reflects a eubacterial origin of the eukaryotic pathway. Indeed, the glycolytic (Embden-Meyerhof) pathway, as it is manifest in eukaryotes (148), is generally lacking in heterotrophic archaebacteria altogether; they use modifications thereof or modifications of the Entner-Doudoroff pathway instead (34, 456). Moreover, archaebacterial enzymes entailing glucose oxidation to pyruvate are often altogether unrelated to their eukaryotic and eubacterial counterparts. Many examples of archaebacterial enzymes with "glycolytic" activities that are unrelated to eukaryotic counterparts were summarized by Siebers and Schönheit (456); these include glucokinase (ribokinase family), phosphoglucoisomerase (cupin family), the ADP-dependent phosphofructokinases (ribokinase family), the archaeal type I aldolase, the phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the ferredoxin-dependent glyceraldehyde-3-phosphate oxidoreductase. Underscoring how different the glucose-to-pyruvate conversions in archaebacteria and eukaryotes are, Say and Fuchs (435) recently discovered the archaebacterial fructose 1,6-bisphosphate aldolase/phosphatase, a bifunctional enzyme

TABLE 1 Enzymes of core anaerobic energy metabolism in eukaryotes

Enzyme	Designation[a]	EC no.	Reference(s)	Fig.
α-Ketoglutarate dehydrogenase (Krebs cycle)	42	1.2.4.2	270, 538	14, 16
Acetate:succinate CoA-transferase, members of subfamilies 1A, 1B, and 1C[b]	2A	2.8.3.8	438, 503, 528, 529, 533	4–9, 12–14, 16, 17
Acyl-CoA transferase	2B			
Propionate:succinate CoA-transferase	2C			
Acetate kinase	40	2.7.2.15	21, 255, 485	18
Acetyl-CoA synthetase (ADP forming)	30	6.2.1.13	428	10, 11
L-Alanine aminotransferase	18	2.6.1.2	28, 169, 404, 445	6–8, 10–14, 16
Glutamate:pyruvate transaminase				
Pyruvate aminotransferase				
L-Alanine racemase	21	5.1.1.1	445	7
Alanopine dehydrogenase	20	1.5.1.17	143	7
Alcohol dehydrogenase (NADH dependent)	33	1.1.1.1	230	16, 18
Alcohol dehydrogenase (NADPH dependent)	34	1.1.1.2	284	12, 13
Alcohol dehydrogenase E	25	1.1.1.1	60, 567	9–11, 18
Alcohol/aldehyde dehydrogenase		1.2.1.10		
Aspartate aminotransferase	19	2.6.1.1	404, 445	6–8
Glutamate:oxaloacetate transaminase				
Glutamate aminotransferase				
α-Hydroxyacyl dehydrogenase	37	1.1.1.35	561	15
α-Ketoacyl reductase	36	1.1.1.35	561	15
α-Ketoacyl synthase	35	2.3.1.94	559	15
Citrate synthase	43	2.3.3.1	441, 538	14
Fumarase/fumarate hydratase	8	4.2.1.2	354	4–9, 13–16
Fumarate reductase (membrane bound)	FRD	1.3.5.1	501	4–8, 15
Fumarate reductase (soluble)	24	1.3.1.6	354	9, 13, 14
Glutamate dehydrogenase	41	1.4.1.2	125	16
Hydrogenase (iron only)	4	1.12.7.2	64, 198, 378	9, 11–13, 16–18
Lactate dehydrogenase	12	1.1.1.27	284	9, 12, 16–18
Malate dehydrogenase	11	1.1.1.37	202	4–15, 17
Malic enzyme	5	1.1.1.39	124	4–17
Methylmalonyl-CoA epimerase/racemase	15	5.1.99.1	42	4–8, 15
Methylmalonyl-CoA mutase	14	5.44.99.2	301, 335, 418	4–8, 15
NADH dehydrogenase, 51-kDa and 24-kDa subunits	31	1.6.5.3	209	12
NAD(P)H:ferredoxin oxidoreductase	23	1.18.1.2/3	317	9
Octopine dehydrogenase	27	1.5.1.11	143, 462	6, 8
Phosphoenolpyruvate carboxykinase (ATP)	10	4.1.1.49	511	4, 6, 7, 9, 14, 15
Phosphoenolpyruvate carboxykinase (ITP/GTP)	17	4.1.1.32	278, 511	5, 8, 11–13, 17
Phosphoenolpyruvate carboxytransferase (PP_i)	28	4.1.1.38	406	10
Phosphotransacetylase	39	2.3.1.8	255	18
Propionyl-CoA carboxylase	16	6.4.1.3	185	4–8, 15
Pyruvate:ferredoxin oxidoreductase	1	1.2.7.1	240	10–13, 18
Pyruvate:formate lyase	22	2.3.1.54	21, 47	9, 18
Pyruvate:$NADP^+$ oxidoreductase	7	1.2.1.51	217, 270, 419	15, 17
Pyruvate:orthophosphate dikinase	29	2.7.9.1	405	10, 11
Pyruvate decarboxylase	32	4.1.1.1	252, 477	12, 13, 16, 18
Pyruvate dehydrogenase complex	6	1.2.4.1	575	4–8, 14–16, 18
Pyruvate kinase	9	2.7.1.40	330	6–18
Strombine dehydrogenase	26	1.5.1.22	388	6–8
Succinyl-CoA synthetase/succinate thiokinase	3	6.2.1.5	179, 226, 227	4–9, 12–17
trans-2-Enoyl-CoA reductase	38	1.3.1.38	197	15

[a] Refers to the enzyme designations in the figures and figure legends.
[b] See reference 503.

that is unrelated to both eukaryotic aldolases and eukaryotic fructose 1,6-bisphosphatase while fulfilling the function of both but probably in the gluconeogenetic direction. The inclusion of representative eubacteria and archaebacteria in Fig. 19 underscores the eubacterial nature of eukaryotic energy metabolism, while the distribution of the genes across major eukaryotic groups underscores their presence in the eukaryote common ancestor.

