Heinrich Heine
Universität Düsseldorf

The role of nitrogen in the onset of metabolism

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Andrey do Nascimento Vieira, M.Sc.

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Supervisor: Prof. Dr. William F. Martin

Co-supervisor: Dr. Martina Preiner

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Statement of declaration

I hereby declare that this dissertation is the result of my own work. No other person's work has been used without due acknowledgement. This dissertation has not been submitted in the same or similar form to other institutions. I have not previously failed a doctoral examination procedure.

Düsseldorf, Andrey do Nascimento Vieira

To my family. The given and the chosen.

Acknowledgements

I never expected to know what I wanted to do by the end of my bachelor's. Curiously enough, the deep and somewhat obscure questions in biochemistry and microbial evolution have always had a fond place in my heart. After my exchange program at Montana State University in the USA, I had an even better glimpse of early evolution through various courses and inspiring people. Perhaps the proximity to the Yellowstone National Park and its natural wonders ignited the spark I needed to set my path towards the origins of life research.

At the end of my master's, already in Germany, I thought I barely managed to grasp the information I was seeking. However, it turned out the technical skills and critical thinking obtained in the fields of microbial physiology, analytical chemistry, and geobiology were decisive for my next step on this journey. The people I met, the labs I have worked, Lisa, Johanna, Mara, Matija, Jan, Cedric, Alberto, Paloma, and many others. All of you were decisive in keeping the flame somewhat alive. On a very ordinary day towards the end of 2018, after discussing my final experimental results with my former supervisor, Prof Dr Alberto Scoma, I received an unexpected email. An open PhD position at the Department of Molecular Evolution from Prof Dr William Martin. The email was short, but the content was straightforward. Alberto said: "If I were you, I would apply. It seems like the position was meant for you. The only problem is that you will have to go back to Germany. Nevertheless, if you are willing to go back, I am sure you would succeed." It was not an easy choice as I was very reluctant to go back to Germany back then. However, a professor I had during my bachelor's once said something that stuck on me – "Choose your supervisor before you chose your project. That is the secret of a successful PhD". Long story short, it took me five minutes to write a page-long email to Prof Bill Martin at 11:30 pm and about five minutes to get a reply. After two days of exchanging information with Bill and Martina, I got an interview.

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Publications in this thesis

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Abstract

Life is a set of chemical reactions that unfolded shortly after the planet's formation 4.5 billion years ago. Although direct geochemical evidence of metabolic origins is scarce, phylogenetic reconstructions of the last universal common ancestor (LUCA) shed some light on what conditions took place before and during the time of origins some 4.2 billion years ago. These reconstructions point toward aqueous, metal-bearing, serpentinizing environments such as those found in modern-day alkaline hydrothermal vents.

The mechanisms of nitrogen assimilation at origins have been under intense debate for the last century due to its low geologic abundance and reactivity. Despite its relative availability, the Redfield ratio, a hypothetical formula for life, indicates the ubiquitous importance of nitrogen to cellular life. As such, a question remains. How did nitrogen get available in substantial amounts to feed early autocatalytic networks? Given the high partial pressure of dinitrogen in the atmosphere, it is plausible that this gas was the starting point to a series of reactions that would result in the formation of highly soluble and reactive nitrogen-bearing species.

In this work, the mechanisms of nitrogen assimilation into prebiotically relevant organics were tested under alkaline hydrothermal conditions. The synthesis of formamide and acetamide from ammonia was attained overnight in the presence of suitable catalysts in the liquid phase, as demonstrated by ^{1}H-NMR at 70 °C. Alanine and glycine, two proteinogenic amino acids, were also observed, but efforts to increase yields and facilitate detection are yet to be optimized. These results indicate that when given the right conditions, a simple mixture of dinitrogen (N_2), carbon dioxide (CO_2), ammonia (NH_3), molecular hydrogen (H_2), and suitable catalysts can yield the formation of organic acids and C-N-bearing molecules. These 16-hour reactions give us a glimpse of what could unfold in geologic timescales.

Zusammenfassung

Das Leben entstand aus einer Reihe chemischer Reaktionen, die sich vor 4,5 Milliarden Jahren kurz nach der Entstehung des Planeten entfalteten. Obwohl es an konkreten geochemischen Beweisen der Entwicklung von Stoffwechselvorgängen mangelt, haben phylogenetische Rekonstruktionen des letzten Urvorfahrens (engl. LUCA) Einblicke in die Bedingungen gegeben, welche während der Entstehung des Lebens vor etwa 4,2 Milliarden Jahren herrschten. Diese Rekonstruktionen deuten auf eine wässrige, metallhaltige, serpentinisierende Umgebung hin, ähnlich der Umgebung, die heutzutage in alkalischen Hydrothermalquellen gefunden werden kann.

Die Mechanismen hinter der Stickstoffassimilation während der Entstehung des Lebens werden seit dem letzten Jahrhundert intensiv diskutiert, da Stickstoff geologisch gesehen in geringen Mengen vorkommt und eine geringe Reaktivität besitzt. Allerdings impliziert das Redfield-Verhältnis - eine hypothetische Berechnung des Lebens - die ubiquitäre Bedeutung Stickstoffs für das zelluläre Leben. Somit bleibt eine Frage offen: Wie wurde Stickstoff in erheblichen Mengen verfügbar, damit es für autokatalytische Netzwerke verwendet werden konnte? Ausgehend vom hohen Stickstoffpartialdruck in der Atmosphäre ist es wahrscheinlich, dass dieses Gas der Ausgangspunkt einer Reihe von Reaktionen war, die in der Bildung von hochlöslichen Stickstoffspezies resultierten.

In der vorliegenden Doktorarbeit wurde die Stickstoffassimilation in präbiotisch relevanten organischen Molekülen unter alkalischen hydrothermalen Bedingungen getestet. Die Synthese von Formamid und Acetamid wurde über Nacht in Anwesenheit von geeigneten Katalysatoren in flüssiger Phase erreicht, bewiesen durch ^{1}H-NMR bei 70 °C. Alanin und Glycin, zwei proteinogene Aminosäuren, wurden zudem nachgewiesen. Allerdings müssen die Mengen noch erhöht werden, um den Nachweis zu bestätigen. Die Resultate dieser Arbeit deuten an, dass bei geeigneten Bedingungen, ein einfacher Mix aus Stickstoff (N_2), Kohlendioxid (CO_2), Ammoniak (NH_3), molekularem Wasserstoff (H_2) und einem geeigneten Katalysator ausreichen, um die Entstehung von organischen Säuren und C-N-Molekülen zu ermöglichen. Die Ergebnisse dieser 16-stündigen Reaktionen geben uns einen Einblick in das Potential, das sich in geologischen Zeitskalen entfalten kann.

Aim of this thesis

Since the dawn of humankind, we have been chasing explanations for the origin of life on Earth. Moreover, although multiple scenarios and hypotheses to explain the origin and evolution of metabolism exist, experimental shreds of evidence are still largely lacking. This dissertation tested the alkaline hydrothermal vent hypothesis to offer a viable explanation for nitrogen assimilation to early organic prebiotic synthesis. While a definitive answer is still not within sight, the 3.8 Ga problem was tackled via a hybrid experimental and theoretical approach to narrow the gap between biology, chemistry, biochemistry, and geology.

Previous attempts to abiotically assimilate dinitrogen (N_2) gas into metabolism focused on purely chemical approaches and failed to approach a biological problem through biological means. Based on phylogenetic reconstructions of LUCA, the conditions where the first cells likely emerged were mimicked using high-pressure steel reactors to create simple organic networks containing nitrogen. During the course of this work, multiple conditions were tested, including the influence of pH, different sources of nitrogen and carbon, the effects of different heterogeneous metal catalysts, the effects of partial pressure of reductants, and the effects of different temperatures on their potential to form carbon-nitrogen bonds.

To better address the early conditions involved in alkaline hydrothermal synthesis, this dissertation aims to delineate the constraints of cellular life and heterogeneous catalysis to shed some light on the mechanism of nitrogen assimilation into metabolism. Other specific goals within this thesis and the publications therein include:

(a) Investigate which nitrogen sources likely were present in the Hadean oceans and their reactivity towards various organic molecules.
(b) Study the role of water in prebiotic organic synthesis and autocatalytic networks
(c) Probe early prokaryotic metabolism for biochemical hints on transamination reactions, nitrogen uptake, and the concomitant reduction of CO_2 and N_2.

1. Introduction

1.1 The moon forming impact – A tale of colliding worlds

Long before life was formed, there was the Sun, the planets, and teeming geology that was far too violent and erratic to foster sustained complex organic synthesis. At some four and a half billion years ago (Gya), there were likely far more than the current placement of eight planets in the solar system resulting in very different planetary dynamics than today. Our planet was still a hot, molten geoid of magma with no liquid oceans or solid land [Kramers 2007; Olson and Sharp, 2019]. The majority of Earth's water content was either in the atmosphere in the form of water vapour or trapped within the bubbling magma therein [Sleep *et al.* 2014]. There was no oxygen nor a stratospheric ozone layer that, together with a faint sun, did not allow solar radiation to reach the first solidifying rocks at the planet's surface [Kasting 1993; Catling 2020]. Meteorites and comets constantly hit the early rocky planets with great intensity at speeds nearing hundreds of kilometres per hour for dozens of millions of years [Bottke and Norman 2017; Morbidelli *et al.* 2001].

This turbulent past significantly enriched magma composition with various elements that would eventually be present in the first solid rocks [Santosh *et al.* 2017]. Due to density and chemical composition, some minerals would be melted and recycled at a higher rate than others. The majority of the heavy metals slowly sank towards the core, forming Earth's inner core [Alfè *et al.* 2007]. The newly arrived elements from comets and meteorites would have gotten slowly mixed and assimilated under the extreme pressures of the mantle, eventually creating a vastly diverse suite of rocks and minerals [Oversby and Ringwood 1971]. In geology, this is called differentiation. This process was responsible for the chemical composition of the Earth's layers. Differentiation is a homogeneous process in which magma gets heterogeneously mixed due to the chemical properties of its elemental constituents. Although extremely important in planetary and geological history, even differentiation and an active geologic cycle did not fuel the Earth with enough carbon and metals at the right places to kickstart the reactions preceding metabolism. An even more powerful event of planetary proportions would be needed to separate the Earth accordingly. Soon after the planet's formation, another event took place with so much kinetic energy that the mantle would almost instantly be separated into volatiles and non-volatiles.

At some 10 to 100 million years after Earth's formation, a tremendous impact took place that would change the complex geology of the planet [Sleep *et al.* 2014]. Isotopic analysis from moon sediments suggests that a planetoid (named Theia), roughly the size of Mars, hit the Earth, creating the biggest and most important chain of effects this planet has ever had [Wiechert *et al.* 2001]. Theia, just like the Earth, had a dense core full of heavy elements [Dauphas *et al.* 2014]. Upon hitting our planet, billions of kilograms of rocks and minerals were immediately vaporised, thus changing Earth's layering that was just beginning to differentiate [Kato *et al.* 2015]. The temperatures on Earth's surface after the impact, now higher than 2500 °C, altered magma by extracting all light and volatile compounds, especially carbon in the form of CO and CO_2, to be in gaseous form in the atmosphere rather than deeply bound to rocks in a rapidly recycling proto mantle [Armstrong *et al*. 2019; Catlin and Zahnle 2020]. Due to these high temperatures, water also left the system and was present primarily in the form of water vapours [Elkins-Tanton 2011; Nakajima and Stevenson 2018]. Another light molecule, nitrogen gas (N_2), mainly remained unchanged during the Thea impact due to its low reactivity and high abundance [Grewal *et al.* 2019; Zahnle 2006]. The triple covalent bond binding two atoms of nitrogen requires high energy to be broken. Although parts of the dinitrogen pool most certainly got split during the impact, the exceptionally high temperatures on Earth resulted in the rapid decomposition of the newly formed nitrogen species back to dinitrogen gas. A significant portion of Earth's mass was ejected with so much violence during this event that it escaped gravity and started orbiting our planet. Over the course of millions of years, this material coalesced into the moon [DeSouza *et al.* 2021].

All other molecules and elements that were either too heavy to get volatile or too unreactive to form complex volatiles ultimately sank to the Earth's crust. It is hypothesised that the carbon in the atmosphere got reduced to organic acids and polyaromatic hydrocarbons during the first millions of years of mantle formation [Morrison *et al.* 2018]. The tug of war between a semi-elastic, highly viscous planet Earth and the newly formed moon would have resulted in increased mixing of minerals at the superficial layers. This impact was so strong that the Earth was left with a 23.5-degree tilt relative to Earth's orbital plane that would stabilise in the next millions of years and result in the four seasons [Kerr 1989]. Tidal effects and differential density by rock differentiation likely contributed to the second round of differentiation resulting in the layering of the Earth as we know of today. From the formation of a predominantly metallic Iron-Nickel-based core to a light Silicate and aluminium-based conglomerate of rocks and minerals in the outer crust [Walter and Trønnes 2004]. The moon

forming impact did not only revamp our planet's geology but also created a chain of conditions that supported liquid water and, eventually, life.

Although it might be hard to believe that such a harsh, troubling past could once bear the first reactions that unfolded to what we now call life, it was likely the violent events involved in the planet's formation that provided the scenarios that will be further discussed in this dissertation. Moreover, before discussing the onset of metabolism, we need to effectively delineate the conditions it likely started. Much of the elements needed for pre-metabolic reactions to unfold were already here before the impact. However, the proportion and differentiation of rocks and minerals resulted from the moon forming impact that kickstarted a widespread set of chemical reactions in a water solution. The mobilisation of light molecules from the mantle to the atmosphere, the concentration of native metals and alloys at the lithosphere resulting from the moon forming impact ultimately led to the formation of simple organic acids [Sleep *et al.* 2011; Arndt and Nisbet 2012]. Current geological data of such deep periods of time are incredibly scarce on our planet but can be found on lunar rocks and sediments and in zircon crystals [Saal *et al.* 2008; Tarduno *et al.* 2015; Borlina *et al.* 2020]. Other pieces of evidence suggest that these first dozens of millions of years were filled with bolide collisions, cooling-melting cycles of magma and a strong influence of water in the formation of the atmosphere in the Hadean Aeon [Harrison 2020].

Our planet's atmosphere was much like Venus's for that brief moment of time [Wordsworth 2016]. Current estimates and scattered pieces of evidence suggest a highly dense atmosphere with over tens of bars of carbon dioxide and about 2.5 atm of nitrogen in the form of dinitrogen gas [Shaw 2008; Kasting 2014; Yang *et al.* 2014]. Water was also likely abundant, whilst no evidence was ever found pointing towards an oxygen-dominated, thus, oxidising atmosphere, as most of the oxygen was likely bound to other elements such as silicon and hydrogen in the form of silicates and water [Yang *et al.* 2014]. The high concentrations of carbon dioxide kept the heat from rapidly escaping the planet, a process that likely resulted in increased organic synthesis due to sustained extreme conditions. Although slow, the heat resulting from the impact eventually slowly radiated away from the Earth, and after consistent magma cooling, a process that took millions of years, the vaporised water once trapped in the rocks and now in the atmosphere slowly condensed and formed the oceans. Even at relatively high temperatures at around 200 °C, liquid water was highly abundant on the planet's surface [Sleep 2010; Kasting 2014]. These vast volumes of water rich in dissolved carbon dioxide, carbonates, and bicarbonates were able to constantly react with freshly precipitated metallic

ores, the first catalysts on Earth. The thick and complex atmosphere above, with a partial pressure of roughly 100 atmospheres rich in carbon dioxide, carbon monoxide, sulfur gases, and dinitrogen, supported an intricate dynamic involving geology and the newly formed liquid oceans.

1.2. Water – Sources and relevance in a geological and biological context

The most conspicuous water source on Earth is accretion itself [Trigo-Rodríguez *et al.* 2019]. This implies that the majority of Earth's water was already present during the planet's formation. The remaining material surviving accretion eventually hit the planet at later stages of planetary history in the form of frozen bolides and comets coming from the far corners of the solar system [Maurette, M. 2009]. This process took millions of years, with many conjecturing the possibility of a heavy bombardment [Chapman *et al.* 2007; Bottke and Norman 2017]. Albeit appealing, final pieces of evidence of such bombardment are still under intense debate [Koeberl *et al.* 2000; Morbidelli *et al.* 2001]. Another exciting aspect of this "late" water from extraterrestrial sources is that it could, in principle, be delivered as bound water in the form of mineral-bound hydroxides and hydrides [Dauphas *et al.* 2000; Beck 2021]. This intriguing postulate has been gaining evidence and support in the past decades. Most meteorites, however, do not carry significant amounts of water in liquid form [Rubin 1997]. For that reason, the hitchhiked water in the form of metal and silicate hydrides would feed the planet not only with water but with activated metal surfaces [Hou *et al.* 2001]. When heated up as a result of atmospheric friction, these hydrides are released together with the meteorite's oxygen content in the form of water.

