



## Review

## Hydrogen, metals, bifurcating electrons, and proton gradients: The early evolution of biological energy conservation

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## ABSTRACT

Life is a persistent, self-specified set of far from equilibrium chemical reactions. In modern microbes, core carbon and energy metabolism are what keep cells alive. In very early chemical evolution, the forerunners of carbon and energy metabolism were the processes of generating reduced carbon compounds from CO<sub>2</sub> and the mechanisms of harnessing energy as compounds capable of doing some chemical work. The process of serpentinization at alkaline hydrothermal vents holds promise as a model for the origin of early reducing power, because Fe<sup>2+</sup> in the Earth's crust reduces water to H<sub>2</sub> and inorganic carbon to methane. The overall geochemical process of serpentinization is similar to the biochemical process of methanogenesis, and methanogenesis is similar to acetogenesis in that both physiologies allow energy conservation from the reduction of CO<sub>2</sub> with electrons from H<sub>2</sub>. Electron bifurcation is a newly recognized cytosolic process that anaerobes use generate low potential electrons, it plays an important role in some forms of methanogenesis and, via speculation, possibly in acetogenesis. Electron bifurcation likely figures into the early evolution of biological energy conservation.

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### 1. Introduction

Life is a process of harnessing energy. All models for the chemical-evolutionary transition from the elements on the early Earth to free living cells recognize the requirement for a sustained source of energy. Among the main possible sources of energy that have been discussed, ultraviolet radiation [1], electric discharge [2], pyrite (FeS<sub>2</sub>) synthesis [3], and molecular hydrogen (H<sub>2</sub>) [4] currently stand in the foreground [5]. Although energy is essential for the origin of life, energy alone is not enough. To be useful for biological systems, energy needs to be conserved, or harnessed, in some form of biochemical currency that can be translated into chemical syntheses and metabolic work. Accordingly, concepts regarding the source of energy that powered early evolution tend to be paired with a concept about carbon metabolism: the ultraviolet radiation and electric discharge theories assume that the first organisms were fermenting heterotrophs while the pyrite and H<sub>2</sub> theories assume that the first organisms were autotrophs that obtained their carbon from CO<sub>2</sub>. Pyrite synthesis as an energy source is conceptually linked with the reverse citric acid cycle [6,7], while the idea that H<sub>2</sub> was the first main biological energy source is paired with the Wood–Ljungdahl (acetyl-CoA) pathway [4,8]. The purpose of this paper is to contrast and explore ideas about the nature of early biological energy conservation in the context of some newer

findings about how modern anaerobic autotrophs conserve chemical energy.

### 2. No soup for starters, but maybe vents

Theories involving organic soup or prebiotic broth [9] entail a two stage process: energy input from ultraviolet light [1], electric discharge [2] or interstellar processes [10] to make organic soup, then a second stage in which biologically useful energy is somehow gleaned from that stable, preformed collection of reduced organic compounds, either via oxidation entailing compounds like Fe<sup>3+</sup> as oxidants [10], or via disproportionations. Both variants correspond to heterotrophy in modern metabolism. Because the simplest forms of heterotrophy involve fermentations, heterotrophy first models have assumed that the first organisms were fermenters that conserved their biochemical energy through substrate level phosphorylations (SLPs) alone. Heterotrophic origin theories thus have two distinct phases of energetic processes at the onset of life: environmental energy spent to reduce CO<sub>2</sub>, a portion of which remains stored in organic molecules, and is later harnessed when organic soup serves as a substrate for fermentation.

The list of problems with heterotrophy first models is long [11,12]. From the bioenergetic standpoint, two problems appear sufficiently severe that prebiotic broth can be excluded from further consideration. First, prebiotic soup models assume that

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after the synthesis of organic soup, the process of life somehow starts from a mixture of carbon, hydrogen, oxygen and nitrogen that already has reacted; organic soup is at or very close to equilibrium. Life, by contrast, is a far from equilibrium process [13]. If we homogenize any organism so as to destroy the cellular structure, but keeping all the molecular constituents needed for life intact, and wait for any amount of time in anticipation of those constituents reorganizing into living cells once again, we will be disappointed. Living systems are open systems undergoing carbon and energy flux through very small volumes in space (corresponding to the size of a cell); the site and chemistry of life's origin should have those properties. Second, anaerobic fermenters like *Clostridia* have traditionally been viewed as representing both ancient lineages and ancient fermentative metabolic types [14], and it was long assumed that their fermentations entail solely SLP. But it turns out that clostridial type fermentations entail chemiosmosis, the generation of ion gradients across the cytoplasmic membrane and the harvesting of those gradients via rotor–stator type ATPases [15]. Fermenters that conserve their ATP via substrate level phosphorylations alone are very rare and even such reduced fermentative specialists as *Mycoplasma genitalium* make use of chemiosmosis and rotor–stator type ATPases to generate membrane potential for import and maintenance of ion homeostasis [16,17]. In modern biology, all free living fermenters are either themselves chemiosmotic [15] or are strictly dependent upon organisms that produce fermentable substrates with the help of chemiosmotic energy harnessing. That circumstance is very problematic for theories holding that the first cells were pure fermenters that emerged from a puddle, a pond, or an ocean of organic soup.

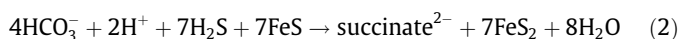
The discovery of submarine hydrothermal vents revealed chemically reactive environments, far from equilibrium conditions, as well as temperature, redox and pH gradients [18] and had lasting impact on theories concerning the origin of life. Such far from equilibrium conditions have more in common with life processes than organic soup, and vents might also provide a window into conditions on the early Earth [18]. While submarine hydrothermal vents brought modern biology and thoughts on life's chemical origins closer together, there remained considerable debate about temperature [19], because the black smoker types of vents harbor water that is 350 °C hot. At the same time, thoughts about the nature of the most ancestral kinds of metabolism gradually shifted away from fermentations and towards autotrophy [11,18], especially with the characterization of prokaryotes living in such chemically reactive environments [20]. The characterization of hydrogen rich alkaline hydrothermal vents with mild temperatures of around 70 °C [21,22] underscored once more the possible relevance of submarine vents in early evolution [23,24]. Geochemistry thus offered fresh chemical, energetic, and thermodynamic perspectives on biochemical origins [25]. During those developments, two concepts emerged with regard to the source of energy at the origin of life: pyrite and hydrogen.