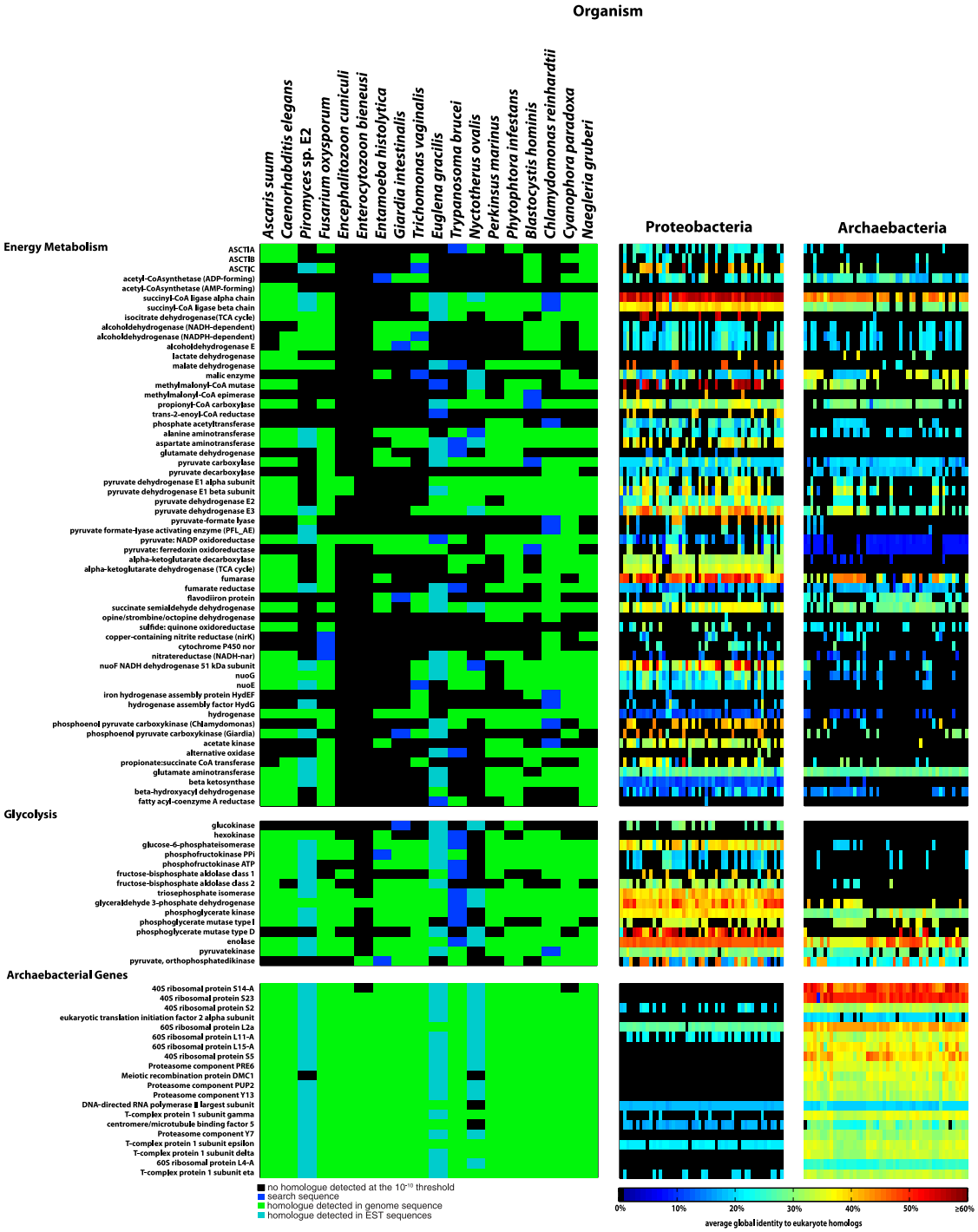


FIG 19 Presence or absence of genes for anaerobic energy metabolism across major eukaryotic groups. For each protein, sequences of suitable functionally characterized enzymes were used to search the databases via BLAST (12); the presence or absence of the gene and the average amino acid identity in pairwise local alignments between the eukaryotic and prokaryotic proteins are color coded as shown. Note the pattern of the presence or absence of genes for anaerobic energy metabolism and glycolysis and those most frequent in archaebacteria across major groups of eukaryota, proteobacteria, and archaea (see Table S1 in the supplemental material). For each protein, suitable functionally characterized sequences (Table 1) were used to search the available sequence data for the organisms shown via BLAST (12). The presence of a certain gene was scored as the best match at an E value threshold of 10^{-10}. For the eukaryotes, the colors show if the genes were found by EST data (light blue) or protein data (green) or comprised the searched sequence itself (dark blue). In the cases of the prokaryotes, the colors indicate the average global pairwise identity of the homolog in the corresponding prokaryote to all eukaryotic homologs (coded as shown). The archaeal genes were included as a control. Eukaryotic proteomes and ESTs were downloaded from RefSeq, GenBank, and, in the case of *Fusarium oxysporum*, the Broad Institute website (www.broadinstitute.org/). Prokaryotic proteomes were downloaded from RefSeq, version March 2011. Data for the archaebacterial genes were reported previously (139) and serve to demonstrate that archaebacterially related genes in eukaryotes are readily detected.