These rock water interactions also explain the water content in magma that greatly contribute to volcanic eruptions [Pokrovski *et al.* 2013; Sharma and Srivastava 2014]. Geologically, many other reactions result in the formation of similar metal-bound hydrides [Smyth *et al.* 2005]. Perhaps the most intriguing ones include native metals and alloys in volcanic and hydrothermal settings as per the metal hydrides that result from such reactions are used both in the laboratory for prebiotic synthesis and in the industry in order to catalyse many relevant reactions, including the Haber-Bosch (HB) and Fischer-Tropsch (FT) reactions [Preiner *et al.* 2020; Maitlis 2004; Humphreys *et al.* 2021]. Many of the non-enzymatic reactions utilised in the industry, particularly those that rely on oxidation and reduction, utilise metal hydrides as their driving force [Bernauer and Halene 1987; Gambini *et al.* 2020]. However, despite the chemical properties of water dissociation and the physical form it likely

arrived in, there is overwhelming evidence that there was enough water in liquid form at a very early stage in planetary formation to form the first oceans [Pinti 2005; Borisova *et al.* 2021]. These evolving proto oceans slowly modified and eroded the rocks and minerals in the crust and mantle of the Earth, and as current evidence suggests that life started some 3.8 Gya, it likely came to happen in a dense, metal, and gas-rich ocean. The same applies to the chemical reactions preceding life.

On a practical level, all known living cells require water to survive. Aside from being ubiquitously present throughout Earth's history, water plays a fundamental role in biochemistry as, without a solvent, solutes would be too far apart in order to interact on the atomic level with each other. In cells, water also plays a major role in biosynthesis. The cell's energy currency, the ATP molecule, is an example where biosynthesis meets hydrolysis. By definition, hydrolysis is the process by which a molecule gets altered by water. In biology, this often involves the gain of a hydrogen atom that disrupts or breaks existing bonds within the parent molecule or fragment of it. By default, hydrolysis is a chemically disruptive reaction. In the cellular environment, the hydrolysis of the phosphoanhydride bonds of the ATP molecule generates energy that is harnessed for biosynthesis. The hydrolysis of ATP results in energy release used for vital activities. The release of the first phosphate group from ATP in the presence of water, for example, resulting in ADP + Pi, generates a ΔG of -30.5 kJ/mol [Ross 2006]. Upon the hydrolysis of the second phosphoanhydride bond, a total of -61 kJ/mol is released [Gropp *et al.* 1999].

The hydrolysis of ATP is the primary driver or biological function. The energy-rich bonds contained within the ATP molecule promote reaction irreversibility while also playing major roles in cellular signalling, synthesis and replication of RNA and DNA, and protein synthesis [Dahan-Grobgeld *et al.* 1998; Thorarensen *et al.* 2014; Pontes *et al.* 2015]. Therefore, it is via the energy of hydrolysis that biosynthetic pathways can take place. When ATP and its derivatives are no longer available, no energy in the form of phosphoanhydride bonds can be harnessed, and the chemical reactions sustaining cellular activity cease. Hydrolysis, however, still takes place intracellularly, contributing to the further degradation of biopolymers following cellular death. Without the hydrolysis of energy-rich molecules, there is no life.

Another example of water function in biology is the role of water in maintaining protein reactivity and stability. Structurally, it is via water interactions that proteins and peptides hold

their stability and function within the cellular space [Jaenicke and Závodszky 1990]. Proteins are polymeric chains of amino acids bound by peptide bonds. Moreover, although there is no consensus regarding terminology, the water of solvation, sometimes also referred to as bound water, is the water that does not freeze under minus forty degrees Celsius [Zayas 1997; Otting 1997]. This water is also not extracted via centrifugation because it is molecularly bound to the three-dimensional structure of proteins. Therefore, making water an integral part of these macromolecules that includes enzymes. These protein-water interactions confer structural stability of peptides and determine the organisation of protein domains in their secondary, tertiary and quaternary structures [Timasheff 1970; Mattos and Ringe 2006]. The exact mechanism in which these interactions occur varies according to amino acid hydrophobicity, its ability to interact with ions and other soluble inorganics such as metals, sulfur, oxygen, sodium, and potassium [Perutz 1978]. Another important aspect dictating protein function is the ability of solvation water to confer the formation of tunnels and other two and three-dimensional structures that involves the coordination of metallic atoms and amino acid residues in their catalytic centre. Therefore, allowing complex multi-step conformational changes and interactions between water, substrate, and proteins [Vargiu *et al.* 2018].

If we take a purely biochemical stance or a geological one, the reactions in prebiotic chemistry can be reduced to a common denominator, water. In geology, the general product of tectonic plate differentiation resulted in mafic oceanic plates with a higher content of basalt and gabbro than continental plates [Fox and Opdyke 1973]. Both of these rocks are rich in iron, magnesium and calcium in contrast to silica, aluminium, and sodium-dominated continental plates. Prebiotically, this means that even in the presence of catalytic metallic surfaces, it is unlikely that significant amounts of biologically plausible precursors were formed without water as a solvent. Aside from the higher abundance of metals in the oceanic crust, serpentinisation, a process where rocks and minerals get modified, producing magnetite (Fe_3O_4) and molecular hydrogen (H_2) as products, is also highly dependent on water (See chapter 1.4). Serpentinization does more than release metals and alloys to the ocean; it also provides a constant supply of energy and a myriad of microenvironments that favours autocatalytic networks and molecular complexity [Russell *et al.* 2010].

1.3. Carbon

Aside from water in bulk quantities, life is predominantly carbon-based. The ability of carbon to bond to four other atoms simultaneously, although not exclusive, makes it an interesting atom to serve as the backbone of life. Carbon and other group fourteen elements in the periodic table have four electrons in their outermost shell. Silicon, germanium, tin, lead, and flerovium make up the other atoms of group fourteen, with flerovium being the only artificial element in the group [Yakushev *et al.* 2014]. For the sake of argument, only silicon and carbon out of group fourteen elements will be discussed in this chapter, as all other integrands of this group are either found in neglectable amounts or are not naturally occurring on Earth's crust.

From a geological perspective, silicon is by far the most abundant group fourteen element, making up to twenty-eight per cent of Earth's crust, while carbon is over a hundred and forty times scarcer [Frieden 1972]. Moreover, although silicon life has been hypothesised, there are no evidences of such. When analysing the bond properties of carbon and silicon, it is clear why silicon is mainly bound to rocks and minerals while carbon is primarily found in the form of gases [Petkowski *et al.* 2020]. From an origin's perspective, the atmosphere of the Hadean had possibly tens of times higher carbon content than today's values, while silicon mainly was bound to oxygen, the most abundant element on Earth's crust, forming silicates in the cooling, differentiating magma [Trail *et al.* 2018]. That is because, although tetravalent, the bond energies of carbon and silicon are widely different. The energy of carbon-carbon bonds is roughly the same as carbon-oxygen bonds, 348 kJ/mol and 360 kJ/mol, respectively. These bond energies define carbon chemistry by its catenation properties [Sanderson 1968; Syakur and Berahim 2013]. That is the ability of carbon to form long polymeric chains with other carbon atoms via covalent bonding. Silicon, on the other hand, has a much stronger bond energy when interacting with oxygen (445 kJ/mol) than while interacting with other silicon (222 kJ/mol), carbon (318 kJ/mol), or hydrogen (318 kJ/mol) atoms [Syakur and Berahim 2013]. These differences in bond energies subject silicon to the formation of silicate-based minerals instead of the long polymeric chains needed in biochemistry. This more homogeneous nature of bonding energies, when put in context, makes carbon a far more interesting candidate at life's origins, where plenty of carbon dioxide, carbon monoxide, and methane were highly abundant both in the atmosphere and as degassing products of teeming hydrothermal activity. At the same time, silicon was almost entirely bound to rocks and, therefore, less available to react.

One of the issues associated to constraining the composition of the Hadean ocean is that the atmospheric composition of the same period is not precisely known. Nevertheless, given that carbon dioxide, carbon monoxide, and methane were highly abundant in the atmosphere,

with carbon dioxide partial pressures (PCO_2) reaching values of tens of atmospheres, it is safe to assume that a significant portion of this carbon was dissolved in the oceans in the form of carbonates, bicarbonates, and carbonic acid [Shibuya *et al.* 2013]. Thus, setting an early carbonate buffer system that presumably kept the pH of the oceans at slightly alkaline values [Morse and Mackenzie 1998]. Bicarbonate is the dominant carbon species at these mildly alkaline pH ranges from 6 to 10 [Middelburg 2019]. The carbonate buffer system goes as follows:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2\ H^+ + CO_3^{2-}$$

The bicarbonate molecule is of particular interest in this case due to the relative angle of insertion of each oxygen atom to its ligand carbon. The angles are 114.1, 113.4, and 132.4 °, starting from the oxygen bonding with hydrogen and carbon concomitantly [Rudolph *et al.* 2006]. For prebiotic synthesis, this angular conformation confers the bicarbonate molecule a structural advantage over carbonate due to its relative distance to other oxygen atoms in contrast to the less reactive nature of CO_2. In alkaline hydrothermal vents, carbon dioxide reacts with dissolved ions from the weathering of rocks and serpentinisation products and is partially converted to bicarbonate [Jones *et al.* 2010].

Carbon dioxide and bicarbonate ions then react at the surface of native metals and alloys such as iron (Fe), nickel (Ni), awaruite (Ni_3Fe), and magnetite (Fe_3O_4) that are freshly precipitated in alkaline hydrothermal vents together with molecular hydrogen and metal bound hydrides to form the first reactive organics including organic acids, alcohols, and ketoacids in autocatalytic networks [Preiner *et al.* 2019]. These molecules, including formate, methanol, acetate, ethanol, and pyruvate, were important for origins because not only they are biologically relevant, but they likely also represent the first simple building blocks of protometabolism. This whole process is still taking place in alkaline hydrothermal vents today, four and a half billion years since the Earth's formation in places such as the Lost City hydrothermal fields [Fruh-Green *et al.* 2003; Allen and Seyfried 2004]. In the laboratory, hydrothermal vent simulations in the presence of heterogeneous metal catalysts and hydrogen have repeatedly shown that the early stages of molecular complexity likely took place in vent-like conditions [Preiner *et al.* 2020; Preiner and Martin 2021]. However, it also showed that a constant supply of reductive energy in the form of hydrogen is necessary for the formation of complex C4 compounds such as pyruvate and potentially more complex organics [Preiner *et al.* 2020; Russel *et al.* 2010].

Carbon dioxide enters metabolism via multiple carbon fixation pathways in biology. The Calvin cycle currently accounts for the highest proportion of biological carbon fixation on Earth but indirectly relies on light to convert carbon dioxide into D-glyceraldehyde 3-phosphate (GAP), and dihydroxyacetone phosphate (DHAP) via ATP and NADPH [Raines 2003; Gong *et al.* 2018]. The reverse TCA cycle, by contrast, is a light-independent pathway that involves the synthesis of acetyl-CoA from two molecules of CO_2 and is found both in archaea and bacteria, the oldest forms of life known [Fuchs 2011; Srinivasan and Morowitz,2006]. The reductive acetyl CoA or Wood-Ljungdahl pathway, another light-independent pathway, converts carbon dioxide to acetate in the presence of molecular hydrogen in the form of H_2. This pathway is also found in both archaea and bacteria, with hydrogenotrophic methanogenesis performed by archaea accounting for around 80% of global methanogenesis [Avery Jr *et al.* 1999].

Although slightly different, one of the branches of the Wood-Ljungdahl pathway, the methyl, is fundamentally the same between archaea and bacteria, which suggests an early presence at the root of these two domains of life [Martin and Russell 2007; Nitschke and Russell 2013; Borrel *et al.* 2016]. Other carbon fixing pathways include the reductive acetyl CoA pathway that requires one ATP for the synthesis of one pyruvate molecule. The 3-Hydroxypropionate bicycle is another light-dependant cycle that converts bicarbonate to pyruvate via a series of intricate redox reactions [Berg *et al.* 2007]. Two variants of this cycle were proposed from archaeal and bacterial isolates [Loder *et al.* 2016; Zarzycki *et al.* 2009]. Nevertheless, due to its complexity and near exclusivity, it is unlikely that such a cycle was present at the root of the evolutionary tree. Aside from these pathways, there are also some prokaryotes capable of reducing carbon dioxide via enoyl-CoA enzymes [Stoffel *et al.* 2019]. For reasons discussed in the previous chapter, it is unlikely that sunlight could reach the seafloor. Even if it could, it would first have to penetrate the dense and turbulent atmosphere of the Hadean. Therefore, all the light-dependent biochemical pathways are ruled out of the equation, leaving the Wood-Ljungdahl and the reverse TCA cycle as the most promising pathways performed by the ancestors of archaea and bacteria.

When probing deep into the evolutionary tree of life, many of the compounds found in alkaline hydrothermal vents play a central role in the ancient Wood-Ljungdahl pathway [Weiss *et al.* 2016]. Phylogenetic reconstructions show that both methanogens (archaea) and acetogens (archaea and bacteria), the oldest known prokaryotes stemming from the last universal common

ancestor (LUCA), are anoxic, light-independent, and capable of performing the Wood-Ljungdahl pathway [Weiss *et al.* 2016; Martin *et al.* 2016]. Enzymatically, these reactions are performed by a series of two-electron transfers involved in the conversion of CO_2 to acetate. The first step of this reaction involves the reduction of carbon dioxide to formate (HCOOH) via the formate dehydrogenase enzyme. Formate is then further reduced to a series of intermediates until acetate is formed. This pathway has a methyl and a carbonyl branch. The methyl branch is an enzymatically complex system where a total of six enzymes are involved in the conversion of carbon dioxide to a metal-bound methyl group in the form of CH_3-CoFeSP. Conversely, the carbonyl branch directly reduces carbon dioxide via a two-electron transfer reaction via the carbon monoxide dehydrogenase and the acetyl-CoA synthetase enzymes. The end product of the two branches then gets converted to acetyl-CoA, Acetyl-P, and, lastly, acetate. Most of these reactions occur via two-electron transfers, in the same manner as the direct conversion performed abiotically in alkaline hydrothermal vents [Preiner *et al.* 2020]. The Wood-Ljungdahl pathway requires ten enzymes to convert carbon dioxide to acetate. Geologically, hydrothermal vents require only metals and hydrogen to perform the same reactions.

Many of the metals performing these reactions in hydrothermal vents, including iron and iron-sulfur clusters, are centrally located in the active centre of the enzymes performing the biological conversion of carbon dioxide to acetate [Belmonte and Mansy 2016]. Interestingly, although remarkably diverse and innovative, biology and evolution cannot perform feats of magic and develop means to perform reactions out of thin air or water. In the beginning, metals and gases reacted together in the core of alkaline hydrothermal vents forming early autocatalytic networks. The system's complexity eventually increased to the point where amino acids were formed, and the products and educts of a yet-to-be enzyme were constantly generated by hydrothermal activity. These networks eventually led to the formation of enzymes that included all the components found in these alkaline hydrothermal vents. For the first time in the history of chemical evolution, a complex chemical system evolved the capacity of self-catalysis and a faster, more efficient conversion of organic intermediates was performed in what would soon become a cell.

1.4. Nitrogen

The Redfield ratio is an empirical chemical formula for life. It states that when carbon, nitrogen, and phosphorus are quantified, there are roughly 106 atoms of carbon to 16 of nitrogen to one

of phosphorus [Takahashi *et al.* 1985]. Although these numbers are not the same for all forms of life, it seems like most cells and organisms deviate little from these rates [Ríos *et al.* 1998]. As discussed previously, from a purely chemical perspective, carbon makes the majority of a cell's dry weight and is not by coincidence as life is carbon-based [Vrede *et al.* 2002]. On the other hand, nitrogen is an indispensable constituent of amino acids, proteins, and the genetic code. As discussed in the previous chapters, nitrogen was primarily present as dinitrogen gas (N_2) in the Hadean atmosphere. Nevertheless, how could such an unreactive molecule such as N_2 be present in all life as we know it? Interestingly, the poor solubility of nitrogen in water hints that its primary reduction mechanism happens in the gas phase [Hidai and Mizobe 1995]. Isotopic data suggests that the Hadean atmosphere had approximately two to three times more nitrogen in the form of dinitrogen gas than Earth's current values of 0.78 atm [Kasting 2014]. This leaves an atmosphere with a partial pressure of 2.35 atm of N_2, where tremendous amounts of volcanic activity and early tectonic plating took place. At the converging zones of the early tectonic plates, significant amounts of water at boiling temperatures get trapped in magma, a process which could, *a priori*, transport significant quantities of dinitrogen to the upper and lower mantle [Mikhail and Sverjensky 2014].