### 3. Pyrite and the reverse citric acid cycle

Wächtershäuser's theory posits that the exergonic synthesis of pyrite (FeS<sub>2</sub>) from iron monosulfide (FeS) and H<sub>2</sub>S fuelled the first metabolic cycles [3,6,7,26] according to the reaction



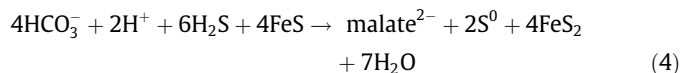
with an estimated  $\Delta G'_0 = -38.4 \text{ kJ mol}^{-1}$  at pH 7 [7]. That idea is closely linked with the idea that CO<sub>2</sub> entered metabolism via the reverse TCA cycle in an overall reaction that was pulled by pyrite synthesis:



with an estimated  $\Delta G'_0 = -429 \text{ kJ mol}^{-1}$  [6]. The thermodynamic favorability of that reaction has, however, come under scrutiny. Schoonen et al. [27] pointed out a number of difficulties with the temperature dependence and kinetic properties of pyrite-dependent CO<sub>2</sub> reduction. Kalapos [28] pointed out some stoichiometric issues with FeS<sub>2</sub> synthesis and obtained less favorable thermodynamic values for the production of oxalacetate



with an estimated  $\Delta G'_0 = -219 \text{ kJ mol}^{-1}$ , and malate



with an estimated  $\Delta G'_0 = -138 \text{ kJ mol}^{-1}$  [28] via the reductive tricarboxylic acid (rTCA) cycle. Michalkova et al. [29] examined the details of acetate synthesis from CH<sub>3</sub>SH and CO with respect to pyrite-pulled reactions and found unfavorable thermodynamic values with regard to pyrite. But it should be recalled that the synthesis of acetate (and the thioester methylthioacetate) did go forward [30], whereby it is often overlooked that some of the best yields reported [30] were obtained with NiSO<sub>4</sub> alone as the catalyst, conditions under which pyrite (for lack of either iron or sulfide in the experiment) could not have been produced.

Wächtershäuser's theory of a pyrite pulled rTCA cycle predicts the presence of the rTCA cycle in the common ancestor of all prokaryotes [26]. But in contrast to earlier assumptions [31] the rTCA cycle is not found among archaeobacteria known so far [32,33]. Another problem is that in modern microbes, the rTCA cycle is, in known cases, always supported by an independent and chemiosmotic energy metabolism. When chemiosmosis was incorporated into Wächtershäuser's theory, it came as a casual afterthought and with the proton gradient backwards at first (acid inside), requiring the assumption that cells somehow inverted their cytosol from outside to inside, and without specifying any redox partners, squeezing the origin of bioenergetics as it exists in the real world into a footnote [26, p. 1797]. Concerning the source of electrons for CO<sub>2</sub> reduction in primordial biochemistry, Wächtershäuser states that the half reaction



can be "excluded as the first source of electrons since its reducing potential is not sufficient for reducing CO<sub>2</sub>" [34, p. 466; 26, p. 1790–1791]. But hydrogenotrophic methanogens and homoacetogens reduce CO<sub>2</sub> with H<sub>2</sub> for a living, they even conserve energy while reducing CO<sub>2</sub> with H<sub>2</sub> [35–37] it is their core bioenergetic process, raising interesting questions as to how they do that.

### 4. H<sub>2</sub> and Wood–Ljungdahl pathway

Several authors have mentioned in passing that H<sub>2</sub> could have been a source of energy for primordial biochemical systems [38–41]. If H<sub>2</sub> was the electron donor, what was the electron acceptor? From the geochemical standpoint, Sleep et al. [42] underscored the energetic potential of geologically produced H<sub>2</sub> stemming from the hydrothermal process of serpentinization to fuel early evolutionary processes, for example methane production. From the chemical thermodynamic standpoint, Shock and colleagues [43,44] underscored the organic-synthetic potential of hydrothermal systems to generate reduced carbon compounds because of the H<sub>2</sub> accrued in the reducing environment provided by massive amounts of Fe<sup>2+</sup> in the Earth's crust, showing for example that amino acid biosynthesis is an exergonic reaction under hydrothermal vent conditions [45]. From the biological perspective, Fuchs [46,47] underscored the simplicity and biochemical antiquity of the Wood–Ljungdahl pathway [48], which is the pathway of CO<sub>2</sub> fixation employed by

homoacetogenic clostridia and by methanogens. The antiquity of the Wood–Ljungdahl pathway in the context of an origin of life in hydrothermal vents [4,8] fits well with the kind of chemistry observed in alkaline vents today [21–25,49].

The Wood–Ljungdahl pathway has several properties that make it attractive as an – or probably the most – ancient pathway of CO<sub>2</sub> fixation currently known [4,46,47], eight are listed in the following.