Comparing Diversity: Eukaryotes versus *Rhodobacter*

The gene presence or absence criterion is a robust source of evidence for the presence of anaerobic energy metabolism in the eukaryote common ancestor. Similar evidence has been found for the distribution and utilization of rhodoquinone (RQ). RQ is a comparatively rare quinone among prokaryotes (366), reported so far only for facultatively anaerobic *Rhodobacter*-related alphaproteobacteria (191) and two betaproteobacteria (50). Among eukaryotes, it has been reported for organisms that use membrane-bound fumarate reductase in anaerobic redox balance, such as parasitic helminths (500), the free-living nematode *Caenorhabditis elegans* (484), and *Euglena* (196). It is required for quinol-based fumarate reduction instead of ubiquinone (UQ) because of its lower redox potential (501). RQ provides a robust biochemical link between the anaerobic energy metabolism found in mitochondria and that found in facultatively anaerobic alphaproteobacteria.

This brings us to a notable observation: the whole spectrum of known energy metabolic pathways in eukaryotes encompasses less diversity than can be found in one single individual typical generalist purple nonsulfur bacterium (alphaproteobacterium). This is illustrated by the example of species such as *Rhodospirillum rubrum* or *Rhodobacter capsulatus*, where aerobic respiration (8, 31, 166) is found, where the same spectrum of fermentation end products is found as among eukaryotes during anaerobic heterotrophy (447) and where FRD and RQ are found (190). However, on top of that, *Rs. rubrum* and *Rd. capsulatus* can also perform anaerobic photolithoautotrophy (479, 550), anaerobic photoheterotrophy (35), nitrate respiration (451), dimethyl sulfoxide respiration (425), or growth on carbon monoxide (239), to name a few of the possibilities, in addition to being able to fix nitrogen in the light or in the dark (425), if needed.

Seen from this perspective, anaerobic energy metabolism in eukaryotes carries the rather unmistakable imprint of a single acquisition via endosymbiosis in that it was acquired once, it represents a very narrow sample of preexisting prokaryotic metabolic diversity, and, at least with regard to an important quinone, RQ, it is traced physiologically to a particular group (facultatively anaerobic alphaproteobacteria). This situation is similar to the cyanobacterial origin of plastids, which also entailed the single acquisition of a small sample of preexisting photosynthetic diversity via a plastid endosymbiont (165), followed by specialization and differential loss (of phycobilisomes in some lineages, for example). Beyond the mitochondrion, there is no evidence for the participation of any other endosymbionts in the origin of the primarily heterotrophic eukaryotic groups, involving microbodies (152), flagella (225), or otherwise (314).

Forests, Trees, and Vertically Inherited Chimerism

The origin of anaerobic energy metabolism in eukaryotes has been studied by using phylogenetic analyses. Investigations have been of two basic types: (i) studies addressing the relationship of mitochondria to free-living prokaryotes, generating inferences about the physiology of the free-living ancestor of mitochondria, and (ii) single-gene phylogenies inferring the phylogenies of individual enzymes involved in anaerobic energy metabolism. There have been more reports of both approaches than we can cover in any depth; hence, we focus on a summary of recent trends.

The origin of mitochondria has been extensively studied from the standpoint of phylogenetic analyses of gene products encoded by mitochondrial DNA (167). Conflicting results have been obtained with mitochondrial genes, which are usually strung together (concatenated) into one big alignment for such studies, with different analyses pointing to three basic kinds of results: an origin of mitochondria from *Rickettsia*-like ancestors (16), an origin from *Rhodobacter*-like facultatively anaerobic alphaproteobacteria (2, 565), or an origin from free-living oceanic alphaproteobacteria of the SAR clade (52, 497). Phylogenetic analyses of mitochondrial genes together with homologs from free-living prokaryotes are technically challenging because of the AT richness and rapid evolutionary rate of mitochondrial DNA. This can lead to artifacts and conflicting phylogenetic results, depending on how these effects are computationally taken into account, especially because the alphaproteobacteria harbor several AT-rich and rapidly evolving lineages (497).

Mitochondrial genes are not, however, the only source of phylogenetic information to investigate the evolutionary affinities of the mitochondrial ancestor, whereby analyses of nuclear-encoded proteins of mitochondrial origin have also produced conflicting results. Trees for pyruvate dehydrogenase subunits pointed to *Rickettsia*-like ancestors (16). Trees for Krebs cycle and glyoxylate cycle enzymes (441) as well as trees for >200 nuclear-encoded mitochondrial proteins from *Chlamydomonas* point more frequently to origins from generalist, facultatively anaerobic alphaproteobacteria (20) than to origins from *Rickettsia*-like ancestors, whereby many proteins indicated a eubacterial but not a specifically alphaproteobacterial ancestry. A recent analysis of 86 yeast nuclear-encoded mitochondrial proteins produced a similar result: some pointed to *Rickettsia*-like ancestors, and some pointed to facultatively anaerobic *Rhodobacter*-like ancestors (2). Clearly, nuclear genes for anaerobic energy metabolism cannot implicate *Rickettsia*-like ancestors, because rickettsias are strict aerobes that harbor no genes of anaerobic energy metabolism for comparison. Using an automated pipeline for phylogenetic trees, Gabaldon and Huynen (153) identified 630 nuclear-encoded protein families that trace to the ancestor of mitochondria, whereby the alphaproteobacteria seldom formed a monophyletic group. Those researchers also found that a large fraction of nuclear-encoded gene products that were acquired from the mitochondrial ancestor now function in the cytosol or other compartments (153), the same result that earlier studies of gene acquisitions from cyanobacteria in plants had uncovered (312). This makes sense, because genes that were transferred from the mitochondrion to the host before the mitochondrial protein import machinery had evolved were expressed in the host's cytosol (309). In a genomewide supertree analysis, nuclear genomes of nonphotosynthetic eukaryotes even branched within the alphaproteobacteria (385), a result most easily explained by the circumstance that eukaryotes acquired many of their nuclear genes from the ancestor of mitochondria (139, 253, 254, 267, 308, 504).