The exact water content in magma varies according to different magma types but ranges from as low as 0.1 to 1.4 wt.% in rhyolitic magma to 3 to 5 wt.% in basaltic magma [Ushioda *et al.* 2014], and 5 – 7 wt.% in andesitic magma [Martel *et al.* 2000]. There is today's water content with a roughly one atm atmosphere. In a denser, warmer and more humid atmosphere, with ubiquitous geothermal activity, it is likely that the magma was less viscous and thus more prone to water and gas intrusions. Such feature alone would contribute to mobilising and exposing at least a part of the atmospheric dinitrogen pool to the great pressures and temperatures of the hot mantle. Once the geological cycle is complete with tectonic plating taking place, the resulting magmatic melts in close contact with hydrothermal fluids would be energy-rich enough to generate and bear reduced forms of nitrogen such as ammonia and transiently other more active species such as hydrogen cyanide and carbamate [Schoonen and Xu 2001; Maheshwari *et al.* 2019]. In the larger scheme of things, it is not unlikely that other energy sources, such as lightning, also contributed to the reduction of dinitrogen into ammonia [Navarro-Gonzalez *et al.* 2001]. However, even in the scenario of millennia-lasting storms, lightning alone would not provide a constant supply of reduced ammonia to the oceans. Additionally, the incredibly high temperatures produced by lightning would *a priori* reduce dinitrogen to ammonia but then immediately dissociate the majority of this ammonia back to

dinitrogen as ammonia dissociates at temperatures as low as 500 °C, while the average lightning temperatures range between 20,000 to 40,000 °C [Perman and Atkinson 1905; Zhivlyuk and Mandel'shtam 1961; Uman 1964]. Therefore, although appealing, this hypothesis is inherently flawed and will not be pursued further in the scope of this dissertation.

Other reduced species such as hydrazine (N_2H_4), cyanoacetylene (C_3NH), hydroxylamine (NOH_3) and hydrogen cyanide (CNH) are also appealing precursors for amino acids and organic amides mainly because the mechanism of dinitrogen reduction to such molecules is achieved in a facile manner [Horton *et al.* 1961; Sanchez *et al.* 1966; Sakurai and Yanagawa 1984; Chang *et al.* 1969]. However, when probing deep into microbial metabolism in its evolutionary roots, it seems evident that if these molecules had a role in early prebiotic mixtures, they would hit a dead end. No known forms of life rely on environmental cyanide or hydrazine, nor electrical discharges as sources for the nitrogen-rich precursors needed to synthesise amino acids. Recent experimental advances suggested that cyanide could have acted as a reductant in the glyoxylate pathway instead of a precursor for amino acids [Yadav *et al.* 2022]. In a broader sense, some of these molecules are so reactive that they are currently used as rocket fuel [Jain 1989]. Moreover, just like the reaction that brings humankind to new heights, hydrazine and hydroxylamine can rapidly dissociate into more stable counterparts while releasing tremendous amounts of energy and reacting with everything in their surroundings [Bremner *et al.* 1980; Le Goff and Ouazzani 2014]. Occam's razor thus favours the far less explosive hypothesis relying on dinitrogen gas instead.

Dinitrogen, probably one of the most stable diatomic known, makes up almost eighty per cent of Earth's atmosphere, and it enters cellular metabolism via nitrogenase enzymes [Schrock 2006]. It was not until the 1960s that FeMo cofactors were found to be responsible for nitrogen fixation in soil [Schrock 2006]. That discovery, paired with the knowledge of the catalytic role of molybdenum in such reactions, gave a glimpse of the possible mechanisms that cells use to incorporate nitrogen into metabolism. In the following years, many other nitrogenases were identified, all of them containing transition metals, including iron (Fe), molybdenum (Mo), and vanadium (V) in their catalytic centre [Shah *et al.* 1977; Miller and Eady 1988]. Once reduced to ammonia, nitrogen is assimilated into metabolism either via reductive aminations of 2-oxiglutarate to glutamate via the glutamate dehydrogenase enzyme [Hasumi *et al.* 1995], or through the two-step conversion of glutamate to glutamine [Temple *et al.* 1998]. Two of the steps happen via the enzyme glutamine synthetase, where the first

conversion requires one molecule of ATP in order to yield the formation of γ-glutamylphosphate from glutamate. γ-Glutamylphosphate then loses a phosphate group (P_i) that is replaced by ammonia, yielding the formation of glutamine [Miflin and Habash 2002].

From a chemical perspective, the primary industrial process utilised to reduce dinitrogen gas to more reduced species, the Haber-Bosch process (HB), requires incredible amounts of energy [Smith *et al.* 2020]. So much so that more than one per cent of all energy produced by humankind goes towards this process [Kibsgaard *et al.* 2019]. In order to convert dinitrogen gas to ammonia, the HB process requires temperatures above 400 °C in pressures above 400 atmospheres in the presence of metal-based catalysts. Nonetheless, the reduction of N_2 to NH_3 has been attained at far milder temperatures in the range of 70 to 80 °C [Dörr *et al.* 2003]. Under these conditions, the reduction of dinitrogen takes a week or more, and yields are as low as 0.1% (3 mmol) based on 3 moles of catalyst. Industrially, this process is inviable due to its meagre yields. However, it hints that the closer we approach milder conditions, the lower ammonia yields. In a different direction, when analysed in more detail, the biological conversion of nitrogen gas to ammonia hints at how such a reaction, while still applying vast amounts of energy in the form of sixteen molecules of ATP, still delivers ammonia with far more energy efficiency than the industrial processes. Compared to the Haber-Bosch reaction, ammonia production via nitrogenase enzymes has far lower energy requirements and higher output and efficiency [Hoffman *et al.* 2013]. For comparison, the conditions reaction and conditions required for both the Haber-Bosch and the biological reduction of dinitrogen go as follows:

Haber-Bosch synthesis of ammonia	Biological synthesis of ammonia via nitrogenase
$N_2 + 3\ H_2 \xrightarrow[Fe_xO_x]{> 400\ °C,\ > 400\ atm} 2\ NH_3$	$N_2 + 8\ H^+ + 8\ e^- \xrightarrow[16\ Mg\text{-}ATP]{25\ °C,\ 1\ atm} 2\ NH_3 + H_2 + 16\ Mg\text{-}ADP + 16\ P_i$

Thermodynamically, the conversion of dinitrogen into ammonia has a negative enthalpy with delta H values of roughly -54.2 kJ/mol, which by definition, makes it an exothermic reaction. However, if we take into consideration the activation energy required for such conversion, we end up with values ranging from 230 to 420 kJ/mol [Modak 2002]. Therefore, nitrogenase enzymes are responsible for reducing this activation energy to perform such

reactions at ambient pressures and temperatures. Although structurally diverse, the reaction mechanism involved in all nitrogenase enzymes is similar in many aspects [Einsle and Rees 2020]. However, seventy years after discovering the first nitrogenase enzyme, the precise mechanism of dinitrogen reduction by the nitrogenase enzymes is still not fully elucidated due to its complexity. For a comprehensive review of the mechanisms used by the nitrogenase enzyme, see [Hoffman *et al.* 2014]. The industrial applications of high heat and pressure in the Haber-Bosch process circumvents a problem our prokaryotic ancestors solved at some point 3.8 Ga.

Another industrial example that, just like the Haber-Bosch, requires tremendous amounts of energy is the Fischer-Tropsch syntheses of liquid hydrocarbons from a mixture of carbon monoxide and hydrogen gas. Although not directly related to nitrogen assimilation, the Fischer-Tropsch (FT) reaction also requires vast amounts of energy in the form of heat (300 - 350 °C) and pressure (20 – 40 atm) with the addition of molecular hydrogen in order to form C1 – C15 hydrocarbons [Gupta and Spivey 2013]. Curiously, the vanadium nitrogenase of the bacterium *Azotobacter vinelandii* seems to possess catalytic abilities germane to both industrial processes by simultaneously reducing CO to hydrocarbons and dinitrogen to ammonia [Lee *et al.* 2010]. As such, it is reasonable to assume that just like the abiotic reduction of carbon dioxide, the reduction of dinitrogen gas is highly dependent on the same denominators: An external energy source in the form of heat, a strong reductant in the form of hydrogen gas, and the presence of vast amounts of active heterogeneous metal catalysts. The existence of such fascinating but unique biochemistry indicates that at some point in the extant past, such reactions could be parallelly performed abiotically by freshly precipitated metals.

1.5. Metals as catalysts

Transition metals make great catalysts due to their ability to exchange electrons in the presence of other molecules. This electron transfer capability is an important property that does not change the equilibria or a given reaction's thermodynamics but directly impacts the reaction rate [Liu and Corma 2018]. Many factors, including redox estate, particle size, atomic radius, reactivity towards the reactants, and solubility, influence how effective each metal is as a catalyst. In biology, only a small portion of these metals are able to perform catalysis, including vanadium (V), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), Molybdenum (Mo), and tungsten (W) [Hagedoorn 2015]. There are multiple functions

performed by organic-inorganic interactions in biology to this day. Among other functions, metals are responsible for transporting small molecules (*i.e.* oxygen, hydroxyl groups, and hydrides), structural stabilisation of organometallic polymers, acting as electron carriers, and catalyse redox reactions [Crans and Kostenkova 2020]. The fact that proteins are still dependent on metallic atoms to perform vital functions such as catalysis and structure stability after billion years of evolution suggests two things: Native metals must have had a central role in early metabolism. And without their inorganic properties, specific reactions would not have been feasible by organocatalysis alone.

By geological abundance and biologic relevance, iron is the most commonly found transition metal in the Earth's crust, making up to 4.32% of its total composition, followed by titanium at 4010 ppm, manganese at 716 ppm, vanadium at 98 ppm, zinc at 65 ppm, and nickel at 56 ppm [Wedepohl 1995]. As previously explored in chapter one of this dissertation, during rock differentiation, most of the heavy elements sunk to the deeper layers of the Earth while the light, silicate-based elements remained afloat due to density and relative weight. However, although most of Earth's iron and nickel are in the core, a portion of these elements also remain in the Earth's crust bound to metal oxides such as FeO, Fe_2O_3, and metal silicates such as fayalite (Fe_2SiO_4). Some of these metal-bound silicates make up the majority of the geology of alkaline hydrothermal vents like olivine ($Mg_{1.8}Fe_{0.2}SiO_4$), orthopyroxene ($Mg_{1.8}Fe_{0.2}Si_2O_6$), serpentine $(Mg, Fe^{2+}, Fe^{3+})_3(Si, Fe^{3+})_2O_5(OH)_4$), and brucite ($(xMg^{2+}, yFe^{2+})(OH)_2$). When in water, these minerals get eroded, thus concentrating their components in metals-rich fluids that can further react with hydrogen to produce alloys and native metals. These metals are constantly supplied and most certainly were crucial for the evolution of prebiotic chemistry and life on Earth.

Not surprisingly, iron is also the most commonly used transition metal in biology. Fe^0 is often the sole electron donor in methanogens, whereas CO_2 is the dominant electron acceptor [Palacios 2019]. Molecular hydrogen is also an important fermentation intermediate, and iron is known to perform homolytic cleavage of water [Pereira 2022]. Both hydrogenases and nitrogenases employ iron atoms in their catalytic site, where molecular hydrogen and molecular dinitrogen get split and stabilised by iron-containing centres [McGlynn *et al.* 2013; Schuchmann *et al.* 2018]. The other transition metals used in biology are far less abundant in current microbial metabolism. However, they still perform vital functions in archaeal and bacterial metabolisms, particularly in acetogens and methanogens. Nickel is also present in some hydrogenases (Ni-Fe hydrogenases) and in the F_{430} cofactor bound to a nitrogen atom in methanogenic archaea [Ragsdale 2014; Ogata *et al.* 2016]. Cobalt is present on cobalamins, a

coordination complex involved in a multitude of physiological processes, including transcription regulation, enzyme catalysis, RNA regulation, adenosilation, and carrier activity of methyl groups in the coenzyme M of methanogens [DiMarco 1990; Rosnow 2018]. Vanadium is involved in a much more exclusive set of reactions. Compared to Fe-only and FeMoCo nitrogenases, Vanadium nitrogenases are capable of performing concomitant reduction of dinitrogen gas to ammonia and carbon monoxide to ethane, ethylene and propane [Lee *et al.* 2010]. Aside from nitrogenase activity, vanadium in the form of vanadate is also known to perform phosphate-like chemistry due to its tetrahedral coordination and similarity to the phosphate ion [Rehder 2015]. Vanadium can be found in different oxidative states such as +V, +IV, and +III in physiological settings. Manganese also has an important regulatory activity in the nitrogenase of *Rhodospirillum rubrum* [Yoch 1979]. Manganese is also involved in many other processes, such as regulating oxidative stress in bacteria, deactivating reactive oxygen species, radiation resistance, and cell growth and development [Bosma 2021].

In the industry, many of these enzyme-mediated processes are mimicked without the constraints of physiology. That means other physicochemical parameters are often exploited to increase product yields while simultaneously reducing reagent usage. Conditions such as high temperatures, high partial pressures of reducing and/or oxidising agents or extreme concentrations thereof, together with the usage of other sources of energy such as microwave radiations or electricity, make up some of the tricks used in industrial catalysis [Wilson and Groot 1995; Larhed e*t al*. 2002; Zhang *et al*. 2014; Huang *et al*. 2019]. Due to these conditions, industrial yields far bypass intracellular concentration ranges and are responsible in return for the production of a multitude of chemicals in an economically viable manner. HB and FT syntheses are two examples, as discussed in section 1.4 [Rofer-DePoorter 1981; Kandemir *et al*. 2013; Smith *et al*. 2020]. Both processes require vast amounts of transition metals acting as catalysts, with cobalt, iron, ruthenium and nickel mainly being used in FT syntheses, while iron and iron-based catalysts are the most common catalysts in the HB process.

On the one hand, many industrial catalytic processes are inspired by physiological mechanisms. On the other hand, biology was inspired by the natural suite of transition metals found in geology to perform catalysis. Ultimately, metabolism started where a different suite of native metals and alloys could be constantly supplied in order to perform reactions with different redox requirements. A constant source of energy and reactants also had to be present

in order to push the equilibria towards product formation and anabolism instead of hydrolysis and catabolism. All these requirements point towards alkaline hydrothermal vents.

1.6. Alkaline hydrothermal vents as motors for molecular complexity

Since the beginning of geological history, as soon as water was abundant in liquid form early in the Hadean Aeon, it completely shaped the geology of our planet. Although water has a low boiling point in comparison to other elements in magma, the water content in magma, also known as magmatic fluids, plays a crucial role in various geological processes, including rock weathering, volcanic eruptions, and ore deposits [Noble 1963; Lasaga 1984; Williams and McNutt 2005]. At the beginning of Earth's history, the high-water content likely resulted in increased hydrothermal activity as this type of magma is more viscous and, therefore, easily moves up the cracks and crevasses in the hard crust above. The built-up of gases such as carbon dioxide and sulfur-rich vapours also tends to migrate from a lower viscosity magma to a higher viscosity magma due to its low density and increased elastic properties compared to its surroundings. If we pair such a system with current water content estimates in the early Earth pointing towards twice as much water as we have in the liquid form today, it becomes increasingly evident that hydrothermal activity was ubiquitous around the Hadean and Archaean Aeons [Dong 2021].

In such a scenario, with twice as much water filling up our oceans, there was very little place for land to begin with. Instead, most, if not all hydrothermal activity happened in the depths of these early oceans. With enhanced geologic activity and great amounts of liquid water in the oceans above, the entirety of the volcanic activity happened underwater. When cracks and fissures in the seafloor expel hot fluids as result of rock water interactions, they are called hydrothermal vents. Presumably, the first hydrothermal vents transported vast amounts of phosphorous, sulfur, and ammonia-rich fluids from magmatic fluids to the oceans, where temperatures were significantly higher than today. The partial pressure of water at such depths would easily reach values of one thousand atmospheres and above. At these conditions, in the presence of a suitable reductant such as molecular hydrogen and metals acting as catalysts, the triple bond of dinitrogen is broken, and ammonia is formed [Kandemir *et al.* 2013]. With the passing of millennia, the planet continued to lose heat resulting from the accretion, the moon

forming impact, and radioactive decay to space. The ocean slowed this process down due to the heat conductivity properties of water [Vega *et al.* 2010]. The intense geologic activity resulting from premature tectonic plating likely resulted in extremely violent hydrothermal vents being ubiquitously present on the seafloor. These types of vents, also known as black smokers, tend to live a short geological life due to the close proximity to magmatic chambers. These black smokers likely contributed to the formation of some polyaromatic hydrocarbons and the conversion of sulfur gases to hydrogen sulphide [Von Damm 1990; Von Damm *et al.* 1997]. However, due to the formation of bigger tectonic plates, fewer spreading zones, the dwelling places for black smokers were available. In low numbers, the surrounding conditions likely favoured breakage of ammonia instead of synthesis. However, although ammonia could likely not be reduced from dinitrogen gas in significant amounts to fuel an early metabolism, the extreme conditions of black smokers could, in principle, for a brief period of time, pump the Hadean oceans with vast amounts of ammonia and its derivatives. Even if ammonia could be synthesised faster than it could get degraded, black smokers still have a short life span in order of decades [Martin *et al.* 2014]. The minuscule time span and extremely high temperatures would result in the immediate decomposition of most of its products, including ammonia and simple organic acids [Holland 2002]. Although it is by no means unlikely that black smokers had the conditions to convert dinitrogen gas to ammonia, in geological timescales, it still seems unlikely that they had other roles in metabolic origin other than feeding the primordial oceans with vast amounts of ammonia for a short amount of time.