1. The Wood–Ljungdahl (or acetyl-CoA) pathway is a linear pathway of CO<sub>2</sub> fixation [48], not a cyclic one, its most complicated intermediate is a methyl group, and from CO<sub>2</sub> through to acetyl-CoA and on to pyruvate and phosphoenolpyruvate (the next steps in methanogen and homoacetogen CO<sub>2</sub> fixation), there are no chiral carbon atoms in any of the pathway intermediate carbon atoms – albeit many in the cofactors, yet not in their active moieties [4].
2. The enzymes of the pathway are replete with FeS and FeNiS centers [48,50], consistent with the notion that transition metal sulfides and clusters such as in ferredoxin are ancient relics from the earliest phases of biochemical evolution [51]. Furthermore, some of its most important reactions, including the reduction of CO<sub>2</sub> [52] or the synthesis of acetyl thioesters [30], have been performed in the laboratory using only transition metals (and sulfides thereof) as catalysts.
3. In the comparison of the pathway as it is manifested in acetogens (eubacteria) and methanogens (archaeobacteria), the pterin cofactors tetrahydrofolate and tetrahydromethanopterin are similar [53], the reactions involved are chemically quite similar and possibly homologous, but the enzymes involved are – for the most part – independently evolved and altogether unrelated at the level of sequence and structure. Exceptions are the starting points (CO<sub>2</sub> reduction) and the endpoints (thioester synthesis). The MoCo binding protein of W-dependent formylmethanofuran dehydrogenase from *Methanothermobacter thermoautotrophicus* is related to clostridial formate dehydrogenase [54] and carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) enzymes from methanogens and acetogens are also clearly related [55]. The lack of obvious sequence similarity for the enzymes catalyzing the steps from formylation of the pterin cofactor up to the methyl transfer to CODH/ACS in acetogen–methanogen comparisons suggests that the basic chemistry of the pathway preceded the origin of genes and proteins [4], consistent with the view that when genes and proteins arose, some of them just added catalysis (hence speed and specificity) to reactions that were already occurring anyway, so that reactions that tend to occur spontaneously could occur more rapidly [31]. The selective/replicative advantage for genetically specified catalysts that increased the flux of carbon into reactive intermediates from which energy could be harnessed is evident for a pathway that generates reactive C1 intermediates, reactive methyl groups, and energy rich thioesters.
4. With the elucidation of several new pathways of CO<sub>2</sub> fixation in archaeobacteria and eubacteria [32,33], the Wood–Ljungdahl pathway is currently the only pathway of core CO<sub>2</sub> fixation known to be present in both eubacteria and archaeobacteria. This argues strongly in favor of its antiquity. This also argues strongly in favor of the view that methanogens and acetogens represent the most ancestral autotrophs within the archaea and bacteria, respectively. If we embrace the concept of autotrophic origins, this would argue for the ancestral position of acetogens and methanogens within their respective domains, consistent with newer phylogenetic reports for the basal position of methanogens among archaeobacteria [56] and the basal position of firmicutes, which contain the clostridial hydrogenotrophic acetogens, among eubacteria [57].
5. In both acetogens and methanogens, which use the Wood–Ljungdahl pathway, there are forms known that lack cytochromes. The acetogens that lack cytochromes lack quinones [35,58]. The methanogens that lack cytochromes lack the quinone functional quinone analog methanophenazine [59]. Acetogens and methanogens are the only autotrophs known (to this author) that harness energy with the help of chemiosmosis but without the help of quinones or quinone analogs. By virtue of simplicity – no quinones, no cytochromes, no soluble electron carriers in the membrane to pump ions, just single ion pumping protein complexes –, that appears to be an ancient energy metabolic configuration, more ancient than with cytochromes and quinones.
6. Abiogenic methane and formate are produced from inorganic carbon at Lost City [49,60,61]. At Lost City geochemical CO<sub>2</sub> reduction is driven by serpentinization, a geochemical process as ancient as water on Earth [42] in which Fe<sup>2+</sup> in the Earth's crust reduces water in hydrothermal systems to H<sub>2</sub> [42,62], and CO<sub>2</sub> to carbon compounds that leave the vent through the effluent. Lost City effluent contains about 1 mM methane of abiogenic origin [49,60,61]. This is important because the theory that life could have arisen at Lost City types of vents carries with it the prediction that such vents should still be able to carry out some of the same chemical reactions today. The circumstance that abiogenic CO<sub>2</sub> reduction is observed at Lost City strengthens the case for hydrothermal origins, because an observable, exergonic geological process at hydrothermal vents – methane synthesis through serpentinization – is similar in overall chemistry to a biological process (methanogenesis). Methane production at Lost City narrows, but does not close, the gap between biological and geological methane production and is consistent with geochemical data indicating evidence for methanogenesis in rocks 3.5 billion years of age [63].
7. The thermodynamics for the overall synthesis of cell mass under Lost City type conditions (alkaline, reducing, moderate temperature effluent interface with ocean water) is favorable. Amend and McCollom [25] and colleagues [64] have found that the overall reaction for the synthesis of the chemical constituents of a microbial cell is exergonic (energy-releasing) at temperatures of around 50–100 °C for exactly the kind of alkaline hydrothermal vent (chemically similar to Lost City) at the source of current consideration. In other words, the synthesis of life's substance (protein, nucleic acids, cell wall, lipids, etc.) from the concentrations of CO<sub>2</sub>, H<sub>2</sub>S, HPO<sub>4</sub><sup>2-</sup>, NH<sub>4</sub><sup>+</sup>, H<sup>+</sup> and electrons (H<sub>2</sub>) as would be found at the vent ocean interface of such an alkaline hydrothermal system releases energy, even without considering main energy metabolic reactions, just considering anabolic cell mass accumulation. That is very important because many people assume that at life's origin one had to add a special kind of energy somehow (for example in the form of lightning), rather than harnessable chemical energy being available naturally, all the time, via H<sub>2</sub> stemming from serpentinization [65].
8. Importantly, the Wood–Ljungdahl pathway is the only pathway of core CO<sub>2</sub> fixation that is also a source of energy conservation [48,66]. All other known pathways of CO<sub>2</sub> fixation require supporting energetic input in the form of an independent energy metabolism, for example photosynthesis, sulfate reduction or other respiration [33,67]. Acetogens and methanogens use the Wood–Ljungdahl pathway not only as their core pathway of CO<sub>2</sub> fixation, it is also their source of energy conservation: they synthesize ATP at the expense of CO<sub>2</sub> fixation, energetically they get a free lunch that they are paid to eat [44]. In the reaction of H<sub>2</sub> with CO<sub>2</sub>, the equilibrium lies on the side of acetate and methane, acetogens and methanogens use this energy to generate ion gradients that are used for

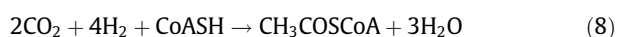
chemiosmotic ATP synthesis. Acetogens and methanogens live from reactions that involve very small free energy changes [68]. The overall methanogenic reaction is summarized as



with  $\Delta G'_o = -131 \text{ kJ mol}^{-1}$  [67]. The overall acetogenic reaction



is somewhat less exergonic with  $\Delta G'_o = -104.6 \text{ kJ mol}^{-1}$  [69]. The reactions of the Wood–Ljungdahl pathway to the level of the energy-rich thioester



are exergonic with an estimated thermodynamic value of  $\Delta G'_o = -59.2 \text{ kJ mol}^{-1}$  [66]; this is enough energy to pump a couple of ions across a membrane, but it is not enough energy to make thioesters for net carbon incorporation and simultaneously to conserve energy through substrate level phosphorylation [69]. That is why acetogens and methanogens are dependent upon chemiosmosis for their energy metabolism.