However, supertrees and concatenated alignments blend often conflicting phylogenetic results from many genes into a single result. If sequence alignments are analyzed individually, rather than as a concatenated alignment, both mitochondrial genes and nuclear genes of a mitochondrial origin trace not to a single apparent donor but to many different apparent donors. The mitochondrial genome of the jacobid *Reclinomonas americana* (268) is still the most gene-rich mtDNA known and, hence, figures prominently in such studies. It contains 63 protein-coding genes, only

about 55 of which are well conserved enough to permit the construction of alignments and trees. Trees for those 55 proteins encoded by *Reclinomonas* mitochondrial DNA give conflicting results, some proteins point to *Rickettsia*-like ancestors, some point to *Rhodobacter*-like ancestors, and some point to *Rhizobium*-like ancestors, while some *Reclinomonas* mitochondrially encoded proteins do not reflect an alphaproteobacterial ancestry at all but, more generally, a eubacterial ancestry (139). A more recent reanalysis of the *Reclinomonas* mitochondrially encoded protein set produced a similar result: some pointed to *Rickettsia*-like ancestors, and some pointed to *Rhodobacter*-like ancestors (2).

The fact that different genes of mitochondrial origin or different genes of mitochondrial DNA can be traced to many different prokaryotic genomes, rather than one single contemporary lineage, is not usually taken to indicate that mitochondria had multiple origins. Indeed, we can be sure that mitochondria had only one single origin (121, 134, 167, 521). Gene trees indicating origins of mitochondria from different lineages have at least two main causes: phylogenetic error and lateral gene transfer (LGT) among prokaryotes. Many branching discrepancies can be readily explained by the errors inherent to phylogenetic inference, such as long-branch attraction, shifts in functional constraints, lineage-specific biases, rate differences, AT richness, and the like (212, 497). As a particularly prominent example of the influence that phylogenetic methods can have on the issue of inferences of mitochondrial and hydrogenosomal evolution, Dyall et al. (128) analyzed the 51-kDa subunit of complex I from *Trichomonas* hydrogenosomes and reported evidence for a separate origin of mitochondria and hydrogenosomes, while Hrdy et al. (209) analyzed the same data using different phylogenetic methods that can recover more ancient evolutionary information from the data and found evidence for a common ancestry for mitochondria and hydrogenosomes.

The second reason why different genes of a mitochondrial origin point to different mitochondrial ancestors is that the role of LGT among free-living prokaryotes complicates the issue in a manner that was long overlooked. The proteobacterium that gave rise to mitochondria lived ≥1.5 billion years (Ga) ago. It had a specific (as-yet-unknown) collection of genes in its genome, and since the time of mitochondrial origin, there has been ample opportunity for its free-living sisters (the immediate relatives of mitochondria that did not become endosymbionts) to undergo gene transfer with other prokaryotes in the wild (2, 140, 409, 494a). The endosymbiont itself remained isolated from further gene exchange, as we see for modern endosymbiotic bacteria (339). The result of this effect as it applies to gene phylogenies involving modern genomes is that different genes in mtDNA and different nuclear genes of mitochondrial origin appear to stem from different and diverse bacterial lineages, even though they were brought in by one and the same mitochondrial endosymbiont (2, 306, 409). A gene acquired via the mitochondrion should thus tend to show a single origin for eukaryotes (131, 134), but it might not show a specifically alphaproteobacterial ancestry, because like other proteobacteria, alphaproteobacteria have been undergoing LGT over evolutionary time (244).

Hence, very many genes of eukaryotes reflect a presence in the eukaryote common ancestor and a eubacterial ancestry but not specifically an alphaproteobacterial ancestry (89, 411, 412, 494a). A recent example for a single origin is pyruvate:formate lyase (PFL) (467), where one can interpret the data as evidence for (i) lateral gene transfer from clostridia to a eukaryote, followed by eukaryote-to-eukaryote gene transfers of an unidentified nature, mimicking a single origin of the eukaryotic gene (467), or (ii) a single acquisition from a mitochondrial endosymbiont in the eukaryote ancestor, followed by the transfer of the gene among prokaryotes (494a). The latter interpretation acknowledges that prokaryotes, especially proteobacteria (244), do indeed acquire and donate genes avidly during evolution (123), via known mechanisms (94, 389). There is no reason to assume that the free-living relatives of mitochondria were immune to LGT.

It is becoming increasingly apparent that the genome of the ancestor of mitochondria was itself a mosaic of genes from different sources (2, 306, 409), just like modern alphaproteobacterial genomes are today (140, 244). Thus, the collection of genes that eukaryote nuclear genomes acquired from the mitochondrial endosymbiont represents a kind of vertically inherited chimerism.

Without forgetting those many trees, there is also a more forest-like observation to be made regarding eukaryotic anaerobes: as lineage and genome samplings for eukaryotic anaerobes have gradually improved over 4 decades since the discovery of hydrogenosomes, the same few genes for anaerobic energy metabolism have been found again and again in the various new eukaryotic lineages sampled (161, 349, 353, 355), pointing clearly to the presence of those genes in the eukaryote common ancestor. During the same time, exactly the opposite trend has been observed for prokaryotic anaerobes: as prokaryotic lineage sampling has increased over the last 4 decades, the diversity of known metabolic types, core energy metabolic reactions, and the genes underlying both have skyrocketed (13). Bearing witness to the burgeoning metabolic diversity of prokaryotes is the circumstance that fundamentally new core metabolic enzymes and pathways are still being discovered among prokaryotes (34, 188, 233, 274, 372, 400, 408, 435). In contrast, the *Chlamydomonas* nuclear genome comes close to containing the complete collection of genes involved in anaerobic energy metabolism across all eukaryotes (21). No prokaryote genome comes anywhere close to containing the full spectrum of genes involved in anaerobic energy metabolism across all prokaryotes.