With the dusk of black smokers, another type of hydrothermal vents dwelling further away from tectonic spreading zones likely populated the seafloor. These so-called white smokers or alkaline hydrothermal vents were first discovered in 2001 near the mid-Atlantic ridge [Von Damm 2001]. Alkaline hydrothermal vent fluids are fundamentally different in composition compared to black smokers in many aspects, including pH, temperature, and effluent mixture. The geology of white smokers is still tied to heat from below. However, instead of a close magmatic chamber feeding a deep-water fume system, white smokers feed on water from their surroundings that seep deep into the oceanic crust and get in contact with hot mantle rocks in close contact with magma. The rock water interactions resulting in alkaline hydrothermal vents generally go much deeper into the mantle than the black smokers due to the absence of a magmatic chamber component. This absence, therefore, results in significantly colder vent fluids ranging from 80 to 200 °C in comparison to up to 400 °C of their counterparts in spreading zones [Jupp and Schultz 2000; Sleep *et al.* 2004].

When the seeping water encounters serpentinising minerals such as olivine and orthopyroxene, serpentinisation occurs. These minerals are rich in metals such as iron and magnesium, and when in the presence of water, they get modified into serpentine and brucite, also metal-rich minerals. The serpentinisation process goes as follows [Preiner *et al.* 2018]:

$Mg_{1.8}Fe_{0.2}SiO_4$ [olivine] + $Mg_{1.8}Fe_{0.2}Si_2O_6$ [orthopyroxene] + $wH_2O_{0.5}$ → (Mg, Fe^{2+}, Fe^{3+})$_3$(Si,Fe^{3+})$_2O_5(OH)_4$ [serpentine] + x(Mg, Fe^{2+})$(OH)_2$ [brucite] + yFe_3O_4 [Magnetite] + zH_2

This process results in the constant precipitation of fresh metals such as elemental nickel and iron and metal alloys such as magnetite and awaruite [Sleep *et al.* 2004; Kitadai *et al.* 2019; Lough *et al.* 2019]. As water interacts with iron in these minerals, it gets disproportionated to hydrogen in the form of protons, metal-bound hydrides, and hydroxyl groups that bind to silicate rich minerals in the crust, effectively sequestering 300 litres of water per cubic meter of serpentinising rock [Müntener 2010]. These oxy- and hydroxy-rich minerals resulted from serpentinisation. Serpentinization generates a steady supply of molecular hydrogen, H_2, one of the most powerful reductants in natural environments with a standard midpoint potential at pH 7 and 25 °C (E_0') of –0.414 V. Hydroxides resulting from serpentinization, such as brucite, result in effluent pH in the ranges of 9 – 11 [Preiner *et al.* 2018; Sleep et al. 2011]. Serpentinization is a constant energy source in the form of dissolved molecular hydrogen, which can interact with marine CO_2, fuelling organic synthesis. When serpentinisation takes place, dissolved hydrogen reaching values of up to 15mM are produced [Kelley *et al.* 2005; Proskurowski *et al.* 2006]. Hydrogen is still the main driver of carbon fixation in subsurface environments today [Sleep *et al.* 2011].

In biology, energy is constantly invested in order to push reactions towards product formation. If equilibrium is achieved, cellular activity stops and death follows. As such, cells employ significant energy maintaining reactant concentrations in physiological ranges. Cellular concentration ranges are often far different than environmental concentrations of the same molecules in nowadays life. This difference in concentration creates an energy flux in the form of chemical gradients between the biotic and the abiotic worlds that in biology are called steady estate equilibrium. One way to concentrate reagents in a given space is by limiting the dissolution rate by limiting water activity. Lower water activities are generally tied to environments with decreased water availability. Although reducing water activities at the

bottom of the oceans seems unreasonable, there are features in the morphology of alkaline hydrothermal vents that enable product concentrations through thermal gradients in small micro and mesoporous structures [do Nascimento Vieira *et al.* 2020]. Alkaline hydrothermal vents thus hint as to how and where steady estate equilibrium first arose whilst also having thermal gradients allowing differential concentrations in small volumes [Wimmer *et al.* 2021].

As mentioned in chapter 1.1, water is of utmost importance for life as we know it. However, water can dissolve products to neglectable amounts when in abundance, effectively moving further from product concentration. This water paradox has perplexed scientists for decades as all life forms are water-based, while too much water leads to uncontrolled hydrolysis and dissolution of complex organics. This seemingly destructive nature of water is counteracted in hydrothermal vents and their micro and mesoporous structures [Kelley *et al.* 2005; do Nascimento Vieira *et al.* 2020]. It is well known that polymerisation of nucleotides occurs in confined spaces when subjected to thermal gradients [Mast *et al.* 2013]. The differential exposure to hot and cold surfaces within small, anastomosing micro and mesoporous structures of alkaline hydrothermal vents generates microenvironments for thermophoresis, thus acting as ideal electrochemical cells and offering a plausible geochemical precursor to steady estate equilibrium [Kreysing *et al.* 2015].

Serpentinization is important for biochemical origins because it leads to product formation by constantly supplying reactants in the form of carbon dioxide, energy in the form of hydrogen and heterogeneous transition metal-based catalysts such as Fe, Ni, Co, Fe_xO_x, and Ni_xFe_x, thus eventually creating one-carbon (C1), two-carbon (C2) and three-carbon (C3) pools. In the presence of suitable catalysts, a constant supply of input (food) molecules and a constant supply of energy, these carbon pools can react further, creating autocatalytic networks that eventually lead to the formation of the main biological polymers [Xavier *et al.* 2020; Wimmer *et al.* 2021]. Current radiometric dating of carbonate-based sediments of the Lost City hydrothermal field suggests that these alkaline vents have been active for 30,000 years [Fruh-Green *et al.* 2003]. These vents were likely far more abundant in the Hadean than today. For millennia, they could pump the building blocks of life from simple molecules such as carbon dioxide, ammonia, methane, dihydrogen, and water in the first electrochemical reactors. Eventually, with increasing complexity, inorganic metal catalysts were replaced by organic catalysts and, eventually, by enzymes that we see in all forms of life today. The diverse suite of

modern microbial physiologies seen today was fundamentally inherited from geology, and hydrothermal vents played a crucial role in this transition 3.8 Gya ago.

2. Experimental summary

In this work, hydrothermal vent simulations were performed aiming to elucidate further the role of different transition metals and alloys acting as heterogeneous catalysts in three categories of reactions that can be seen as follows:

(a) The concomitant reduction of dinitrogen and carbon dioxide gases and their potential to form C-N bonds.
(b) The reductive amination of organic acids using ammonia and urea as nitrogen sources and carbon dioxide, bicarbonate, and carbonate as carbon sources.
(c) Reductive transaminations of formamide and acetamide to organic acids synthesised in hydrothermal vent simulations.

All experiments were performed in aqueous solutions where the role of water, varying temperatures, hydrostatic pressures, and the effects of molecular hydrogen in the mixture were tested. For an easier visualization or results, a table with experimental conditions disclosing the presence or absence of products will be presented in each section.

2.1. The concomitant reduction of dinitrogen and carbon dioxide gases and their potential to form C-N bonds.

As previously discussed, dinitrogen gas is the most ubiquitous nitrogen source on Earth. As such, N_2 was chosen as a nitrogen source in various reactions aiming to concomitantly reduce dinitrogen gas to ammonia and CO_2 to organic acids for further incorporation of nitrogen into organics. However, the solubility of dinitrogen gas is low even at high partial pressures, meaning that even at a few hundred bars, dinitrogen fails to get solubilized in substantial amounts in the liquid phase at low temperatures [Wiebe *et al.* 1933]. The presence or absence of dihydrogen and different heterogeneous metal catalysts were also tested in order to activate and catalyze these possible conversions. Temperatures ranging between 25 and 200 °C as well as N_2 partial pressures of up to 25 bars were applied in hydrothermal vent simulations performed

in high-pressure steel reactors. All experiments were carried out for up to 72 hours of reaction time. An overview of the experimental conditions tested can be found in the table below.

A) Experimental conditions	Temperature (°C)	Carbon source	Nitrogen source	Catalyst of choice	Reaction time (h)	pH before reaction
	25 - 100	CO_2 - HCO_3^-	N_2	Ni	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	NiO	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	Ni_2O_3	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	Ni:Fe	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	Ni_3Fe	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	Fe	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	FeO	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	Fe_3O_4	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	Co	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	Mo	4 - 72	7 - 8
	25 - 100	CO_2 - HCO_3^-	N_2	Mn	4 - 72	7 - 8

Due to the technological limitations and the stability of N_2 under these conditions, no nitrogenated compounds could be identified by nuclear magnetic resonance spectroscopy (^{1}H-NMR) in these laboratory simulations. However, based on speciation models of carbonate, bicarbonate, and carbamate in ammonia solutions, carbamate most certainly was produced [Mani *et al.* 2006]. Nonetheless, due to an absence of a proton in its chemical structure, no peaks could be generated by ^{1}H-NMR spectroscopy. An overview of the main experimental results can be found in the table below.

2.1.		Result summary										
		Catalyst of choice										
		Ni	NiO	Ni_2O_3	Ni:Fe	Ni_3Fe	Fe	FeO	Fe_3O_4	Co	Mo	Mn
Reaction products	Carbamate	?	-	-	?	-	?	?	?	-	-	-
	Bicarbonate	✓	-	-	✓	-	✓	✓	✓	-	-	
	Alanine	-	-	-	-	-	-	-	-	-	-	-
	Glycine	-	-	-	-	-	-	-	-	-	-	-
	Formamide	-	-	-	-	-	-	-	-	-	-	-
	Acetamide	-	-	-	-	-	-	-	-	-	-	-
	Ammonium carbonate	-	-	-	-	-	-	-	-	-	-	-
	Urea	-	-	-	-	-	-	-	-	-	-	-
Reaction condition	pH after reaction	8 - 9	7 - 8	7 - 8	7 - 8	7 - 8	8 - 9	7 - 8	8 - 9	7 - 8	7 - 8	7 - 8
	H_2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

	Carbon source	CO_2	CO_2	CO_2	CO_2	CO_2	CO_2	CO_2	CO_2	CO_2	CO_2	CO_2
	Nitrogen source	N_2	N_2	N_2	N_2	N_2	N_2	N_2	N_2	N_2	N_2	N_2
	Temperature (°C)	100	100	100	100	100	100	100	100	100	100	100

Despite the absence of amino acids and bioactive C-N containing molecules, the possibility of a mutual CO_2 and N_2 reduction cannot be excluded. Instead, these results hint that conditions currently used by the Haber-Bosch process (temperatures above 400 °C and partial pressures above 400 atm) to synthesize ammonia might be necessary. Although recent advances have successfully achieved N_2 reduction to ammonia at room temperatures [Dörr *et al.* 2003], it is noteworthy that tremendous amounts of reactants were needed for extended reaction times in order to have a 0.1% ammonia yield. Over geologic times and product concentration by means of thermophoresis, it is plausible to consider that similar reactions might have taken place on the early earth. A low ammonia yield could, in principle, also take place in our setup. However, even if ammonia gets produced, only a small fraction of it would react further with pyruvate to yield alanine. In such a scenario, the concentrations of ammonia and amino acids would be far too low to be detected by ^{1}H-NMR. Given the low reactivity of ammonia, both the application of a more sensitive analytical technique such as isotope ratio mass spectrometry (IRMS) and longer reaction times should be explored further.

Alternatively, although a simple common mechanism for reducing N_2 and CO_2 is appealing, the activation of dinitrogen gas requires substantially more energy than the activation of carbon dioxide [Gambarotta and Scott 2004; Ma *et al.* 2009]. This disparity suggests that a one-pot synthesis of ammonia and organic acids might not be achieved in a facile manner: while N_2 requires high temperatures and pressures to be activated, CO_2 does not. When coupled to the thermal decomposition of formate, acetate, and pyruvate, the primary organic acids resulting from CO_2 reduction at mild temperatures [Mars *et al.* 1963; Rajadurai, S. 1994; Preiner *et al.* 2020], it seems evident that by approaching the necessary conditions to activate dinitrogen gas, most likely all organic acids are degraded to CO_2 and CO.

In the presence of hydrogen gas, these gases would either go through methanation, the process of converting carbon monoxide to methane or condense to form alkanes, alkenes, and aldehydes [Van Santen *et al.* 2013]. While alkanes have been shown to increase the stability of

membranes under extreme temperatures and pressures [Misuraca *et al.* 2021], and aldehydes have been hypothesized as potential organocatalysts under prebiotic conditions [Closs *et al.* 2020], there are no pieces of evidence suggesting that these molecules could be used as carbon sources to early autocatalytic networks. Phylogenetic reconstructions of LUCA and core metabolic reactions also hint us to organic acids and their central role in early metabolism rather than alkanes and aldehydes [Weiss *et al.* 2016; Wimmer *et al.* 2021]. For a one-pot synthesis of organic acids and ammonia from CO_2 and N_2 in relevant quantities, either product concentration by means of thermophoresis should be applied, which would also prevent thermal decomposition of organic acids, or a flow-through system should be used where reactants are constantly supplied. Such a flow-through system would force reaction equilibria towards product formation and simulate a steady-state equilibrium in such prebiotic settings. In the absence thereof, the reduction of N_2 and CO_2 could not be detected by ^{1}H-NMR under the tested laboratory conditions.

2.2. Reductive amination of organic acids using ammonia and urea as nitrogen sources, and CO_2, bicarbonate, and carbonate as carbon sources.

Once ammonia is formed, the narrative changes significantly. When reacting (0.1 M) ammonia and (15 bar) carbon dioxide for 16 hours at 70 °C in the presence of tea-templated awaruite (Ni_3Fe), micromolar amounts of formamide and acetamide are formed. Ammonia is a stable product of dinitrogen reduction but also relatively inert under mild conditions. The reactivity of ammonia is highly dependent on pH. At pH values higher than 9, there is a prevalence of ammonia ions over ammonium [Langenfeld *et al.* 2021]. When using ammonium hydroxide and bicarbonate, the initial pH of these solutions was predominantly above 12, which ensures an abundance of ammonia over its less reactive unionized form ammonium. An overview of the experimental conditions tested can be found in the table below.

B) Experimental conditions	Temperature (°C)	Carbon source	Nitrogen source	Catalyst of choice	Reaction time (h)	pH before reaction
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Ni	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	NiO	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Ni_2O_3	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Ni:Fe	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Ni_3Fe	4 - 72	7 – 14

	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Fe	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	FeO	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Fe_3O_4	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Co	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Mo	4 - 72	7 – 14
	25 - 100	CO_2 - HCO_3^-	NH_3 - CH_4N_2O	Mn	4 - 72	7 – 14

Except for the previously mentioned conditions, ammonia as a nitrogen source did not significantly improve product yields in these hydrothermal simulations. Instead, it limited the catalytical activity of metal nanoparticles. It is well known that ammonia in the form of NH_3 and NH_2 strongly binds to metal surfaces at high temperatures, thus limiting the effectivity of metal catalysts towards their CO_2 and HCO_3^- ligands [Gajbhiye *et al.* 2002; Niwa *et al.* 2017]. However, when an excess of carbon dioxide is established at the lower temperatures used, copious amounts of metal carbonates are formed in these conditions. Ammonia accelerates carbonate precipitation because it has a pH of 11.3 at 0.1 M solution. An overview of the main experimental results can be found in the table below.