### 5. The early formyl pterin problem revisited

The notion that the Wood–Ljungdahl pathway is a viable model for prebiotic chemical evolution is not without energetic problems [4]. The problem concerns the synthesis of formylated pterins in early biochemical evolution, because the synthesis of  $N^{10}$ -formyl- $\text{H}_4\text{F}$  in acetogens and of  $N^5$ -formyl- $\text{H}_4\text{MPT}$  in methanogens from  $\text{H}_2$  and  $\text{CO}_2$  each entails thermodynamically very unfavorable steps. The problem remains interesting, but a new and exciting solution (exciting from the standpoints of physiology and early chemical evolution) is now in sight.

Specifically, for acetogens, the synthesis of  $N^{10}$ -formyltetrahydrofolate from formate and tetrahydrofolate ( $\text{H}_4\text{F}$ ) is endergonic with  $\Delta G'_o = +22 \text{ kJ mol}^{-1}$  [53]. This is an extremely steep bioenergetic barrier. The reaction catalyzed by  $N^{10}$ -formyl- $\text{H}_4\text{F}$  synthetase goes forward only because it is coupled to ATP hydrolysis. That ATP hydrolysis – by analogy to *Clostridium cylindrosporium*  $N^{10}$ -formyl- $\text{H}_4\text{F}$  synthetase [70,71] – at the  $N^{10}$ -formyl- $\text{H}_4\text{F}$  synthetase step appears to involve the synthesis of formyl phosphate as the active intermediate. In the absence of enzymes, formyl phosphate will spontaneously formylate  $\text{H}_4\text{F}$  specifically at the  $N^5$ -position [72]. But without chemiosmotic coupling, no net ATP synthesis for any (anabolic) process other than acetate synthesis is possible [58]. And although the exact nature of coupling in acetogens growing on  $\text{H}_2$  and  $\text{CO}_2$  is still not known [73], it almost certainly involves Rnf, a protein complex that pumps ions while reducing  $\text{NAD}^+$  with electrons from ferredoxin [74,75], and possibly involves an important and newly characterized mechanism of energy conservation, first described in *Clostridium kluyveri* [76], but turning up in many anaerobes [36,77,78] called electron bifurcation [15,73].

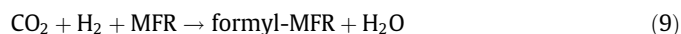
In methanogens, the synthesis of formylmethanofuran from methanofuran (MFR),  $\text{CO}_2$  and  $2[\text{H}]$ , catalyzed by formylmethanofuran dehydrogenase, is also highly endergonic ( $\Delta G'_o = +20 \text{ kJ mol}^{-1}$ ) if  $2[\text{H}]$  is  $\text{H}_2$  [36]. The subsequent transfer of the formyl group to tetrahydromethanopterin ( $\text{H}_4\text{MPT}$ ) by formylmethanofuran: $\text{H}_4\text{MPT}$  formyltransferase is slightly exergonic, with  $\Delta G'_o = -3.5 \text{ kJ mol}^{-1}$ . Even though free formate does not occur in the methanogen pathway, the synthesis of  $N^5$ -formyl- $\text{H}_4\text{MPT}$  from formate is endergonic with  $\Delta G'_o = +9 \text{ kJ mol}^{-1}$  [53]. The reaction catalyzed by formyl-MFR dehydrogenase is coupled to later steps of methane synthesis, which involve chemiosmosis, but the nature of that coupling is, significantly, now known [36] to be different to what was recently thought; an earlier paper [4] assumed that the formyl-MFR dehydrogenase reaction mechanism in hydrogenotrophic methanogens involves chemiosmotic flux of ions into the cytosol through the

formyl-MFR dehydrogenase protein, but that was incorrect, as newer findings show [36].

The problem – the early formyl pterin problem [4] – is this: If the Wood–Ljungdahl pathway is genuinely ancient, and if we turn back the clock to the time before early metabolically competent and replicating systems could harness chemiosmosis (which requires genes and proteins), how did they overcome the steeply uphill energetic barriers to synthesizing formyl pterins as critical intermediates in their core  $\text{CO}_2$  reduction and energy conservation pathways? The answer appears to lie in a newly recognized aspect of bioenergetics in methanogens and in clostridias (and probably in clostridial acetogens): electron bifurcation.

### 6. Electron bifurcation in methanogens and clostridial fermenters

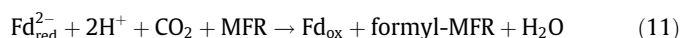
The principle of electron bifurcation is familiar. It is a means to drive an endergonic reaction forward by coupling it to an exergonic reaction, but the “energetic currency” of coupling is neither a high energy bond such as a thioester or an anhydride, nor is it a chemiosmotic ion gradient, it is a soluble reduced low potential ferredoxin [15,36,73,76–78]. A simple explanation of electron bifurcation, which was only recently proposed as an energy conserving mechanism in clostridial-type fermenters [15,76], is given by Kaster et al. [36], who addressed the problem of how modern hydrogenotrophic methanogens synthesize formylmethanofuran at the highly endergonic formylmethanofuran dehydrogenase step



with  $\Delta G'_o = +20 \text{ kJ mol}^{-1}$  [36]. They note that reaction (9) consists of two partial reactions involving Fd (ferredoxin) as an electron carrier