There is clearly a fundamental difference between prokaryotes and eukaryotes with respect to how they generate lineage-specific genetic novelty: prokaryotes use LGT, allowing different lineages to assemble virtually limitless new combinations of genes, while eukaryotes use genome duplications and sex (26), allowing them plenty of room to generate new morphological types but little room to improvise on energy metabolism. LGT among prokaryotes operates with known mechanisms of conjugation, transduction, transformation (367, 389), and gene transfer agents (318). Similar mechanisms do not exist in eukaryotes, which have meiosis instead. Accordingly, two individuals of a eukaryotic species will tend to have the same numbers of genes and loci, but this is not so for prokaryotes. *E. coli* individuals typically harbor 4,500 genes, but this is only about 25% of the pangenome of the species, which, at current count, contains some 18,000 genes (293). Except for gene transfers from organelles to the nucleus, which are well characterized at the mechanistic level (186, 210, 461, 504), mechanisms of LGT from prokaryotes to eukaryotes remain unknown and uncharacterized at the molecular level.

None of this is to say that there have been no lateral gene transfers whatsoever in eukaryotic evolution. For example, a careful phylogenetic analysis of the *Entamoeba* and *Trichomonas* genomes uncovered evidence for LGT from prokaryotes, possibly affecting

about 1% to 2% of protein-coding genes (71, 290), a far lower LGT frequency for eukaryotes than other surveys might imply (15, 236). All things being equal, one might then expect that LGT could have affected 1% to 2% of the genes involved in anaerobic energy metabolism in eukaryotes, but with more samplings of eukaryotic lineages, those numbers should decline. From studies of eukaryotic anaerobes that unfolded after the discovery of hydrogenosomes in trichomonads (279) and chytrids (570), one very clear central trend has emerged: as lineage sampling increases, we are not discovering fundamentally new energy metabolic pathways; rather, we are finding the same genes over and over again, and more and more genes trace to the eukaryote common ancestor. Lineage-specific acquisitions in core anaerobic energy metabolism—if there are any at all—are clearly the exception rather than the rule. Eukaryote nuclear genomes acquired a colorful collection of genes from the ancestor of mitochondria, but they acquired it only once and then inherited that chimerism vertically.

Functional Modules and Their Compartmentation

The function of core energy metabolism is to supply ATP and to afford redox balance. In anaerobic energy metabolism in eukaryotes, this entails a small number of discrete functional modules that are differentially distributed across the cytosol and in mitochondria in different lineages, as summarized in Table 2. Taking an updated look at the eight main carbon flux modules recognized previously among anaerobic protists (353), here we distinguish electron-donating processes, electron-accepting processes, and ATP-generating processes as well as their occurrences in the cytosol and the organelle. In anaerobes, the most widespread oxidative process in the cytosol is, of course, the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (77, 304). The oxidation of malate to pyruvate usually occurs in mitochondria but has been transferred to the cytosol in trichomonads, with a parallel route existing in its hydrogenosomes. In *Giardia* and *Entamoeba*, the mitochondrion has undergone reduction to the level of a mitosome and has lost its function in core carbon and energy metabolism; as a consequence, pyruvate:ferredoxin oxidoreductase (PFO) in those lineages has been relocated to the cytosol, where it catalyzes pyruvate oxidation to acetyl-CoA.

Electron-accepting processes in the cytosol lead to the formation of the five major end products of cytosolic fermentations covered here: lactate, opines, ethanol, succinate, glycerol, and, as a minor cytosolic product only for *Giardia* so far but suspected for *Entamoeba*, H_2. The list of major cytosolic ATP-generating processes is devoid of lineage-specific variants (Table 2). The list of organellar processes contains many processes that also occur in the cytosol, and in several cases, the same enzymes are involved; exceptions are the acetyl-CoA-to-acetate pathways, which entail acetyl-CoA synthase (ADP forming) in the cytosol but phosphotransacetylase and acetate kinase (PTA/ACK) in the *Chlamydomonas* mitochondrion. Also of note for *Chlamydomonas* is that the [Fe]-Hyd is localized to the chloroplast in that species, at least under some conditions (560), and that an additional set of PTA/ACK isoenzymes appears to operate in the chloroplast (331). In a similar vein, the ethanol-producing enzyme in *Chlamydomonas*, ADHE, is chloroplast localized but is mitochondrial in a very close algal relative, *Polytomella* (22).

The examples of cytosolic PFO localization in *Giardia* and *Entamoeba* versus the hydrogenosomal localization in trichomonads and *Piromyces* versus the chloroplast localization in *Chlamydomonas*, in addition to the example of the ADHE localization shift for *Polytomella* (mitochondrial) versus *Chlamydomonas* (chloroplast), point to another important evolutionary aspect: compartmentation. During evolution, enzymes and pathways can readily undergo recompartmentation between mitochondrial, cytosolic, and, in the case of H_2-producing algae, plastidic compartments. This has been evident since studies of chloroplast-cytosol isoenzymes (315), comparative studies of the anaerobic protists (353), studies of the differential localization of isoprenoid biosynthesis to plastids and the cytosol of algae (276), studies of starch metabolism in algae (104), studies of the localization of fatty acid metabolism in peroxisomes of some species and tissues (466), studies of peroxisomal pathways in general (151), and—the classic example—studies of the glycosome in trypanosome evolution (172).