2.2.		Result summary												
		Catalyst of choice												
		Ni		NiO	Ni_2O_3	Ni:Fe	Ni_3Fe		Fe	FeO	Fe_3O_4	Co	Mo	Mn
Reaction products	Carbamate	✓	-	-	-	-	-	N/A	✓	✓	-	✓	-	✓
	Bicarbonate	✓	-	-	-	-	-	N/A	✓	✓	-	✓	-	✓
	Alanine	✓	-	-	-	-	?	N/A	-	-	-	-	-	-
	Glycine	?	-	-	-		?	N/A	-	-	-	-	-	-
	Formamide	✓	-	-	-	-	✓	N/A	?	-	-	-	-	-
	Acetamide	✓	-	-	-	-	✓	N/A	?	-	✓	-	-	-
	Ammonium carbonate	✓	✓	-	-	-	-	N/A	-	-	-	-	-	-
	Urea	-	-	-	-	-	-	N/A	-	-	-	-	-	-
Reaction	pH after reaction	9 - 10	11 - 12	8	10	8	11 - 12	-	10	9 - 10	11 - 12	9 - 10	11	9 - 10
	H_2	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

	Carbon source	$C_3H_4O_3$	$C_3H_4O_3$	CO_2	CO_2	CO_2	CO_2	N/A	CO_2	CO_2	CO_2	CO_2	CO_2	CO_2
	Nitrogen source	CH_4N_2O	NH_3	Either	Either	Either	NH_3	Either	Either	Either	NH_3	Either	Either	Either
	Temperature (°C)	90		90	90	90	70	N/A	90	90	90	90	90	90

At pH ranges of 6.5 to 10, bicarbonate is the dominant carbon species in solution, but above 10.5, carbonate becomes the dominant ion in the system [Schwarzenbach and Meier 1958]. This pH shift limits the product spectrum and potentially sequesters carbon, and metal particles in an amalgamate where ammonia has no surface to react. In order to circumvent this pH problem, a mixture of ammonium chloride, which has a pH of roughly 4.6 at 0.1 M, and ammonia with a pH of 11.3 at 0.1 M was used. The final pH of such a mixture was set to 9. Despite reducing the pH, the less alkaline ammonia solution did also not improve product yields. Although pH regulation is important for nitrogen assimilation, these results suggest that catalyst availability plays a pivotal role in these reactions. In the case of awaruite (the reactions yielding formamide and acetamide), no carbonate deposition could be observed after 16 hours. This indicates that although carbonate has a lower reactivity than bicarbonate and is likely the dominant ion in these experiments, it reacted at metallic surfaces with metal-bound hydrides forming formate and acetate. These organic acids likely reacted with ammonia hydroxide in order to form formamide and acetamide, as first described more than 150 years ago [Hofmann, A. W. 1863]. It is well known that by heating pure formamide to 160 °C, a number of nucleobases are formed [Yamada and Okamoto 1972; Saladino *et al.* 2008]. Furthermore, although formamide yields were bleak, concentrations of up to 85 wt % of formamide were reached when a diluted 10^{-3} wt % solution of formamide was applied to thermal gradients [Niether *et al.* 2016]. This could indicate that although a low yield of formamide might not kickstart the formation of amino acids and nucleobases, the action of temperature gradients over geologic times could have been enough to form the first biologically plausible nitrogen-containing molecules.

Constraining pH fluctuations during the reactions might be another decisive step toward improving product yields. As ammonia has great potential for hydrogen bonding, not only NMR spectroscopy is unfit to detect and identify possible reaction products, but it also fails to detect molecules at lower concentration ranges. Replacing carbon dioxide for bicarbonate might improve the system because the pH fluctuates less due to a lesser abundance of carbonic acid

(H_2CO_3). When CO_2 gets exposed to water, it slowly gets dissolved and forms carbonic acid that rapidly speciates to carbonate and bicarbonate. However, due to the low solubility of bicarbonate and the high solubility of carbonic acids, a higher concentration of CO_2 usually translates to a more acidic mixture. The low solubility of bicarbonate can also easily be improved by exposing the mixture to heat. As such, experiments with 1 mmol awaruite at 70 °C, 0.1M of ammonia in a mixture of acidic and alkaline forms (*i.e.* NH_4Cl and NH_4OH), and 0.1M of bicarbonate should be performed at various reaction times and subsequently analyzed by NMR and mass spectrometry. By replacing CO_2 with HCO_3^- as the carbon source, the pH of the mixture would be more stable due to an absence of carbonic acid in the mixture. The parallel use of bicarbonate and ammonia mixtures should be set, so the final pH before the reaction ranges between 10 and 12, as observed in the reactions with awaruite yielding formamide and acetamide. Preliminary data with such an ammonia mixture did not increase product yields. However, a thorough exploratory study with stepwise changes in pH should be performed for their potential to improve product yields, given that a suitable catalyst is present. Such experiments would help elucidate the role of pH and, most importantly, help solve the mechanism involved in these reactions.

Given the alkaline nature of these hydrothermal simulations, when copious amounts of carbon dioxide and ammonia are present, ammonium carbamate can also be formed [mani *et al.* 2006]. Ammonium carbamate is an interesting molecule because, just like bicarbonate, it reacts on metallic surfaces to form urea [Hanson *et al.* 2021]. Conversely, hydrothermal simulations using 0.1M of urea as an amino donor in the presence of 1 mmol of elemental nickel nanopowder and an atmosphere of 15 bar carbon dioxide and 10 bar hydrogen yielded micromolar amounts of formamide, acetamide, and alanine. Theoretically, these reactions can also yield formaldehyde [Gahlaut and Paranjothy 2018], although it was never observed by ^{1}H-NMR under the tested conditions. The reactivity of aldehydes such as formaldehyde and acetaldehyde would significantly increase side reactions and add complexity to these mixtures [Kopetzki and Antonietti 2011; Omran *et al.* 2020]. However, opposed to natural alkaline hydrothermal vents, such simulations lack a constant supply of reactants, thus, yielding products with increasing complexity but decreasing concentrations.

2.3. Reductive transaminations of formamide and acetamide to organic acids synthesised in hydrothermal vent simulations.

Formamide and acetamide are of great interest for metabolic origins because they incorporate C - N bonds in stable molecules. Moreover, both organic amides have been synthesized from 0.1 M of ammonium hydroxide, 1 mmol of awaruite, and 100 bar of carbon dioxide over 16 hours of reaction at 70 °C. In particular, formamide has been extensively suggested as a possible precursor for complex biopolymers [Saladino *et al.* 2012; Bizzarri *et al.* 2021] – although formamide is highly toxic to cellular life due to its denaturing properties [Celeda *et al.* 1992]. A possible formamide-rich scenario is interesting because these molecules could act as amino donors for transamination reactions. In the case of formamide concentration via thermal gradients, it could even act as a direct precursor to more complex biopolymers [Saladino *et al.* 2012]. Based on these premises, an overview of the tested experimental conditions can be found in the table below.

C) Experimental conditions	Temperature (°C)	Carbon source	Nitrogen source	Catalyst of choice	Reaction time (h)	pH before reaction
	25 - 100	CO_2 - HCO_3^-	CH_3NO - C_2H_5NO	Ni	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3^-	CH_3NO - C_2H_5NO	NiO	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3^-	CH_3NO - C_2H_5NO	Ni_2O_3	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3^-	CH_3NO - C_2H_5NO	Ni:Fe	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3^-	CH_3NO - C_2H_5NO	Ni_3Fe	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3^-	CH_3NO - C_2H_5NO	Fe	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3^-	CH_3NO - C_2H_5NO	FeO	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3-	CH_3NO - C_2H_5NO	Fe_3O_4	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3-	CH_3NO - C_2H_5NO	Co	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3-	CH_3NO - C_2H_5NO	Mo	4 - 72	7 - 14
	25 - 100	CO_2 - HCO_3-	CH_3NO - C_2H_5NO	Mn	4 - 72	7 - 14

Given the possibility of converting aldehydes and ketones to amines at 120 °C in the Leuckart reaction [Alexander and Wildman 1948], a series of experiments were performed to test the feasibility of organic amine formation from carboxylic acids. When reacting (1 mmol) of iron-based catalysts (Fe and Fe_3O_4), 10 bar of H_2, and 15 bar of CO_2 at 100 °C for 16 hours, the main product of carbon dioxide reduction is acetate. Conversely, when (1 mmol) of Nickel is used under the same reaction conditions; formate is produced in greater abundance. Given

the different potential for yielding C1 and C2 compounds according to the catalyst used, formamide could either undergo methylation and form acetamide or act as an amino donor for the reductive amination of acetate. Thus, also yielding acetamide in the presence of a suitable catalyst.

Acetamide, however, is also cytotoxic and denaturing [Lozoya-Colinas *et al.* 2021]. The cytotoxicity of formamide and acetamide is attributed to their ability to disrupt hydrogen bonding [Schermann, J. P. 2007]. The formation of complex biopolymers such as polypeptide chains, proteins, and DNA is only possible if peptide and nucleotide bonds are stable enough to sustain continuous molecular complexity. In principle, a high concentration of formamide or acetamide could disrupt these bonds and result in a mixture of unreactive monomers. This might suggest that just as formamide, acetamide either played exclusively the role of an amino donor in Leuckart-like reactions, or the high concentration of these organic amides would not be homogeneous throughout the prebiotic mixture. Based on these premises, formamide and acetamide were tested for their potential as amino donors to organic acids (reductive transamination) under alkaline hydrothermal conditions. An overview of the main experimental results can be found in the table below.

2.3.		Result summary											
		Catalyst of choice											
		Ni		NiO	Ni_2O_3	Ni:Fe	Ni_3Fe	Fe	FeO	Fe_3O_4	Co	Mo	Mn
C) Result summary	Carbamate	?	?	-	-	✓	N/A	✓	-	-	?	-	✓
	Bicarbonate	?	?	-	?	✓	N/A	✓	-	-	?	-	✓
	Alanine	-	-	-	-	-	N/A	-	-	-	-	-	-
	Glycine	?	?	-	-	?	N/A	-	-	-	-	-	?
	Formamide	-	-	-	-	-	N/A	-	-	-	-	-	-
	Acetamide	✓	-	-	?	?	N/A	-	-	-	-	-	**?**
	Ammonium carbonate	✓	-	?	?	✓	N/A	?	-	-	?	-	✓
	Condensation products	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Urea	?	-	-	-	-	N/A	-	-	-	-	-	-

Reaction conditions													
	pH after reaction	12	10 - 11	9 - 11	9 - 11	9 - 11	N/A	9	8 - 9	9 - 11	9 - 11	8 - 11	9 - 11
	H_2	✓	✓	-	-	-	N/A	-	-	-	✓	-	✓
	Carbon source	HCO_3^-		Either	Either	HCO_3^-	N/A	Either	Either	Either	Either	Either	Either
	Nitrogen source	CH_3NO	C_2H_5NO	Either	CH_3NO	CH_3NO	N/A	Either	Either	Either	Either	Either	CH_3NO
	Temperature (°C)	70		Either	Either	100	N/A	Either	Either	Either	Either	Either	100

Under temperatures of 25–100 °C and for reaction times between 4 and 72 hours, formamide failed to act as a suitable amino donor to organic acids freshly produced from bicarbonate and carbon dioxide as measured by ^{1}H-NMR. The limited reactivity of formamide can be greatly attributed to the highly aqueous environment it was in [Nguyen *et al.* 2011; Bada *et al.* 2016]. At lower temperatures (below 80 °C), formamide has a greater degree of association than water [Bipp and Kieczka 2000]. Furthermore, the addition of water leads to uncontrolled side reactions that often favour organic acid formation and limit formamide's reactivity [Bipp and Kieczka 2000]. Nonetheless, by employing thermal gradients in a porous environment such as vent systems, formamide could be concentrated and react further in the presence of suitable catalysts and a suitable reductant in order to either undergo reductive transamination or to act as a precursor for amino acids [Niether *et al.* 2016]. These thermal gradients, however, were not tested under laboratory conditions due to technological limitations of the high-pressure steel reactor setup.

Applying different *in situ* temperatures while maintaining the high pressures of reactants in one run would be ideal for yielding the formation of amino acids and potentially nucleobases from formamide or acetamide in aqueous solutions. One way to confer these conditions would be using a thermoelectric cooling apparatus inside the reactor. A device such as a Peltier cooling module could, in principle, be adapted to the high-pressure reactor and generate extreme temperature gradients within a few millimetres and potentially concentrate products by thermophoresis.

Another interesting observation in these systems is that product profiles changed significantly when using formamide or acetamide as nitrogen sources. When reacting formamide and bicarbonate for 16 hours and different metal catalysts at 70 and 100 °C, only

formate and acetate could be observed via ^{1}H-NMR. These two major products were found in millimolar amounts, whereas all other products of bicarbonate reduction observed in similar conditions without formamide were not detectable. Other products expected in these reactions include pyruvate, acetamide, ethanol, and methanol. This sharp decrease in the product spectrum indicates that formamide may limit the reactivity of metal surfaces and impact the formation of organic acids. Formamide may also undergo hydrolysis and yield formate and ammonia, limiting reaction products to either be exclusively acetate, or to a greater extent, render the quantification of formate as a direct product of bicarbonate reduction imprecise. However, the expected product spectra of CO_2 fixation could be observed when using acetamide as a nitrogen source. Acetamide is stable at 320 °C in the absence of a metal catalyst [Jiang *et al.* 2020], whereas formamide degrades significantly at temperatures as low as 160 °C [Bipp and Kieczka 2000]. The stability of acetamide in a broader temperature range may be beneficial in hydrothermal pores. For reactions performed at 70 and 100 °C for 16 hours using (0.1M) acetamide and (0.1 M) bicarbonate, micromolar amounts of formate, acetate, ethanol, and propionic acid were produced. However, no amino acids could be observed from either formamide or acetamide reactions as measured by ^{1}H-NMR. Although formamide and acetamide did not yield measurable amounts of amino acids, the results with acetamide are promising because it suggests that the carbon fixation part of the reactions is not negatively affected.

A possible solution to the low yield quandary would involve improved analytics and some means to concentrate products. In the future, analyzing probes of similar experimental conditions with other synergic analytical techniques such as LC-MS would be vital. Mass spectrometry can be over a hundred times more sensitive than ^{1}H-NMR, making it more suitable for such a reaction system [Wishart, D. S. 2011]. This increased sensitivity could give us a glimpse into what side reactions would occur in a mixture containing carbonate, bicarbonate, carbamate, urea, aldehydes, organic acids, alcohols, and amino acids. In the absence of thermal gradients or other means of molecular thermophoresis potentially found at alkaline hydrothermal vents, these simulations can only yield a limited number of very diluted products. Because if life started in an alkaline hydrothermal vent, products must have been available at high concentrations. Such a system can only be achieved by (complex) conditions that involve a constant supply of reactants and catalysts. In these simulations, we use a far simpler system where the effects of hydrostatic pressure and temperature could not be fully exploited. Despite the experiments' simplicity, their product spectrum, including formamide, acetamide,

ammonia, and carbamate, gives us a glimpse in 16 hours of what could unfold in geologic timescales.

3. Publications

3.1. To what inanimate matter are we most closely related and does the origin of life harbour meaning?

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Authors: William F. Martin, Falk S. P. Nagies, and Andrey do N. Vieira

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Contribution: Last author. Conceptualization, writing, review and editing.

Summary: This publication aims to delineate the constraints of cellular life, its place, and meaning from a philosophical and scientific perspective. Historically, questions of meaning were often addressed by a purely philosophical approach. However, natural scientists are better acquainted with the nuances of biology to define life and how it likely started. In order to bridge the gap between the epistemologies of life's origins and the philosophical hypotheses behind it, we conducted an in-depth review of the leading scientific hypothesis for origins and their philosophical repercussions. Ultimately, we created a guideline of seven parameters in which all hypotheses aiming to elucidate cellular origins must fulfil.

philosophies

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Article

To What Inanimate Matter Are We Most Closely Related and Does the Origin of Life Harbor Meaning?

William F. Martin *, Falk S. P. Nagies and Andrey do Nascimento Vieira

Department of Biology, Institute for Molecular Evolution, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, Germany; falk.nagies@hhu.de (F.S.P.N.); nascima@uni-duesseldorf.de (A.d.N.V.)
* Correspondence: bill@hhu.de

Abstract: The question concerning the meaning of life is important, but it immediately confronts the present authors with insurmountable obstacles from a philosophical standpoint, as it would require us to define not only what we hold to be life, but what we hold to be meaning in addition, requiring us to do both in a properly researched context. We unconditionally surrender to that challenge. Instead, we offer a vernacular, armchair approach to life's origin and meaning, with some layman's thoughts on the meaning of origins as viewed from the biologist's standpoint. One can observe that biologists generally approach the concept of biological meaning in the context of evolution. This is the basis for the broad resonance behind Dobzhansky's appraisal that "Nothing in biology makes sense except in the light of evolution". Biologists try to understand living things in the historical context of how they arose, without giving much thought to the definition of what life or living things are, which for a biologist is usually not an interesting question in the practical context of daily dealings with organisms. Do humans generally understand life's meaning in the context of history? If we consider the problem of life's origin, the question of what constitutes a living thing becomes somewhat more acute for the biologist, though not more answerable, because it is inescapable that there was a time when there were no organisms on Earth, followed by a time when there were, the latter time having persisted in continuity to the present. This raises the question of where, in that transition, chemicals on Earth became alive, requiring, in turn, a set of premises for how life arose in order to conceptualize the problem in relation to organisms we know today, including ourselves, which brings us to the point of this paper: In the same way that cultural narratives for origins always start with a setting, scientific narratives for origins also always start with a setting, a place on Earth or elsewhere where we can imagine what happened for the sake of structuring both the problem and the narrative for its solution. This raises the question of whether scientific origins settings convey meaning to humans in that they suggest to us from what kind of place and what kinds of chemicals we are descended, that is, to which inanimate things we are most closely related.