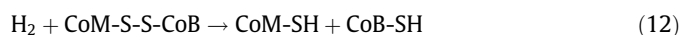


with  $\Delta G'_o = +16 \text{ kJ mol}^{-1}$  [59] and

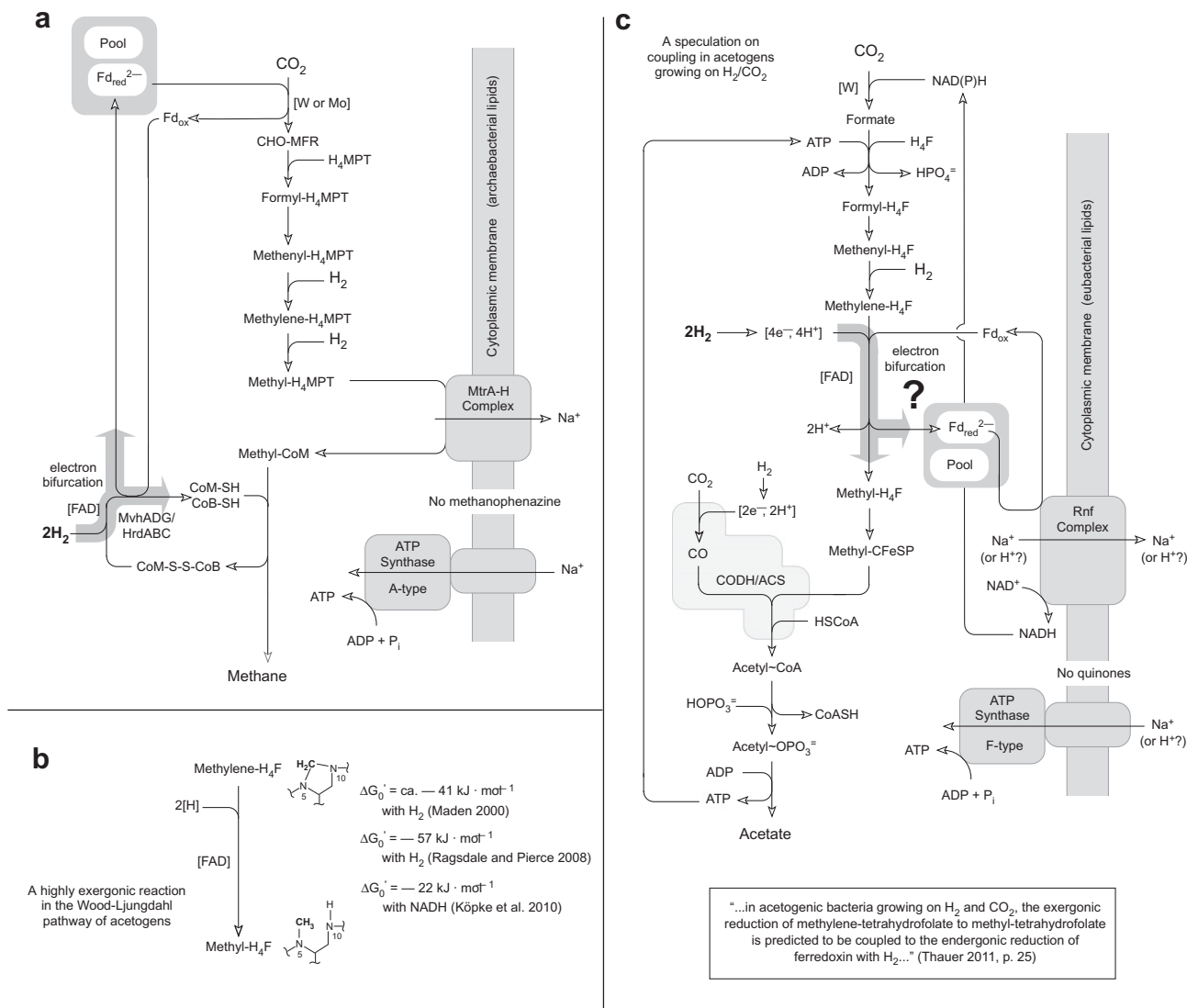


with  $\Delta G'_o = 0 \text{ kJ mol}^{-1}$  [59], and point out that the ferredoxin involved in reaction (10) is so far not characterized, whereby *Clostridium pasteurianum* ferredoxin with two reduced FeS clusters ( $\text{Fd}_{\text{red}}^{2-}$ ) was used in their enzyme assays [36]. In (over)simplified terms, the synthesis of formyl-methanofuran with electrons from a reduced low potential ferredoxin (instead of from  $\text{H}_2$  directly) is not an energetic problem, but the generation of reduced low potential ferredoxin with electrons from  $\text{H}_2$  is, which is why many organisms use chemiosmotic coupling (ion influx) to reduce ferredoxin when the electrons stem from  $\text{H}_2$  [59]. So where on Earth does the  $\text{Fd}_{\text{red}}$  for the formyl-MFR dehydrogenase reaction come from?

Kaster et al. [36] show that the energetic problem posed by reaction (9) is solved by coupling the endergonic reaction (10) to a highly exergonic reaction (12) catalyzed by heterodisulfide reductase



with  $\Delta G'_o = -55 \text{ kJ mol}^{-1}$  [36], whereby CoM and CoB are the thiol cofactors coenzyme M and coenzyme B, respectively. The mechanism of coupling entails neither ATP nor chemiosmosis, it entails electron bifurcation. The soluble, cytoplasmic enzyme complex that catalyzes reaction (12) in *Methanothermobacter marburgensis* (the MvhADG/HdrABC complex) contains FAD as a cofactor, and one of the electrons in the reduced FAD ( $\text{FADH}_2$ ) goes in the direction of the highly exergonic reaction (12) while the other electron from  $\text{FADH}_2$  goes to a low potential ferredoxin in the direction of the endergonic reaction (10). Repeating those uphill and downhill electron routes gives the gray arrows in the scheme shown in Fig. 1a which is redrawn in simplified form from Fig. 4 of Ref. [36].



**Fig. 1.** A sketchy comparison of energy metabolism in hydrogenotrophic methanogens and acetogens. (a) Simplified scheme of energy metabolism in *Methanothermobacter marburgensis* redrawn from Kaster et al. [36]. Electron bifurcation is indicated by a broad gray arrow. (b) A highly exergonic reaction in the Wood-Ljungdahl pathway with some estimates from the literature for the corresponding  $\Delta G_0'$ . (c) Simplified scheme of energy metabolism in acetogens growing on H<sub>2</sub> and CO<sub>2</sub> compiled from references [36,37,50,58,74,75] with an attempt to incorporate the suggestion of Thauer [73] that electron bifurcation might accompany the reduction of methylene tetrahydrofolate (see quote at bottom); the scheme was not endorsed by anyone.

Thus, the overall reaction of formyl-MFR synthesis from H<sub>2</sub> and CO<sub>2</sub> is thermodynamically favorable because reaction (12) is still exergonic even when driving reaction (10) on the side. But this CO<sub>2</sub>-fixing reaction, though ultimately driven by H<sub>2</sub>, requires Fd<sub>red</sub> to go forward efficiently, and the reduction of low potential ferredoxin with H<sub>2</sub> requires energy input. That energy is gleaned, and conserved as Fd<sub>red</sub>, from more favorable steps later in methanogenesis.