Each of these examples underscores the power of natural selection to bring forth different and useful variations of enzymatic compartmentation and sometimes variation in metabolic function as well, starting from the one basic set of enzymatic tools. The vast majority of mitochondrial and plastid proteins, and all hydrogenosomal proteins, are nuclear encoded, and many of those nuclear-encoded gene products are acquisitions from the endosymbiotic ancestors of organelles (504). When the gene is transferred from the organelle to the chromosomes of the host during evolution, there is no guarantee or homing device to ensure that its product will be targeted back to the donor organelle; natural variation and natural selection determine the outcome of the enzyme compartmentation process. Moreover, during the early phases of organelle origins, before the evolution of an organelle-specific protein import machinery, any genes that were transferred to the host's chromosomes, became expressed, and ultimately became fixed gave rise to cytosolic products (315). This process of endosymbiotic gene transfer is how whole pathways could easily be transferred from DNA-containing organelles to the cytosol (309). Slightly more puzzling was the problem of how to transfer whole pathways (or major segments thereof) consisting of several enzymes from the cytosol to a new organelle, for example, in the case of glycolysis moving to the glycosome (172). This is because a single enzyme in a new compartment is of no use and, hence, hardly selectable in evolution; the whole functional unit has to move. One solution to this problem involves the concept of minor mistargeting (307). If protein targeting in eukaryotes is not 100% specific (and many examples of dual targeting of enzymes are known), then minor amounts of whole pathways could conceivably end up in the wrong compartment. Such minor mistargeting is all the more likely for highly expressed proteins, such as those involved in core energy metabolism, and if a small amount of a whole pathway is present, it could readily become a unit of function and, hence, a unit upon which natural selection could act to generate more or less of that specific compartmentation variant. Clearly, pathways and enzymes are often recompartmentalized during evolution, and the minor mistargeting mechanism (307) could, in principle, explain how whole pathways can attain new states of subcellular localization (160).

Ecological Implications over Geological Time

The uniform nature and broadly shared conservation of genes for anaerobic energy metabolism across the major eukaryotic groups indicate their presence in the common ancestor of eukaryotes, which can be viewed from the physiological standpoint as a "garden-variety" facultatively anaerobic heterotroph. When available, oxygen could have readily served as a terminal electron acceptor,

TABLE 2 Functional modules and their compartmentation in anaerobic energy metabolism in eukaryotes[a]

	Presence of enzyme													
	Opisthokonta						Amoebozoa	Excavata				Chromalveolates		Archaeplastida
Compartment, process, and enzyme	*Fasciola*	*Arenicola*	*Sipunculus*	*Mytilus*	*Ascaris*	*Piromyces*	*Entamoeba*	*Giardia*	*Trichomonas*	*Tritrichomonas*	*Euglena*	*Nyctotherus*	*Blastocystis*	*Chlamydomonas*
Cytosolic														
Electron donors														
Glyceraldehyde-3-phosphate→1,3-BPG	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Pyruvate→AcCoA							●	●						
Electron acceptors														
Pyruvate→lactate						●			●			●	●	●
Pyruvate→opines		●	●	●										
Pyruvate→ethanol									●	●		●		●
AcCoA→ethanol						●	●	●						
Oxaloacetate→malate	●	●	●	●	●	●	●		●	●	●		●	
Fumarate→succinate (NADH)						●				●				
Pyruvate→malate								●	●	●				
$H^+ \rightarrow H_2$								●						
DHAP→glycerol									●	●				
ATP production														
Phosphoglycerate kinase	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Pyruvate kinase		●	●	●		●	●	●	●	●	●	●	●	●
PEP carboxykinase	●	●	●	●	●	●	●	●	●	●	●		●	
AcCoA→acetate (ACS [ADP])							●	●						
Organellar														
Electron donors														
Malate→pyruvate	●	●	●	●	●	●			●	●	●		●	
Pyruvate→AcCoA (PDH)	●	●	●	●	●						●	●		
Pyruvate→AcCoA (PFO)									●	●				●[b]
Pyruvate→AcCoA (PNO)											●		●	
Pro/Glu→αKG→succinate												●		
Electron acceptors														
Fumarate→succinate (RQ)	●	●	●	●	●						●	●		
$H^+ \rightarrow H_2$						●			●	●		●	●?	●[b]
AcCoA→ethanol														●[b]
Acyl-CoA→lipids					●						●			
Nitrate→nitrite*														
Sulfur compounds*														
ATP production														
ASCT/SCS cycle	●	●	●	●	●	●			●	●		●	●	
AcCoA→acetate (PTA/ACK)														●
Propionate cycle	●	●	●	●	●						●			
Ox Phos (anaerobic)	●	●	●	●	●						●			

[a] An asterisk for the pathways involving nitrate and sulfur as terminal acceptors refers to denitrification in foraminiferans (384, 410) and *Fusarium* (241, 242, 359) and sulfur reduction in *Fusarium* (1). A question mark indicates that hydrogen production in *Blastocystis* (270) has not yet been directly demonstrated. BPG, bisphosphoglycerate; AcCoA, acetyl-CoA; αKG, α-ketoglutarate; Ox Phos, oxidative phosphorylation.

[b] H_2 production and ethanol production via ADHE in *Chlamydomonas* appear to be plastidic, but not mitochondrial, processes.

either with energy conservation in mitochondria via the respiratory chain or in the cytosol using diaphorases that support redox balance without conserving energy (see "Anaerobes and Microaerophiles: Redox Balance with a Pinch of O_2") or in the mitochondrion without conserving energy, for example, via the alternative oxidase (AOX). This leads to three broader questions. (i) How old are eukaryotes as a group? (ii) How much oxygen is required for oxygen-dependent pathways? (iii) How available was oxygen when the eukaryote common ancestor lived and the earliest, facultatively anaerobic eukaryotic lineages were diversifying?