Keywords: origins of life; epistemology; hydrothermal vents; warm little pond; site of life's origin

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1. Introduction

The question of how life emerged is older than science. Historically, various cultures have found satisfactory explanations for how life emerged. Such explanations have been and continue to be of great importance both to society as a whole and to individual members thereof. We will make no attempt to present the origins narratives of different cultures here; every reader will know some examples against which to vet the merit of our claim. The explanations that different cultures have handed down over generations differ in their principles and narrative, though all fulfil a common goal of providing an account of how living things in general and humans in particular fit into the continuum of time. Scientists also have explanations for how life emerged. The explanations that different scientists offer also differ in their principles and narrative, although it is unclear whether scientific origins narratives generally provide an account of how humans fit into

the continuum of time, because most scientific origins narratives do not directly connect to specific kinds of living cells that we can observe today that could serve as a starting point of biological evolution. That is, most scientific origins narratives either offer an account of an abstract or idealized cell or, more often, strive to explain some specific aspect or individual component of cells, rather than addressing the origin of the whole cell. For example, many origins narratives present an account for where RNA came from and what it can do if one assumes the existence of an unlimited supply of activated RNA precursors on the face of the early Earth [1,2]. That is a much simpler task than presenting a narrative for where a whole living cell came from under the premise that there was no unlimited supply of activated RNA precursors anywhere on the early Earth [3,4]. RNA is a molecule: a nucleic acid with three simple components—phosphate, sugar, and bases—that are iterated in a linear polymer that can assume different conformations via folding. It is easier to explain the origin of a molecule than it is to explain the origin of a prokaryotic cell: a small volume of space roughly one micrometer on a side, containing about ten thousand ribosomes, each consisting of about 50% RNA by weight, plus five million individual protein molecules that catalyze on the order of 2000 different chemical reactions that harness environmentally available energy and nutrients to make an imperfect copy of itself in a period of time that might last 20 min or 2000 years. It is also unclear whether scientific origins narratives, whether for molecules or cells, even qualify as science, because the object of investigation lies irretrievably buried in the past, such that even if we were to recreate life from chemicals in a laboratory, we would have no way of ascertaining whether the first cells, our ancestors, arose that way.

Should we then just give up on studying origins? There are good reasons to do so, but at the same time, humans apparently have an innate curiosity about where we came from and how living things came to be the way that we observe them to be in nature. It is part of our human condition to seek and find answers to the question of where we came from. This might relate to the circumstance that humans seem to generally fear things that they cannot explain and tend to find comfort in explanations of otherwise inexplicable phenomena. Scientific narratives for origins do not provide comfort, though they do strive to soothe our curiosity about the living past. The interest of the general public in origins would, of itself, seem to justify origins research.

During the Enlightenment, European science sought independence from religious doctrines, but its scientific revolution had no immediate impact on scientific concepts about origins because that first generation of people whom we would tend to call scientists by today's measures, while taking a pragmatic rather than dogmatic approach to natural phenomena, had no grasp whatsoever of what makes living things alive and what they are made of, let alone how such might have arisen. Priestly's discovery of oxygen [5] was a step towards identifying chemical requirements for life, but offered little help towards understanding what living things are or where they come from.

Though today's libraries can boast much progress in organic chemistry, microbiology, geoscience, astrobiology, and computational biology, the question of how life began remains perennially near the top of the ten most wanted list of major (and underdetermined) problems or questions facing science. Traditional approaches to the problem start from the simple and work forward to the complex. If we place the origins problem in the context of chemistry that was known around 1900, scientists knew that atoms form molecules and that cells contain molecules, though most of the molecules were too large and complex to identify. Scientists knew that cells transform substrates into other molecules in some manner, but the nature of such transformations was obscure, as was the question of whether any chemical transformations were universal among cells. Otto Warburg helped to unravel a good many of the chemical transformations germane to the reactions that keep cells alive [6,7]. Today, we know a great deal about how cells transform molecules during the process of growth (physiology, the chemical reactions of growth) and how the information that directs the synthesis of a new cell is stored and retrieved, but the origins problem of how such reactions started remains, although some newer findings do harbor hints of

progress in that they identify a distinct chemical connection between geochemical reactions and what might have been the first biochemical reactions [8,9].

Tracking down the first chemical reactions that led to metabolic pathways would allow us to pin a starting point on the map of physiological reactions. If we had a robust clue about the starting point of metabolism, we could probe the concept further by experiment. However, if we consult the literature about the myriad ways in which cells make a living physiology, the undertaking of assigning a starting point might seem like a task without chance of success. There are hundreds of biochemical pathways used by countless prokaryotic lineages, many with unique traits, and most microbes that we know to exist (because we can see them) are still vastly under characterized because they do not (yet) grow in pure culture. But is the search for a starting point of physiology really futile? Among all the pathways we know, whereby hundreds are used for harvesting energy alone [10], common sense has it that they cannot all be equally old. Some pathways must be older and some must be younger. They cannot all have arisen at once. There must have been a temporal progression in metabolic evolution [11]. Finding a suitable starting point for the evolution of biochemical reactions would help constrain an otherwise unwieldy problem, because it would identify the chemical reactions at the very base of the life process as it arose.

Given the daunting diversity of microbial metabolism, finding its starting point appears as a typical needle in a haystack problem: hopeless. However, let us suppose that the needle really *is* in the haystack, that is, that among the thousands of pathways we know, one really is the oldest. To find it, all we have to do is use a really strong magnet, and suddenly, finding that needle is easy, a day's work for one person—with that we can identify the starting point of metabolism, the origin of the chemical reactions that produce cells. Where can we obtain such a magnet? We will return to that at the end of this paper.

1.1. Narratives Are Set in Places

In recent years, rapid progress in the field of genome sequencing and analytical biogeochemistry has enabled the creation of robust molecular libraries that allow a far more precise categorical classification of life forms than anything we had previously. In the past however, many of these categorizations were performed via generalization derived from complex and systematic observation of macroscopic animals and their traits over many generations in a populational context. In most cases, evolutionary experiments were not feasible. In the 1800s, the rise of evolution as a theory changed everything about the way scientists thought about the history of life, and brought with it the possibility of a single origin from which all else might have emerged via time and change. Though no one in Darwin's time could have provided a satisfactory answer to the fundamental problem of origins, his speculations on the first organism's environment, the "warm little pond" in his correspondence with Hooker, paved the way to the proposal of the first concrete experiments in the field many decades later [12]. Darwin left a hint about the conundrum of origins in a time where geochemical data for the deep past were scarce, if not completely unavailable. Still today, the answers to origins are buried irretrievably deep in the past. In Darwin's day, no experimental approach was robust enough to spark progress on the issue, thus leaving most hypotheses to the sheer taste of personal belief systems. To some extent, that is still true today.

Darwin set a rarely recognized tradition in origins research; he started the origins narrative exactly the same way as traditional cultural and religious narratives usually have, namely he started with a place and a setting, a warm little pond: "But if (& oh what a big if) we could conceive in some warm little pond with all sorts of ammonia & phosphoric salts,—light, heat, electricity &c. present, that a protein compound was chemically formed, ready to undergo still more complex changes . . . " [13]. Curiously, origins research still works that way today in that scientific origins narratives usually start with a place.

Of course, it would have helped Darwin to know more about the chemical reactions of cells. In his day, the most severe impediment to approaching origins in concrete language was a lack of knowledge about what cells are made of in detail and how they work as a

chemical reaction. In Darwin's day and well into the 20th century, biologists relied on the concept of protoplasm to explain the seemingly inexplicable properties of life [14]. The term protoplasm traces to the middle of the 1800s and the Czech physiologist Jan E. Purkinje and the German physiologist Hugo von Mohl [14,15]. At the heart of the protoplasm concept was the notion that a special vital force, a vis vitalis, is associated with living substances but is lacking in non-living substances, creating, in essence, two different kinds of matter. Strong proponents of protoplasm were called vitalists, their opponents were called mechanists [16]. In Darwin's time and thereafter, biologists had no chemical understanding of the life process within cells. Vitalists held that protoplasm represented a special kind or organization of matter that bestows the property of life and distinguishes living from non-living things. In his book on protoplasm, Drysdale [17] characterized protoplasm as follows " ... the elements are in a state of combination not to be called chemical at all in the ordinary sense, but one which is utterly sui generis. That, in fact, no albumin, fibrin, myosin, protagon, or fats exist at all in the living matter, but that the sum of the elements of all these is united into a compound, for which we have no chemical name, and the complex mode in which the atoms are combined we can form no idea; and it is only at the moment of death that those chemical compounds, with which we are familiar, take their origin. [...] Vitality is thus a property inherent in each particle of the living matter, and all the parts of a complex organism differ in function, each part has a specific kind of vitality peculiar to itself." Clearly, if one held that life was chemically distinct from other forms of matter, then the key to understanding the origin of life was understanding the origin of protoplasm, a substance immune to direct investigation, but whose properties, in theory, remained stable enough over the eons of life's history to distinguish major lineages in the living world [18]. Protoplasm might be seen as a kind of dialectic capitulation before the severity of the origins problem—its nature is too complex, hence unknowable.

Despite a lack of understanding of what cells are and how they function, and despite the absence of an empirically supported concept of the conditions on early Earth, Mereschkowsky [18] inferred that the first cells arose as the young Earth was still hot and covered in boiling water. Like Darwin, he had a setting, but it was much harsher than a warm little pond, because he thought the first cells must have been extremophiles. In his view, they were extremely small, they could thrive at temperatures of 100 °C, they were anaerobes, they had the ability to synthesize proteins and carbohydrates from inorganic substances without the help of chlorophyll and they were resilient against alkaline solutions, concentrated salt solutions, sulfur compounds, and diverse toxins [18]. Those extreme conditions sound much like those of modern theories for an autotrophic origin of life in hydrothermal vents, theories that we can find in modern college textbooks [19]. Haeckel [20] espoused similar but much less detailed thoughts about the nature of the first cells. However, even today, the thought of origins in a dark, deep, hot and oxygen-free abyss, from gasses that react all by themselves in the presence of catalysts [9], conjures a hellish notion that has almost a demonic character for proponents of the warm little pond [21].

By the time of Oparin's book [12], and by Miller's [22] experiment at the latest, biological and microbiological renderings of origins gave way to chemical renderings of origins; beginnings from CO_2 gave way to beginnings in some form of pond. For the majority of the last century, Darwin's hypothesis, enriched by Oparin's [12] lengthy book and Haldane's [23] tersely argued narrative of a prebiotic broth, or organic soup, inspired many researchers to embrace a simple explanation to origins: The action of sunlight on carbon in the ocean could, in principle, generate all sorts of organics, which could somehow assemble themselves in solution into something more complex. This approach assumed that at the early stages of planetary evolution, Earth's atmosphere was poor in oxygen (O_2) but rich in reducing gasses such as ammonia (NH_3), methane (CH_4), and hydrogen (H_2), based on studies of other planetary bodies like the outer Jovian planets in our solar system. This kind of atmosphere, which was used in Miller's experiment, generated amino acids and other organics using energy supplied by an electric spark, simulating lightning.

Many subsequent hypotheses built upon this foundation, adhering to the premise that, in essence, the origins problem consisted of two components: an initial process or phase of molecular synthesis to obtain the basic building blocks of life (the origin of soup) in a particular setting, followed by a subsequent process or phase of molecular organization that arranged pre-existing components into more highly structured state (self-organization). In the 1970s, a concept emerged that RNA molecules could compete with one another for activated RNA monomers (resources), such that the fastest replicating molecules became the most fit in a Darwinian sense [24] by bringing forth the most progeny. This idea was exceptionally well suited to empirical endeavor and experimental tests. It gave rise to over five decades of productive research on a concept and a field that have become widely known as the RNA world. In many modern papers, one can read about the RNA world as if it were an established "fact", a known whose properties merely require further characterization [1]. In other papers, the term RNA world is used more or less synonymously with the origins of life [25]. There is now much evidence underpinning the view that RNA molecules can multiply if they are provided with a steady stream of biochemically pure precursors, so much evidence in fact that some have begun to ponder the "ecology" of RNA molecules in such a world [1] as if it, the RNA world, were an observation in nature as opposed to a premise.

Fascination with RNA has, however, distracted from the more important and still unanswered question of whether an RNA world ever existed and if it existed [26,27], whether it had anything to do with the origin of things that are actually alive—microbial cells [28]—notwithstanding the sobering observation that RNA, given inorganic substrates, is clearly no more alive than an isolated protein, a fat droplet, or a grain of starch. A critic will immediately interject that we are constraining the issue too much by imposing the criterion of living from "inorganic substrates", but if we accept the evidence indicating that the moon forming impact converted the Earth to a ball of boiling magma, converting all carbon to CO_2, then CO_2 was the initial form of carbon from which life emerged [29]. Another critic will complain that after the magma oceans cooled, there was a late heavy bombardment that brought a veneer of new organics from space, countering the CO_2 dictum [30]. We would counter that the late heavy bombardment probably never even occurred; it is more likely an artefact of (mis)interpreting lunar craters [31]. Another critic might complain that we are countering philosophical critique with appeals to evidence rather than logic. Yes, as we said at the outset, this is an informal essay about origins and meaning.

1.2. Molecules or Cells?

For the biologist, it is sometimes more useful to discuss the origin of "microbial cells" than to discuss the origin of "life" because if one debates the origin of life, one can debate in very open terms and with a long literature the question of what life is, leading to philosophically fertile but biologically barren fields of discourse. If we constrain the issue to concern the origin of things that are obviously alive, microbial cells, and without whose existence there would be no creatures that debate, then we get closer to the issue, the dimension of which causes many researchers to give up: how do we get from the early Earth to a fully fledged free living microbial cell whose main function it is to first convert environmental energy into expendable chemical currency for survival and then, if resources permit, to grow from inorganic compounds all by itself. The hard part of seeing the origin of life as the origin of microbes is that one first has to learn a lot of biology, the nuts and bolts of what cells are and how they work, so as to be able to verbalize specific processes underpinning the origins of the components and the whole. That is why it is much more convenient to reduce the origins problem to the origin of RNA, which is a very simple explanandum compared to a cell, or to detour into definitions of life where there are almost no constraints in observation to guide our reasoning. If we insist on defining the problem as the origin of microbes, we can get straight to work on that problem without having to debate the existence of an RNA world or define life before starting. Solving the problem

of the origin of microbes would then be left up to biologists, where it arguably belongs (and where it began), because (with *pro domo* immodesty) nobody knows the individual chemical reactions that compose living things and the ~2000 enzymes that catalyze those reactions in a given microbial cell better than biologists.

We also face the problem that the origin of microbes was a singular event by all logic, because of the universality of central metabolism [32] and the universality of the genetic code [33], and that origin event occurred roughly 4 billion years ago according to isotope data [34]. Again, even if we performed an experiment in which microbial cells demonstrably arose de novo from inorganic compounds in a laboratory experiment, we would still have no evidence in hand that life (our unicellular microbial ancestors) actually arose that way, we would just have a narrative richer than our current ones on how it might have occurred. A priori, we have no access to a more systematic approach to natural problems than of hypothesis, experimentation, observation and interpretation. It seems clear that we can use the scientific method to explore aspects related to the origins problem, but the problem might be without solution. No final answers to be had at origins? That would be an honest admission, but it would not satisfy the curiosity of scientists and the public when it comes to wanting to know where we come from. It is part of our human nature to want to know about the past. All human cultures have a natural interest in the question of where living things came from, a question that, in contrast to agriculture or medicine, has no obvious practical importance unless, of course, concepts of origins help provide meaning, and meaning simultaneously has practical importance, a question that is well beyond the scope of this paper.

If we want to probe problems rooted deep in the past, such as the emergence of the first prokaryotic cells and the diversification of primordial prokaryotic lineages some 4 billion years ago, we have to make some assumptions for the sake of moving forward, and we have to state the assumptions explicitly. Darwin started in a pond, but he never justified why he chose to start in a pond. There might be several ways to recreate life in the laboratory, but the universal laws of thermodynamics that govern the chemical reaction that we call biology apply across the tree of all life. All cells require proteins that are made of amino acids; cells are about 50% protein by dry weight. Genetic material is made of nucleotides; cells are about 20% RNA and 3% DNA by dry weight [35]. Cells require a constant far from the equilibrium system from which to harness energy; cells always synthesize much more ATP than they need, often three times more [36,37], because ATP is the main energy currency of the cell and the converse would violate the 2nd law of thermodynamics. Armed with a few observations of this type, we can discuss the origins problem in the comfort that we are just thinking about it, not trying to solve it.