Herrmann et al. [15] list several examples in which ferredoxin reduction is coupled to various exergonic reactions via electron bifurcation, and they suggest that electron bifurcation can be viewed as a third form of energy conservation, in addition to substrate level phosphorylation and chemiosmotic coupling. The principle of bifurcating electrons from a reduced cofactor to two different acceptors, one energetically uphill and the other energetically downhill, is not fundamentally new, because the same thing occurs with quinones in the Q-cycle [79] at the cytochrome *bc*<sub>1</sub> complex of respiration and at the cytochrome *b*<sub>6</sub>*f* complex of photosynthesis [80]. The idea that reduced ferredoxin is a currency of energy was formulated over four

decades ago by Margaret Dayhoff, who wrote about ferredoxin from *Clostridium pasteurianum* [51, p. 363–364]:

*This protein seems to have arisen at an earlier times than many others which have been studied. We draw this inference from the following considerations. (1) Ferredoxin occurs in primitive anaerobic organisms, both photosynthetic and non-photosynthetic. It must have been present in simpler organisms, the extinct common ancestors of these. (2) Ferredoxin contains iron and sulfur, bonded to the protein at its active site. Ferrous sulfide, FeS, is a widely dispersed mineral, a catalyst which would have been readily available to the most primitive organism. (3) The functions of ferredoxin are basic to cell chemistry. The reduction of ferredoxin is the key photochemical event in photosynthesis by chloroplasts. All the energy is channeled through this compound to other cellular energy storage mechanisms. Ferredoxin is the most highly reducing stable compound so far found in the cell, having a reducing potential near that of molecular hydrogen. This suggests that its function may have evolved at a very early time when the earth's atmosphere*

was still strongly reducing. It reduces nicotinamide adenine dinucleotide (NAD), a ubiquitous reducing agent in the cell. Therefore, it may be even more primitive than NAD. It catalyzes adenosine triphosphate (ATP) formation by radiation. This indicates possible relation to primitive energy transfer processes. It catalyzes the synthesis of pyruvate from carbon dioxide and acetylcoenzyme-A. This indicates its involvement with one of the simplest, most primitive synthetic processes in intermediary metabolism, the fixation of CO<sub>2</sub>. It participates in nitrogen fixation and hydrogenase-linked reactions.

Dayhoff (then) expressed the (now) conventional wisdom that iron–sulfur centers represent catalytically active, inorganic relics from the mineral origins of life. Her comment that in photosynthesizers, all the energy is channeled through ferredoxin to other cellular energy storage mechanisms, now curiously applies to hydrogenotrophic methanogens, too. The downhill flow of electrons from Fd<sub>red</sub> in cyanobacteria and hydrogenotrophic methanogenesis harbors something very familiar, but the chemical means of generating Fd<sub>red</sub> could hardly be more different – photochemistry at photosystem I vs. electron bifurcation driven by (hetero-)disulfide reduction. Proteins involved in electron bifurcation are turning up in an increasingly diverse spectrum of prokaryotic anaerobes [81], suggesting that this mechanism of generating low potential reduced ferredoxins is even more widespread than initially suspected.

## 7. Early bioenergetic evolution and electron bifurcation

The prevalence of electron bifurcation in the energy metabolism of clostridia and relatives [15], its prominent essentiality in hydrogenotrophic methanogens [36] as sketched in Fig. 1a, and its predicted significance for acetogens growing on H<sub>2</sub> and CO<sub>2</sub> [73] as sketched in Fig. 1b and c, prompts a brief evolutionary reflection. Could the first free-living cells really have lived from H<sub>2</sub> and CO<sub>2</sub> alone and was the Wood–Ljungdahl pathway really at the root of carbon and energy metabolism at an alkaline hydrothermal vent?

In hydrogenotrophic methanogens, carbon metabolism and energy metabolism are not independent of one another because the anabolic hydrogenases used for net carbon fixation depend upon the ion gradient generated from methane production [36]. In acetogens the situation is very similar, net carbon and energy gain from H<sub>2</sub> and CO<sub>2</sub> are only possible through chemiosmosis [58,82]. In both physiological groups, the Wood–Ljungdahl pathway stands central. Assuming for the sake of argument that biochemistry and life did indeed arise at an alkaline hydrothermal vent [4], we can explore the sequence of events.

The universality of chemiosmotic coupling via ATP synthases among eubacteria and archaeobacteria leaves little (or no) doubt that the first *free-living* cells were able to harness ion gradients. The naturally chemiosmotic nature of alkaline hydrothermal vents – roughly pH 9–10 on the vent effluent side [21,22] and roughly pH 6 in the Hadean ocean [83] leaves little doubt such proton gradients did exist at the vent–ocean interface of Archean (>3.8 Ga old) alkaline serpentinizing systems. Geochemists furthermore tell us that serpentinization has been going on since there was water on Earth [42]. So we can assume (safely) that chemiosmotic potential was available in alkaline hydrothermal settings before there were genes and proteins. But the view that naturally preexisting chemiosmotic potential could be harnessed in a biochemically relevant manner without the help of genetically specified proteins [84] is problematic for this author, who contends that we have to get to genes and proteins without bioenergetic help from chemiosmosis. Once genetically specified proteins exist, harnessing geochemically generated chemiosmotic potential is not a problem [4]. But without proteins, the mechanism of coupling remains problematic.

The hydrothermal delivery of chemically reactive methyl groups, such as methyl sulfide, to the vent–ocean interface could, in principle, solve the energetic problem presented by the synthesis of formyl pterins in a chemiosmosis-independent manner [4] – we just leave the hard chemistry of reducing CO<sub>2</sub> to higher temperature and pressure processes (serpentinization, Fe<sup>2+</sup>-dependent reductions) deeper in the Earth's crust. The circumstance that Lost City effluent contains about 1 mM methane of a biogenic origin, derived from serpentinization [49,60,61], provides evidence supporting that view. But if all the hard chemistry of CO<sub>2</sub> reduction is taking place 3–5 km deep in the Earth's crust, what use is the H<sub>2</sub>/CO<sub>2</sub>(H<sub>2</sub> from serpentinization and very high marine CO<sub>2</sub> levels from volcanoes) redox couple at the vent ocean interface? Electron bifurcation appears to provide the solution.