Let us first look at eukaryote age. Statements that eukaryotes are about 2.5 billion years (Ga) old are common (294) but are not well supported by data. Fossil microorganisms with a size and morphological complexity clearly representing eukaryotic microbes appear in rocks 1.45 Ga of age (223, 246), with the first fossils that clearly represent eukaryotic algae appearing slightly later, in rocks 1.2 Ga of age (67). Thus, by at least 1.2 Ga ago, eukaryotes had emerged and had commenced—or completed—diversification into the six major lineages recognized today. Molecular dates for the major events in eukaryote evolution (83) are consistent with that view. In terms of the geological time scale (548), eukaryotes arose and diversified during the Proterozoic eon, 2,500 to 542 million years (Ma) ago. Current studies integrating molecular and fossil data indicate that eukaryotes started diversifying about 1.8 Ga ago, at a time when the Earth's chemistry, especially with regard to oxygen, was much different from today's (376). Earlier reports, based on sterol biomarkers (geologically preserved organic compounds), that eukaryotes can be assumed to be 2.7 Ga old (55) turned out to be doubly incorrect because (i) the sterol biomarkers were younger than the rocks in which they were found (147, 401) and (ii) several groups of prokaryotes, including proteobacteria, synthesize sterols (379); sterols are synthesized by eukaryotes but are in no way specific to eukaryotes.

Sterols bring us to the second question, regarding how much oxygen is required for oxygen-dependent pathways. Sterols figure into issues both on eukaryote age and on the relationship of eukaryotes to oxygen. This is because oxygen is required as a cofactor in known eukaryotic sterol biosynthesis pathways: a squalene epoxidation step, three demethylation reactions, and other modifications consume about 12 molecules of O_2 per sterol backbone (547). Accordingly, sterols are widely used as geochemical proxies for the presence of oxygen in the ancient Earth environment, but the crucial question is, How much oxygen does biological sterol synthesis indicate? Waldbauer et al. (547) recently addressed this question by using yeast and found that yeast requires only trace amounts of O_2 for sterol biosynthesis. Unimpaired sterol accumulation was observed at 7 nM, the lowest dissolved oxygen concentration tested (547). In contrast, current atmospheric oxygen levels correspond to about 250 μM dissolved O_2, and the Pasteur point (the concentration at which facultative anaerobic eukaryotes start to respire O_2) is about 2 μM dissolved O_2. This indicates two things. First, sterol biomarkers indicate the presence of oxygen at levels corresponding to a trace gas in the atmosphere (547) and, hence, make no statement about the oxygen requirements of energy metabolism. Second, the ubiquity of sterol synthesis across diverse eukaryotic lineages (105) does not indicate that eukaryotes arose in oxic environments; it merely suggests that the eukaryote common ancestor arose subsequent to the origin of oxygenic photosynthesis (547).

What ecological setting confronted the emerging, pioneer eukaryotic lineages? The geosciences are currently delivering a new, detailed, and differentiated view of global ocean chemistry and ecology during the Proterozoic era, one that meshes well with our present understanding of anaerobic energy metabolism in eukaryotes. This view, based on abundant findings, as reviewed elsewhere (111, 296) and supported by additional newer findings (229, 273, 362), is outlined in Fig. 20. As Lyons et al. (296) formulated it, the available data "point to global oxygen deficiency in the deep ocean and perhaps euxinia throughout most, if not all, of the Proterozoic, and likely extending into the Paleozoic."

On the bottom line, the current view of Proterozoic ocean chemistry has it that prior to the appearance of oxygen in the atmosphere 2.4 Ga ago (295), the oceans were (of course) anoxic but, in marked contrast to older views, remained anoxic for almost 2 billion years after the onset of atmosphere oxygenation. More specifically, during the time from 2.4 to 1.8 Ga ago, the oceans below the photic zone were anoxic (19, 297) and ferruginous (containing much dissolved iron). In the time from 1.8 Ga to ~750 Ma ago, the oceans below the photic zone were euxinic (anoxic and sulfidic) (394), whereby Planavsky et al. (386) recently made the case for mixed ferruginous and sulfidic subsurface waters throughout the Proterozoic era. At ~750 Ma ago, ferruginous conditions persisted with continued anoxia (68), and at about 580 Ma ago, the oceans finally became oxygenated below the photic zone (14, 68, 450). The geological and biological processes that generated these three apparent phases of Proterozoic marine anoxia are far from pinpointed (386), and current discussions involve the interplay between a plethora of factors, including the oxygen-dependent weathering of continental sulfide deposits causing an influx of sulfate to the oceans, the growth of marine sulfate-reducing bacteria that produce H_2S, marine anoxygenic photosynthesizers that consume H_2S, diversity among marine primary producers, global glaciations, carbon burial rates, stratified ocean chemistries (229, 273, 296), and various elemental cycles. With regard to eukaryote ecology, and given the age of eukaryotes, this suggests that early eukaryotic evolution was influenced by anoxic and sulfidic water masses in the oceans of their day (324, 376). Today, the oxygen-poor sulfidic waters of hypersaline Mediterranean basins (129) or the Black Sea (565a) abound with eukaryotic microbes from the six supergroups, providing windows into protist ecology from the ancient past; the cultivation of these organisms and studies of their pathways provide exciting challenges for the future.

The late oxygenation of the oceans at about 580 Ma ago is generally brought into connection with the sudden appearance of large, diversified animal lineages in Ediacaran (630 to 542 Ma ago) fauna (69, 273, 452, 566); that is, the evolutionary lineages were already in existence, but with the advent of oxygenated oceans, the animals just increased in size (245, 247) and were furthermore able to form more rigid bodies with the help of oxygen-dependent collagen synthesis (509), consistent with the appearance of collagen in the earliest metazoan lineages (46). By inference, the smaller metazoan ancestors from which the Ediacaran fauna arose (361) must have been well suited to life with low or no oxygen, in line with the biochemical findings presented here. The mitochondrial sulfide-oxidizing metabolism of animals such as the annelid *Arenicola* (496) or *Urechis* (299) that still inhabit sulfidic environments can be seen as a relic from early metazoan evolution.