1.3. Teleology and the Notion of Epistemological Obstacles

Epistemology (the theory of knowledge, the methods to obtain knowledge, and the scope of knowledge) is generally traced to ancient Greece with the works of Plato and Aristotle, who pondered what we know and the distinction between what exists and what does not. In the seventeenth century, John Locke's philosophic categorization and understanding of knowledge became a branch discipline of philosophy per se. Gaston Bachelard [38] contextualized the fields of scientific knowledge and its intrinsic robustness and is regarded by some to be one of the founding fathers of modern epistemology [39–41]. Bachelard [38] proposed that the scientific mind must develop against human nature and that epistemological obstacles are natural stressing points to scientific knowledge that hinders scientific progress. He proposed obstacle categories that have affected science. These include the first experience, or how the first object of research can establish a bias for further experiments, leading to mischaracterization of the subject of study. This can snowball to generate a premature generalization of the problem, another obstacle. The limitations of natural language, the broad use of scientific terms or analogies and the restrictive nature of explanatory words can further impede understanding as can animism as an explanation to natural problems or indiscriminate use of knowledge with its often-

overlooked teleological conception. Few scientific questions lend themselves more readily to animism and hidden teleology (the explanation of phenomena in terms of the purpose they serve rather than of the cause by which they arise) than the issue of how inorganic material became living cells.

According to Mayr [42], no other ideology influenced biology as much as teleology. Mayr categorizes the teleological obstacle into five subsections: (i) Teleonomic processes, referring to phenomena with a goal-directed purpose guided by an implicit program. (ii) Teleomatic processes that are not guided by a pre-established program but instead follow passively a directed path of events as a result of the action of natural laws [43]. (iii) Cosmic teleology refers to processes guided by a supreme force. (iv) Adaptive programs, or processes in which there is a direction of events towards a posteriori outcomes such as those observed in non-Darwinian evolutionary theories. Lastly, there is (v) purposeful behavior in which the subject of change is directing its behavior towards a certain need. Mayr proposes that the use of teleological arguments must be avoided at all costs as no phenomenon in nature is innately teleological. In Masatoshi Nei's mutation driven theory of evolution [44], teleological components are forbidden by mechanistic means, the primary process-driving vectors of evolution being mutation, which all things being equal can be (somewhat safely) assumed to be blind. As such, the use of teleology in the field of origins fuels the discussion of whether there is a role to dogma in science and if such concepts hinder the acquisition and transmission of knowledge by (falsely, we assume) giving purpose to the natural phenomena permeating chemical and biological evolution.

1.4. Could Nei's Conjecture Be True?

Yet before we look into stumbling blocks of how we think about evolution, let us consider something that has bothered the senior author of the present paper for some time. Is Nei right? Does mutation really set a vector in evolution [44], or is mutation unlimited in scope with selection doing the work of bringing forth new forms from the set of all possible? Worse, is there any way we can even approach an avenue towards obtaining an answer to the problem? We know more about our world than in Plato's day, maybe we can get an estimate. We start with the question of how many cells have ever lived and the estimate that roughly 10^{30} microbial cells are alive today [45]. Most of these cells are living in the subsurface or marine sediment where they are growing very slowly, if at all, some with doubling times estimated as hundreds or even thousands of years [46]. We generously give them fast growth and an average doubling time of ten generations per year. Ten generations (doublings) per year would mean a 1024-fold increase of 10^{30} microbes or 10^{33} new microbes per year, but global biomass cannot increase 1000-fold every year, as nutrients are limiting (fortunately, for us all). However, we are still generous and say that each microbe nonetheless manages 10 generations per year (accompanied by many microbes that simultaneously die). For 10^{30}, microbes that gives us 10^{33} individual doublings per year, or, summing up across 4 billion years, we have about $4{\cdot}10^{42}$ generations on the books, which we conveniently round to 10^{42} new microbes in history because for most of earth's history, fewer microbes existed per year than today, owing to the lack of oxygen. How many mutations have there been in those ~10^{42} cells? Let us say that a microbe has a mutation rate similar to that in *Escherichia coli*, on the order of 10^{-3} per generation per genome [47,48]. At that rate, for every 1000 cells that undergo one cell division, one new mutation will accrue. Over 4 billion years, that means that every 1000th cell gets a new mutation, or about 10^{39} new mutations in all of evolution. That sounds like a big number. Yet maybe we underestimated the number of cells badly, for example that we should be using much faster doubling times, so we throw in a factor of 10^9 for good measure, giving a rather generous estimate of 10^{48} mutations that have occurred in evolution—a big number, close to the number of water molecules in the oceans, but smaller than the approximate number of protons in the universe, ~10^{80}. How does the number of roughly 10^{48} mutations in evolution stack up against the number of possible mutational states for microbial genomes? Are they roughly equal?

For that, we have to estimate how many mutational states are possible. If each microbe has one genome of only 1000 genes with avg. 1000 bp each, then there are 10^6 bp in the genome, with four possible bases per position, or $4^{1,000,000}$ possible sequences, which is about $10^{602,060}$, or close enough to $10^{600,000}$ to round it for our purposes. We can also count gene transfers into the mutation category, which does not generate sequence variants beyond the $10^{600,000}$, although it could increase the number of sites in the genome, raising the number of possible mutational states. The number of possible sequences that could be realized for small genomes during evolution, ~$10^{600,000}$, is hundreds of thousands of orders of magnitude greater than the number of mutations that took place, generously estimated at ~10^{48}. One might interject that many sequences cannot be realized because of the nature of the genetic code, the size of proteins, the occurrence of stop codons, etc. To conservatively take that into account, let us say that the structure of genes, reading frames, proteins and the genetic code constrains evolution so tightly that only one single base per gene is allowed to vary by mutation. That is an extreme exaggeration of reality, but we are just trying to get an estimate. Allowing only one base per gene to mutate per genome, keeping all other bases constant throughout evolution, reduces the number of possible sequences from $4^{1,000,000}$ to 4^{1000} possible sequences because we have assumed 1000 genes per genome, and that still translates to 10^{603} possible sequences, such that even then, the number of possible sequences, over-conservatively estimated, that could be realized for small genomes during evolution, is still 10^{500} times greater than the number of mutations that took place, overgenerously estimated, recalling that the number of protons in the universe is roughly 10^{80} for comparison.

Regardless of how we cut the cake of possibilities, mutation rules, it would seem. Does Darwin's natural selection even figure into this? Yes, in the big picture, selection is important, obviously, as it weeds out unviable sequence variants in specific environments. However, the point here is that mutation never even had a ghost of a chance to explore what combinations are possible with genomes in four billion years. Nei once said in a lecture to 1000 evolutionary biologists in Puerto Rico "Natural selection is overrated". The audience gasped, some quietly scoffed. The person sitting next to one of us (WM) asked "Did he really just say that?", "Just listen and pay attention" was the reply.

The foregoing back of the envelope calculation shows that the vectors of evolutionary lineages from origins to the present were mechanistically limited by mutation (and driven forward in time by thermodynamics, we know without the calculation). There was never a world on this planet where life explored all possibilities, with selection pruning all viable states from the set of all possible. If mutation is blind, which we generally hold to be true, then the course of evolution is only one of (for practical purposes) an infinite number of possibilities. The path that mutations, not selection, took brought us to where we are. That is possibly irrelevant to the issue of how we think about origins, early evolution, and meaning, but possibly not. Stephen J. Gould famously asked whether if we could replay the tape of evolution, a similar result would unfold. Some argue yes [49]. The foregoing indicates that the answer is a clear no, although life still has to obey the 2nd law, meaning that the finite number of chemical reactions on Earth (there are only 92 natural elements and they have $\leq$8 oxidation states each) that can be harnessed to support life sets constraints. Is natural selection overrated? It very possibly could be, and we are hardly the first to suggest that it is so.

1.5. The Concept of Epistemological Obstacles in Hypothesis Pervading Origins

We can briefly consider the role of epistemological obstacles and how they influence the progress of a heavily polarized field such as origins. Given the influence of teleology in evolutionary biology, we can ask how the categories proposed by Mayr might perpetuate a dogmatic line of thought that was deeply rooted in evolutionary biology. Although abundant in the humanities, scientific case studies of epistemological obstacles in the fields of origins and early evolution are rare by comparison [50–54]. We contextualize the concept of epistemological obstacles in origins and early evolution by identifying

teleological arguments in origins and estimating their impacts. Being biologists by training, our philosophical scope is narrow.

The year 2020 witnessed continued progress in the chemistry of origins. The gap between opinion-based to experiment-driven hypothesis laid a concrete environment for new theoretical and experimental work. However, the origins field is divided into schools with conflicting, mutually incompatible viewpoints and heated debates as to how life started. This is a sign that the problem is underdetermined, like the origin of eukaryotes [55], suggesting a role for philosophy of science to further progress. In simplistic terms, experimental work on the topic should have an explicit theoretical basis that generates predictions, that is based on robust methods, that involves the careful observation of data that lends itself to meaningful interpretation. In practice, there is no way to perform such work without personal bias. We are the product of our experience, never genuinely objective. The literature is vast and nobody knows all of it. How to even begin forming hypotheses under such suboptimal conditions? Even in the absence of knowledge, humans are blessed with curiosity. Curiosity can lead to good experimental questions, regardless of whether we have any idea of how curiosity works. Plain curiosity can work in favor of progress. In our favor also, good scientific work ethics has it that we want to do sound work that will stand the test of time, that will make a difference as science moves forward. We all know papers that had an impact on our own view of the world, papers that made a difference in how we approach scientific work. Often, we want our papers to be like those positive examples that, together with curiosity, led us down the path that we have taken. No objectivity there. Curiosity leads investigations like mutation leads evolution.

A problem is how to reduce human bias in a field like origins. Given the multitude of theories on origins, it is only natural that scientists rely on intuition, which might be driven by curiosity. Although highly subjective, it is intuition that tells us what clues to follow and which directions to pursue further. Curiosity sets a course, intuition decides among alternative paths forward. Of course, intuition is also influenced by beliefs. With the ever-growing information pool in the context of schools following mutually incompatible hypotheses, it is only human to follow the information that meshes most harmoniously with what we "think" we already "know", leading different schools in different directions, which is perhaps the best thing that can happen to a field, for if everybody in a field is following the same idea, there is little opportunity for discovery.

1.6. Settings in Origins Theories: Where to Start?

There are currently a number of competing hypotheses about origins that, despite their differences, have a few things in common. For example, all theories have to assume a constant supply of carbon, nitrogen, sulfur, phosphorus and trace elements as all life is made of these; hence, we can presume that the first cells were as well, such that a constant element supply was needed to replenish reserves and to permit growth. In addition, these elements had to be supplied in such a way that they could react to form covalent bonds and simple primordial molecules, which needed to become concentrated enough to react further and form more complex molecules. All the above can be achieved in a variety of ways under certain assumed primordial earth conditions, such that the assumptions concerning the early Earth and the specifics of geochemical conditions at the site of origins constitute a main criterion by which the alternative hypothesis differs. Concerning the transition from simple chemicals to systems of molecular self-organization, few hypotheses have clear concepts, help coming from network theories and concepts of autocatalysis [56,57]. The flux into and out of a steady state pool of reproducibly formed molecules (a metabolic "identity") has to be or become stable enough for them to form increasingly complex structures, ultimately seeding a process that we would describe as evolution today. This leads to roughly seven phases in origin of life hypotheses.

1. The initial setting and medium including soluble materials and catalysts;
2. Generation of organic molecules—substrates and energy;

3. Concentration of organics;
4. Increased molecular system complexity;
5. Stable but far from equilibrium environment fostering the newly formed system;
6. Emergence of the first free living cells;
7. The lifestyle of those first cells.

The setting that one assumes for origins bears upon all other aspects of the molecular process that leads to life or components of life or cells or microbes, so it is worthwhile to look at settings and what they bring to the origins issue.

The pond. As mentioned in the introduction, the first hints of chemically driven origins came from Darwin himself. He proposed that life started as a set of molecules that changed over time to what we consider life today. This gave rise to Oparin's [12] and Haldane's [23] primordial soup concept. This hypothesis assumed that as there were no primary consumers for the first organic molecules, over time and the action of continuous synthesis, these molecules would accumulate to high concentrations in the primordial oceans while also being free to interact with each other until life emerged. A few decades later, Miller [22] experimentally demonstrated the synthesis of some basic building blocks of life by applying electric spark to a gaseous mixture in order to simulate the early atmosphere. The demonstrable synthesis of organic molecules in the Miller experiment had broad impact because it left no doubt that basic molecules of life are nothing special (in the sense of protoplasm, for example)—they can arise through simple reactions of inorganic compounds under imaginable prebiotic conditions. There was also no specific mechanism yet that could bridge the gap between simple molecules and a living, replicating and evolving system. A possible scenario was that by chance, the first gene emerged and then life began [58]. Yet, this would make the emergence so improbable that even Oparin [59] criticized the idea [60]. Nevertheless, that did not stop researchers from building upon these ideas and realizing that, at some point, simple organic molecules were probably created from gases and that over time, these molecules had to assemble into cell-like states [61].

Clay. A possible role of clay minerals in origins was proposed in 1951 by Bernal [62], according to which life could have possibly emerged from a clay matrix [60]. In this hypothesis, organics could be adsorbed into minerals, ultimately concentrating them. A first step into how the necessary concentrations for life's reactions might have been formed apart from a primordial soup. Cairns-Smith [60] then changed this idea to what can be boiled down to self-replicating crystal systems evolving by virtue of their structural changes, which is guided by the specific organic compounds they adsorb. Eventually, these clays would undergo "a genetic metamorphosis" to purely organic units of heritage and thus, life. The incorporation of clay or mineral matrices into the theory has several advantages that would be reused in several later origin of life hypotheses. Nevertheless, another problem with several assumptions on this and other hypotheses slowly became apparent. The primordial soup was deemed unrealistic if not synthesized under increasingly specific circumstances. For example, due to the equilibrium of reactions, it would be impossible for organics to accumulate in the concentrations often assumed [63,64]. This constraint prompted the postulation of drying out phases. That is, the pond is a site for chemical synthesis and then dries up to concentrate and products of synthesis so that they might react further. One round of drying is clearly not sufficient to drive the process forward, leading to the introduction of wet–dry cycles at the site of origins [65]. Several experiments showed potential to polymerize activated RNA monomers using clay matrices as condensing agents [66].

Space. Regardless of how they polymerized, life's basic molecules had to come from somewhere [67]. Besides electricity and UV light, a popular scenario began with the discovery of amino acids (albeit mostly non-proteinogenic) in meteorites [68]. This seeded the panspermia hypothesis, a relatively old idea [69], that life could travel between planets by virtue of interstellar radiation [70] or hitchhike by meteorites [71]. This, of course, did not solve the problem of how life or its basic molecules were created in the first place.

However, it succeeded in motivating some to change the setting for origins to Mars, for example [72], using a reasoning that a few particular chemical reactions relevant to life (polymerizations) would work better in the absence of water, such that a planet with less water might be a more likely site of origin. Cells are about 90% water by weight; cells have no problem with water, they have a problem when there is not enough water.

Black smokers. With the first reports of deep-sea hydrothermal vents in [73], there was immediate discussion of the possibility that these structures played a major role in the origin of life [74,75]. The initial proposal was promptly criticized as the temperatures at the black smoker types of vents, the first ones discovered, were around 400 °C, too hot to sustain life [76,77]. Life is able to survive and even thrive at temperatures of 110–120 °C [78,79], but not 400 °C, which left this particular hypothesis initially with few supporters. Vents, however, impacted the origins issue deeply in that a different setting for the origins problem gave rise to different paths of origins narrative development [80]. The initial focus on temperature at black smokers was unfortunate in that it distracted from the more important observation that they presented a continuously far from equilibrium chemical setting with many metals that play a role as catalysts in microbial metabolism and with many sources of chemical energy to fuel physiological reactions [75].

Volcanic iron and sulfur. In the wake of debates about black smokers, volcanic settings for origins were proposed, where minerals once again played a central role, but not in the form of clays that could absorb chemicals, rather in the form of iron sulfur minerals that performed catalytic activity [81]. As the start of metabolism, Wächtershäuser proposed a citric acid cycle that was energetically driven forward by pyrite synthesis [82]. This harkened back to the conditions proposed by Mereschkowsky [18], and a start of metabolism from CO_2, or autotrophic origins. Volcanos offered an alternative to ponds as a possible site where one could imagine origins, and it led to a different narrative, one that started with surface metabolism, or protobiological reactions in two dimensions before moving to three-dimensional cells [83]. As with the temperature aspect of black smokers, critics honed in on one specific aspect of the theory that seemed particularly vulnerable to criticism—the concept of two-dimensional life [84].