Electron bifurcation is a means to generate low potential Fd<sub>red</sub> in hydrogenotrophic methanogens (and probably acetogens [73]) and Fd<sub>red</sub> appears to be the key compound linking carbon and energy metabolism in both groups, which lack cytochromes and quinones. At the kind of alkaline vent upon which the present considerations are founded, the business end of reduced ferredoxin – iron monosulfide – comprises the substance of the hydrothermal mound in which, for the purposes of this paper, the origin of gene and proteins is assumed to have taken place to begin with [4,83]. So naturally forming inorganic compartments consisting of freshly precipitated FeS in a highly reducing environment replete with H<sub>2</sub> are not a problem, as they are hard wired into the model. But a chemistry that depends upon physical contact to a hydrothermal mound for catalysis and electrons can only go so far en route to free living cells. At some point electrons have to get from H<sub>2</sub> to CO<sub>2</sub> via proteins and cofactors. That requires electron bifurcation in modern methane and acetate producing biochemistry. Maybe the same was true at biochemical origins.

Can electron bifurcation possibly operate in the absence of aromatic cofactors such as FAD or quinones? It has been suggested [84] that other compounds in addition to quinones and FAD, including W<sup>IV</sup>, Mo<sup>IV</sup>, and possibly even H<sub>2</sub> might be capable of electron bifurcation, by virtue of their two-electron donating redox reactions [85]. Indeed, the entry of carbon into metabolism in acetogens and methanogens starts with W- (or Mo-) dependent reactions. Formylmethanofuran dehydrogenase catalyzes the first CO<sub>2</sub>-reducing step in methanogens, accepts electrons from Fd<sub>red</sub>, and requires either Mo or W [86]. Formate dehydrogenase (FDH) in acetogens is a soluble enzyme that catalyzes the energetically unfavorable synthesis of formate from CO<sub>2</sub> and NADPH [87] and requires W [88]; the enzyme from other sources requires Mo [50]; the enzymes in methanogens (formyl-MRF dehydrogenase) and acetogens (FDH) share related components [54] even though they catalyze different reactions. Yet even if W<sup>IV</sup> and Mo<sup>IV</sup> could provide electron-bifurcating catalysis, what would serve as the exergonic reaction to permit the generation of Fd<sub>red</sub> from H<sub>2</sub>? Nitrogen oxides [83] is one suggestion, but neither methanogens nor acetogens require nitrogen oxides for growth. Geochemically delivered disulfides of simple organic thiols (such as methane thiol or similar), in analogy to the case of methanogens (Fig. 1a) would be another.

If so, that would explain why Wächtershäuser [26] was partially right concerning the claim that the half-reaction (5) (H<sub>2</sub> as a reductant) cannot reduce CO<sub>2</sub>. By itself, it probably cannot, but in the presence of simple organic disulfides, it certainly can in methanogens; the reoxidation of the thiols is achieved through an exergonic carbon reduction step (reduction of a methylthiol to methane). The implication is that in laboratory regimens the same should be true, because enzymes and cofactors do not make impossible reactions possible, they just allow reactions that tend to occur anyway to occur more rapidly. There can be no question that acetogens and methanogens do make cell mass out of H<sub>2</sub>, CO<sub>2</sub>, and (in diazotrophic forms) N<sub>2</sub> and that the overall reaction is exergonic. But some

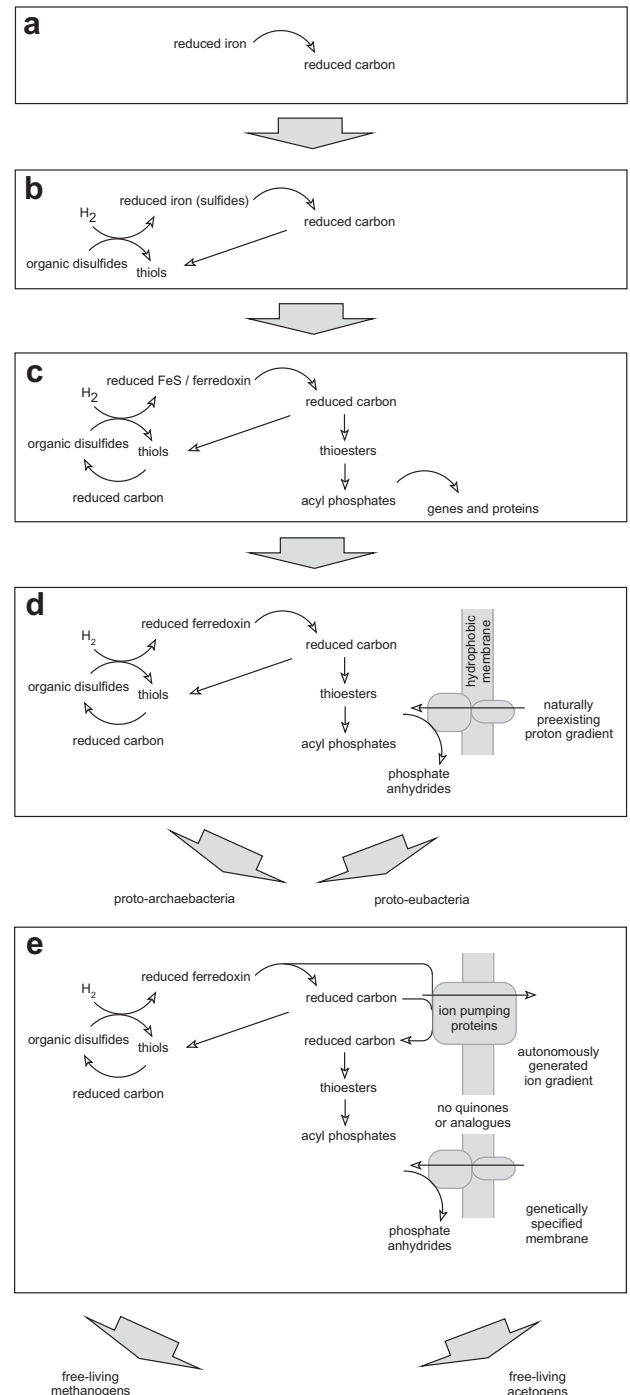
individual steps can be steeply uphill, and the mechanistic details of how modern anaerobes that live from  $H_2$ ,  $N_2$ , and  $CO_2$  overcome those hurdles ought to hold insights into the very ancient past.

Taking that thought one step further, and looking at Kaster et al.'s [36] scheme for energy metabolism in methanogens (Fig. 1a) in comparison to what the situation in acetogens might approximate (Fig. 1c), the simplicity of the methanogen pathway is striking. For generation of the ion gradient, no ATP is involved, no NAD(P)H is involved, not even thioesters or acyl phosphates. Were the methyl group transfer from  $H_4MPT$  to CoM to be short circuited so as to omit ion pumping we would have an overall reaction identical to methane production at Lost City, which has its own natural pH gradient of ca. 10 (effluent) vs. ca. 8 (today's ocean). At face value, this simplicity speaks for a greater antiquity of methanogenesis (Fig. 1a) compared to the scheme for acetogenesis (Fig. 1c).