The recognition that anaerobic energy metabolism in modern eukaryotes is an ancestral character calls for a general rethinking of

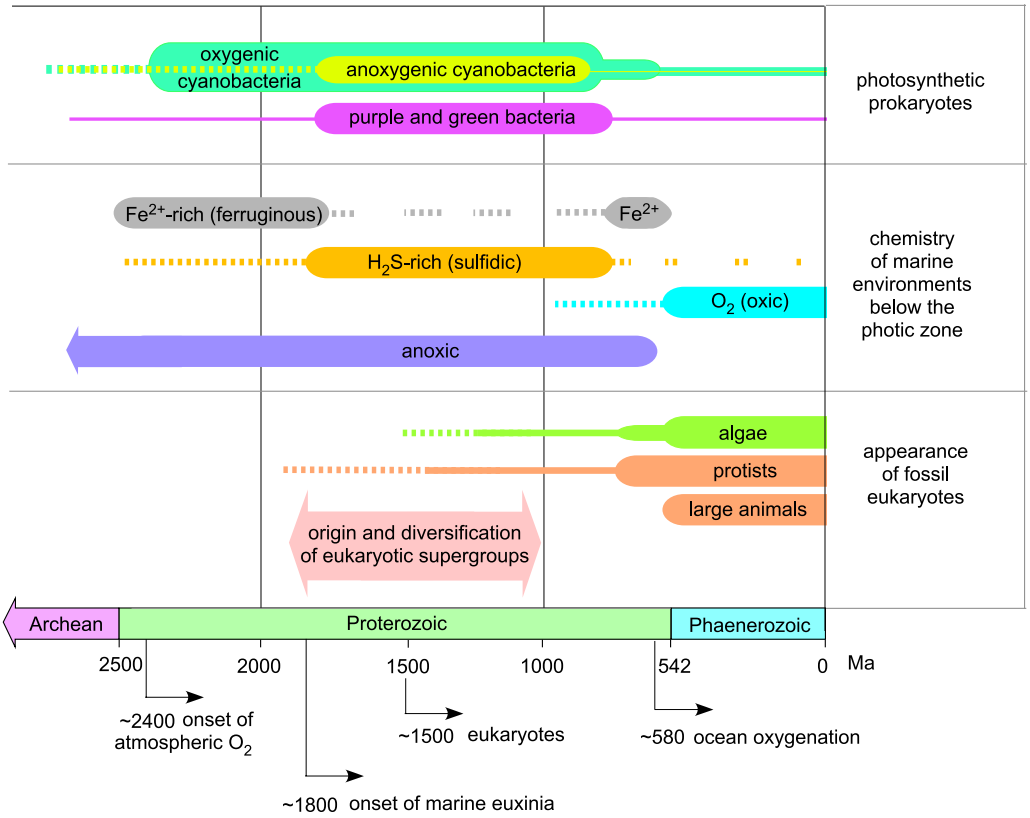


FIG 20 Summary of major events in Earth history relating to the appearance of eukaryotic groups and the appearance of O_2 in the atmosphere and in marine environments (see the text, and see also references 376 and 386). Ma, million years. (Reprinted from reference 229 with permission.)

traditional views of eukaryote ecology in the context of the fossil record. Conventional wisdom in the geochemical literature has it that "animals require oxygen" (69) or that global oceanic anoxia would make animal life impossible and "eliminate marine animals" (361). If eukaryotes in general and metazoans in particular (a derived and comparatively young lineage of eukaryotes) were fundamentally unable to survive or inhabit anoxic environments 600 million years ago, modern hypoxic and euxinic environments should also be devoid of animal life. However, modern animals inhabit many hypoxic and even euxinic environments. Metazoan examples are loriciferans from a Mediterranean hypersaline basin (99), recently discovered nematodes from environments 1 to 4 km deep within the crust (44), and various metazoan species that inhabit sulfidic cave waters (137). Nobody would seriously argue that animal lineages of the Ediacaran fauna independently acquired genes to allow them to use oxygen as a terminal electron acceptor. That ability was present in their common ancestor; accordingly, the ability of eukaryotes to inhabit anoxic or euxinic environments was also present in their common ancestor. Indeed, the various hypoxic and anoxic zones that exist in today's oceans are teeming with anaerobic eukaryotic microbes (9, 38, 291, 473), and the study of their energy metabolism will enrich our understanding of these vast and important habitats. It is possible that the unusual and diversified animal lineages from the Ediacaran period (361) were not only making a first exploration of macroscopic morphological space (452) from microscopic beginnings but also making—with the help of their facultatively anaerobic mitochondria—the first steps of the animal kingdom from hypoxic beginnings to oxygen-rich environments. In a similar vein, it is possible, if not probable, that the ecological interactions of eukaryotic anaerobes with prokaryotes in their aquatic environments are not fundamentally different from those manifest in the protistan founders of a young eukaryotic kingdom 1.5 billion years ago.

CONCLUSION

In summary, 4 decades of investigations into eukaryotic anaerobes have uncovered the same basic set of genes and enzymes for anaerobic energy metabolism among all major lineages sampled. This is in contrast to what one might have expected in 1973, when the investigation of a new anaerobic lineage uncovered not only new enzymes but also a new organelle (279). What has emerged is a picture of unity in eukaryotic anaerobic energy metabolism across all major lineages, indicating in straightforward terms a single origin and a common ancestry of the underlying genes, tracing back to the eukaryote common ancestor. The corresponding genes for anaerobic energy metabolism were lost in those lineages that underwent specialization to oxic ecological niches and the aerobic life-style or that underwent reductive evolution toward parasitism, as in the case of the microsporidians (512). It long seemed puzzling that the same basic set of genes for anaerobic energy metabolism could have been retained from eukaryotic origins up to the present in independent eukaryotic lineages (352). However, the late oxygenation of marine environments only some 580 Ma ago (68, 144, 229, 273, 296, 386, 450) readily explains why members of all major eukaryotic lineages should have retained the genes germane to the anaerobic life-style: the strictly aerobic life-

style (ours) is the one that arrived late on the scene of eukaryotic ecological specialization.

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