It is noteworthy that the acetogen *Moorella thermoacetica* encodes a heterodisulfide reductase of unknown function (HdrABC homologs of the *M. marburgensis* enzyme) directly next to the genes for acetyl-CoA synthase [37] and genes with sequence similarity to the subunits HdrABC and MvrADG of the soluble *M. marburgensis* heterodisulfide reductase are readily found in database searches among firmicutes and other eubacteria that use the acetyl-CoA pathway. Given that heterodisulfide reductase supports electron bifurcation in methanogens [36], its presence in acetogens raises the question of whether electron bifurcation plays more than one role in their carbon and energy metabolism. This issue is all the more pressing with regard to the path of electrons from  $H_2$  to CODH, which is sometimes said to entail  $Fd_{red}$  [50], yet leaving the issue open whether chemiosmotic coupling is tapped to move electrons uphill from  $H_2$  to reduce ferredoxin, or whether electron bifurcation coupling might be used [36]. This potential commonality between acetogens and methanogens points to a very ancient role for disulfide reduction in metabolism, as it might play a key role in the energetics of organisms other than methanogens [81]. In the present context, the connection of disulfide reduction to  $CO_2$  reduction as outlined in Fig. 2 seems most important.

Only the energy metabolic routes are sketched in Fig. 1. Both groups have to assimilate net carbon via carbon monoxide dehydrogenase/acetyl CoA synthase (CODH/ACS), which accepts methyl groups from methyl- $H_4MPT$  [85] or from methyl-CFeSP (corrinoid iron sulfur protein [50]), generates CO from  $CO_2$  with electrons (from  $H_2$ , but via  $Fd_{red}$ ?), catalyzes condensation of the carbonyl and methyl groups to an enzyme bound acetyl group that is removed via thiolysis with CoASH yielding the thioester acetyl-CoA [50]. Although  $N_2$  fixation has not been shown for Lost City or similar vents, the transition metal nature of nitrogenase catalysis and laboratory results [90] suggest that it should, in principle, be possible.

In methanogens and acetogens, net energy conservation with concomitant net carbon assimilation from  $H_2$  and  $CO_2$  strictly depends upon chemiosmotic coupling [89,91]. Because chemiosmotic coupling requires proteins, the ability to harness natural chemiosmotic potential at a Hadean alkaline hydrothermal vent should be an genetic invention in the world of genes and proteins. Harnessing chemiosmotic potential also requires semipermeable membranes consisting of a hydrophobic barrier; such hydrophobics are today synthesized in metabolism, but in Lost City effluent alkanes up to pentane are detected [49], suggesting that abiotic synthesis of hydrophobic compounds in the Hadean capable of generating an organic membrane should not pose an insurmountable problem. Net carbon and energy gain through ancestral (inorganically catalyzed) versions of the Wood-Ljungdahl pathway entailing substrate level phosphorylation via thioesters and acyl phosphates [4] would appear possible at a Hadean hydrothermal vent, pro-



**Fig. 2.** A hypothetical series of events in carbon and energy metabolism, based on earlier schemes [4] but taking newer data from methanogens and acetogens into account (see legend to figure 1). Reduced iron refers to  $Fe^{2+}$  minerals (iron magnesium silicates) that reduce  $H_2O$  and  $CO_2$  during the process of serpentinization [49,60–62]. The starting reaction “reduced iron yields reduced carbon” refers both to  $CO_2$  reduction via serpentinization, and to the circumstance that  $CO_2$  reduction process in acetogens and methanogens involves ferredoxin, even though the electrons ultimately stem from  $H_2$ . The arrows indicate associated processes and flux.

vided that chemically accessible methyl groups are delivered via serpentinization, for example in the form of methyl sulfide or similar. In that case, the origin of genes and proteins could have been energetically feasible based on soluble reactions (and with time, enzymatically catalyzed versions thereof) that do not require membrane-associated chemiosmotic coupling. Electron bifurcation

coupled to highly exergonic reactions without recourse to membrane potential is an alternative to chemiosmotic coupling for generating reduced ferredoxin, and probably represents a relic from the time of carbon and energy metabolism before there were free living cells. The involvement of electron bifurcation to reduce ferredoxin in the carbon and energy metabolism of clostridia [15], methanogens [26] and probably acetogens [73] speaks in favor of its antiquity. A rough general sketch for a possible sequence of stages in early carbon and energy metabolism is given in Fig. 2, the arrows indicate associations as much as flux.

## 8. Conclusion

Some modern cells overcome the energetically uphill reaction of generating low potential FeS clusters (ferredoxin) with electrons from H<sub>2</sub> via electron bifurcation, it seem possible that early biochemical systems did as well. While modern electron bifurcation entails FAD (or quinones), there is room for (testable) speculation that simpler inorganic catalysts (transition metals) might have been able to do the job. In very early chemical evolution, the problem of whence reduced carbon has priority over whence RNA-like bases, whose origin is conspicuously disregarded here. From thermodynamics, the synthesis of amino acids is the main energy releasing reaction when it comes to the synthesis of cell mass under reducing hydrothermal conditions [25,45], their synthesis compensates for the endergonic synthesis of bases [25]. In metabolism, the bases come from amino acids and carbamates [4], in early evolution a similar sequence can be envisaged. However, in the beginning there is just no way that base synthesis could have been highly specific. The early real RNA world (assuming it existed) must have consisted of messy bases synthesized spontaneously without the help of genes. The chemical environment at the heart of the present considerations is full of reactive C1 and methyl groups (on their way to becoming methane, acetyl groups and acetate), and it is noteworthy that ribosome-tRNA interactions at the heart of the genetic code will not work properly without the myriad base modifications in tRNA and rRNA, the vast majority of which are methylations [92]. That a chemical imprint of the environment in which genes and proteins arose is preserved in the chemical structure of modern tRNA and rRNA bases is something to consider. Biological energy conservation, anaerobes, ferredoxin, and Fe-S proteins are topics that stood central in the interests of Antonio V. Xavier, to whose memory this volume is dedicated. It is hoped that he would have enjoyed this paper.

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