A nuclear geyser. Another setting was suggested by Ebisuzaki and Maruyama [85], a nuclear geyser in which natural radioactive elements provided a great deal of energy in the form of heat and radiation. In the nuclear geyser hypothesis, such sites could provide so much ionizing and thermal energy that organic molecules would easily be generated. This scenario emphasized the energy requirements of early life and that the combination of wet–dry cycles should allow molecular concentration and synthesis of more complex organics. Yet, they make no specific proposals for how LUCA emerged from this new setting. In addition, the damaging effects of radiation from intense radioactive decay for biological molecules are well known. Along the same lines, the minimotif synthesis hypothesis [86] does not try to claim how LUCA emerged nor how the primordial soup was created; rather, this hypothesis tries to bridge the gap between the primordial soup and the first evolving RNA world system by creating a scenario of continuously interacting and thus, evolving minimotifs of the first macromolecules.

An RNA world, somewhere. Minimotifs are examples of addressing the origin of one component of a cell. The paradigm for this principle is the RNA world. In the 1960s, many hypotheses involving a seemingly central role of RNA as a simple, possibly self-reproducing component were emerging [87,88]. With the recognition that RNA molecules had catalytic activity, the primordial soup hypothesis morphed into an RNA world hypothesis [89,90]. The term "RNA world" itself was coined by Gilbert [91] in the context of Cech's discovery of self-splicing introns [92], with RNA molecules envisaged as recombining in a phase of early evolution of RNA genes preceding the advent of DNA genes. The notion of an "RNA world" quickly gained a much broader meaning in the context of chemical origins however, by incorporating aspects of White's suggestion that coenzymes preceded proteins [93] and the enzyme catalyzed in vitro RNA replication experiments of Eigen from the 1970s [94]. This gave rise to the concept of natural selection among exponentially

replicating molecules before the advent of natural selection among cells. It is an enticing idea, but it might be wrong [95]. Proponents of the idea that RNA was the starting point of evolution have gone so far as to propose the existence of specific mountainside settings on the early Earth with individual meteorite impacts and a variety of atmosphere-derived cyanide compounds and temperature ranges across hundreds of degrees as Archaean incarnations of chemical reaction conditions that lead to highly specific RNA monomer synthesis in the laboratory [2]. That is, a set of (geochemically quite questionable) Earth conditions are proposed based on the need for their existence in order to accommodate RNA synthesis for the RNA world, creating a curious kind of cart (RNA) and horse (Earth) problem. The early Earth conditions that geochemists infer are not in the slightest conducive to the specific synthesis of RNA bases [29,96] requiring a different early Earth than the one that geochemists have to offer for the RNA world to work. Some researchers question the need for an independent replicator at any point in prebiotic evolution [97,98]. Others build entire theories upon the premise of its existence [1], but the question of what natural early Earth setting would accommodate the specific synthesis and operation of a replicator is usually unanswered. Nisbet's proposal of hydrothermal springs to harbor an RNA world [99] with organic synthesis starting from methane and ammonia found little resonance among RNA world proponents. Hydrolysis of RNA has traditionally been seen as an insurmountable problem for hydrothermal vents, and it still is in some circles [21]. Yet it is only a problem if one believes in an RNA world that exists in free aqueous solution, as in typical laboratory experiments, because alkaline hydrothermal vents harbor many local environments of low water activity [8].

H_2-producing hydrothermal vents. Deep sea hydrothermal vents that emerge from serpentinizing systems have a chemistry that is fundamentally different from that of black smokers in that their effluent is alkaline and ~100 °C rather than acidic and ~400 °C. The existence of such alkaline vents on the early Earth was inferred from studies of metal ore deposits [99,100]. The first modern deep sea alkaline hydrothermal vent system discovered was the Lost City hydrothermal field [101]. The term alkaline is important because the alkalinity in vents is generated in the process of serpentinization: an unfamiliar word for a philosophy journal but an important one because it produces H_2, a well-known currency of chemical energy, in the crust. H_2 exits the vent in the effluent; the more alkaline the water is, the more H_2 (chemical energy) it contains [102]. The effluent of modern serpentinizing vents contains about four orders of magnitude more H_2 than modern H_2-dependent microorganisms need to grow. The overall chemistry at alkaline vents shares long overlooked similarity with the main energy harnessing reactions of microbes that live from H_2, which can react with CO_2 to generate organic compounds spontaneously because the reaction releases energy (it is an exergonic reaction) as recently demonstrated in the laboratory [9]. Alkaline vents continuously synthesize systems of inorganic microcompartments that can concentrate the products of organic synthesis where they are made, offering a means to concentrate the products so that they can react further. The continuous supply of H_2 interfacing with inexhaustible reserves of CO_2 on the early Earth provides a continuous source of chemical energy to drive the system forward towards higher complexity. In continuity of this reaction, the first cells to emerge from the vent have a carbon and energy metabolism that is based on the exergonic reaction of H_2 and CO_2, in which the cells synthesize acetate (acetogenic bacteria) or methane (methanogenic archaea) [4,103,104] as the main product of energy metabolism. In that view, the first free living cells were acetogens and methanogens, the starting point from which further physiological evolution took place [11]. This narrative addresses the seven criteria outlined above in a level of explicit detail that others do not; it is compatible with the LUCA inferred from genomic reconstructions [105], while at the same time, connecting the origin of life narrative with real microbial cells that still grow in such environments today.

Variations on a vent. There are variants of the H_2-dependent vent narrative for origins. Some replace the chemical energy of the vent with ultraviolet light [106], but the theories do not connect to modern cells because UV light kills cells and no cells can harness energy

or live from UV light. This generates a hybrid between a pond and a vent, relying on UV light as an energy source like Oparin and Haldane did at a time before anyone knew how cells conserve energy or grow. The hot spring hypothesis [107–109] goes back to the warm little pond, with the settings changed in that the pond is fed by hydrothermal springs (providing thermal energy and wet–dry cycles with first molecules possibly coming from meteorites). There is no chemical role for the vent in the hot spring narrative, it just supplies water and salts, nor is there a connection to the lifestyle of the first cells. Other variants replace the chemical energy of the reaction between H_2 and CO_2 with chemical energy of the reaction of methane with nitrogen oxides [110], the problem being that in that narrative, the vent is no longer needed because the source of nitrogen oxides is lightning [111], effectively translocating the setting of the vent to the surface, as in the UV model [106] or the hot spring pond. While the H_2 + CO_2 reaction of the H_2 theory produces essential compounds of central metabolism using only hydrothermal catalysts like awaruite or magnetite overnight [9], no similar laboratory reaction is available to which the methane nitrogen oxide narrative could appeal. Other settings include tidal cycles that provided wet–dry cycles [112] for cyclic DNA (or RNA) synthesis. A different hypothesis that relies heavily on the RNA world is the hydrogel hypothesis [113], where the authors explain the stability that was needed for the system to evolve into primordial life by relying on the physicochemical features of a hydrogel. These structures would be similar to today's cytoplasm in retaining its form until cell-like structures with lipid encapsulation could form. This hypothesis proposes that LUCA possibly emerged from a biofilm-like hydrogel formation [114].

2. Conclusions

The foregoing provides some examples to underpin the claim in this paper that scientific origins narratives tend to start in a setting, a place that we can imagine. How do those settings connect to the chemical reactions of life? In an earlier passage, we said that trying to find the starting point in the evolution of metabolic pathways is like trying to find a needle in a haystack, but that the problem becomes manageable for one person in one day (given an average sized haystack) if we have a very powerful magnet. One of us has been claiming that we have found that starting point. If so, what was the magnet? The magnet would appear to be physiology: the reactions that cells use to make a living are the reactions that make ATP, which is in turn the molecule whose hydrolysis drives all else in the cell forward (thermodynamics). For very few organisms, the reactions that synthesize ATP are the same reactions that supply carbon to metabolism from CO_2: acetogens and methanogens [11,102–104]. If we look for simple forms of energy metabolism, we find that the simplest ones (anaerobic, H_2-dependent, CO_2 reducing, using linear pathways instead of cycles, many transition metals as catalysts, lacking cytochromes and quinones hence, older than heme) are similar to naturally occurring exergonic geochemical reactions. When we react H_2 with CO_2 in the laboratory in water in the presence of a simple hydrothermal mineral as a catalyst—either the pure nickel metal alloy awaruite, the iron oxide magnetite, or the iron sulfide greigite—the backbone of carbon and energy metabolism unfolds all by itself: formate, acetate and pyruvate accumulate in physiologically relevant amounts [9]. All coincidence, possibly, but possibly not.

There is no shortage of settings for origin of life hypotheses and new proposals are constantly emerging. At the most basic level, these fall into two categories with regard to setting: surface or subsurface, with energy coming from the heavens or energy coming from below. This might or might not have a bearing on our unspoken preferences for scientific origins narratives because it would bear rather directly on the possible meaning of origins, facing us with a fairly distinctive choice for what we would rather call our closest relatives among objects of inanimate matter—pointing to the heavens above us or pointing to the Earth beneath us. Genomic reconstructions of LUCA, that is, inferences of the habitat and lifestyle of the last universal ancestor of all cells based on the evidence for

early life preserved in genomes, recover the physiology of a cell that lived from H_2, CO_2, N_2, and H_2S in a hot metal-rich environment [105].

In the beginning of our solar system, the Earth accreted from remnants of a supernova, so says the planetary narrative. In that sense, our closest relative is stardust from the heavens. We live on the surface. In that sense, our closest relative is the surface. We are buried in the Earth, and when the microbes have had the last word, our carbon becomes CO_2 that becomes life of future generations. That was true for our ancestors, in the sense that we come from below. Looking way back, before there was photosynthesis, all life on this planet depended on H_2 from serpentinization [115,116]. In terms of energy for life, H_2 (chemical energy) was the precursor of light (electromagnetic energy). In that sense, our closest inanimate relative would be the reaction of rocks with water in the Earth's crust. Were that so, does it have meaning? Worse, does it have purpose? These are questions that, as a rule, biologists cannot readily answer. However, we can pose them.

In the abstract, we surrendered to the challenge of defining either life or meaning. Greater courage was displayed by Cleland and Chyba [117], who delved into both definitions, concluding that there is no easy definition of life and that the issue regarding the definition of meaning is no simpler, although exploration of both can enrich the way we approach the problems to which these definitions pertain, whereby caution is warranted because many things we now hold to be true as a reference system for definitions might turn out to be false. Cleland [118] later concluded that current approaches to deriving definitions of life are all "deeply flawed". Though not well versed in the long literature of life's definitions, we tend to concur. We furthermore contend that one can study processes relevant to the origin of things that are obviously alive (microbes) and even obtain useful insights into the problem without the strict need for definitions of the process (life) whose origin is under study.

By all accounts, life arose when the Earth was still young. Carbon isotopes indicate the existence of autotrophs by 3.95 [34] or even 4.1 [119] billion years ago, although such studies also point out the possibility that the kind of carbon isotope fractionation found in such ancient samples might be the result of geochemical H_2-dependent CO_2 reduction rather than the biological process. For proponents of H_2-dependent autotrophic origins under hydrothermal conditions, the difference between geochemical and biological CO_2 reduction via the most ancient pathway [9,103] boils down to a matter of grade concerning the sophistication of the catalysts.

There is an issue at origins concerning what the Earth "really" looked like >3.5 billion years ago when life was already up and running, having already evolved a sulfur-based energy metabolism [120,121]. In the least human-friendly versions, the oceans were 10 km deep because the roughly one (or more) ocean volume of water that is sequestered in the crust and mantle today was still in the ocean [122], meaning that there was no land, a severe problem for some theories about the site of origin. The continents did not form until about 3 billion years ago, long after there was life and they were made of rocks that are very low in silicate content (mafic) meaning that they were particularly prone to generate H_2 via serpentinization [123,124]. The atmosphere was like that of Venus, mainly CO_2 and N_2 [29], meaning that the interface of H_2-producing vents with a CO_2-containing atmosphere and ocean would have generated sites with a good fit to the H_2-producing hydrothermal vent idea.

There is also an issue of whether life's origin (less so its meaning) might have something to do with dissipative structures of the kind that Prigogine [125] described. Dissipative structures are discussed in the context of the origin of physicochemical order, as in Zhabotinsky reactions, and in the context of life [126]. Though skeptical that life could be seen as belonging to a category that would fall under the label of dissipative structures, we can say that living cells might have some properties in common dissipative structures, as the latter only arise in far from equilibrium systems, whereby both living things and H_2-producing hydrothermal vents are far from equilibrium systems. Dissipative structure or not, what is the source of life's order? It of course depends on who one asks and where

one thinks how life might have arisen. If life arose from the reaction of H_2 and CO_2 in hydrothermal vents [103,125], as we contend, then its structure emerges from the geometry of carbon bonds as carbon oxides undergo reduction with electrons and hydrides from H_2, reacting to generate longer and more diverse carbon chains [9] while reacting with other elements like nitrogen and sulfur to produce the amino acids, nucleosides, and cofactors that comprise life [127,128], through overall reactions that are thermodynamically favored [129] under the conditions of hydrothermal vents.

In that way, the form and function of molecules of life emerge from the geometry of orbitals in carbon, and the order in cells emerges from the geometry and properties of its molecules as they are synthesized with the help of microbial energy metabolism. That is how life arises today. This order does not conflict with entropy. Measurements of entropy change during growth have repeatedly shown that the entropy change in cells is always zero or close to zero because, "cells are assembled in a spontaneous process" [130]. That is, if a cell has what it needs to grow, it organizes environmentally available components into more of itself as an effortless by-product of the exergonic growth process [103]. The circumstance that central compounds of life arise from H_2 and CO_2 without enzymes [9] might be taken to mean that that life has a natural tendency to emerge under suitable conditions—but which conditions? We have listed a few of the proposals here. Microbes say: H_2-producing hydrothermal vents. Humans have more diverse views. Identifying inanimate matter to which we are most closely related would help us anticipate what to expect, chemically, during the search for life elsewhere—even if it finds us first.

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3.2. Catalysis, autocatalysis and the origin of metabolism

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Authors for correspondence:
Martina Preiner
e-mail: preiner@hhu.de
Joana C. Xavier
e-mail: xavier@hhu.de

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Catalysts, autocatalysis and the origin of metabolism

Martina Preiner[1], Joana C. Xavier[1], Andrey do Nascimento Vieira[1], Karl Kleinermanns[2], John F. Allen[3] and William F. Martin[1]

[1]Institute for Molecular Evolution, and [2]Institute for Physical Chemistry, Heinrich-Heine-University, 40225 Düsseldorf, Germany
[3]Research Department of Genetics, Evolution and Environment, University College London, Darwin Building, Gower Street, London WC1E 6BT, UK

MP, 0000-0001-5137-5556; JCX, 0000-0001-9242-8968; ANV, 0000-0003-4468-7724; JFA, 0000-0002-0950-0429; WFM, 0000-0003-1478-6449

If life on Earth started out in geochemical environments like hydrothermal vents, then it started out from gasses like CO_2, N_2 and H_2. Anaerobic autotrophs still live from these gasses today, and they still inhabit the Earth's crust. In the search for connections between abiotic processes in ancient geological systems and biotic processes in biological systems, it becomes evident that chemical activation (catalysis) of these gasses and a constant source of energy are key. The H_2–CO_2 redox reaction provides a constant source of energy and anabolic inputs, because the equilibrium lies on the side of reduced carbon compounds. Identifying geochemical catalysts that activate these gasses en route to nitrogenous organic compounds and small autocatalytic networks will be an important step towards understanding prebiotic chemistry that operates only on the basis of chemical energy, without input from solar radiation. So, if life arose in the dark depths of hydrothermal vents, then understanding reactions and catalysts that operate under such conditions is crucial for understanding origins.

1. Introduction

When the Earth was formed 4.5 billion years ago, it was formed without life, we can safely presume. If there was any life on the freshly accreted Earth, it was destroyed at the moon forming impact, which converted the Earth into a ball of boiling magma [1]. By about 3.95 billion years ago, there was life on Earth [2]. The question of how it arose is of substantial interest. Hydrothermal vents play an important role in the question of life's origin, because they were present on the early Earth [3–7] and because they harbour continuously far-from-equilibrium conditions in an environment where H_2 and CO_2 interact in such a way as to generate reduced carbon compounds [8–15]. In the discussion of possible sites for life's origin, hydrothermal vents are unique by that criterion: hydrothermal vents harbour far from equilibrium conditions over geological timescales, and the approach towards equilibrium releases energy in the synthesis of reduced carbon compounds. This sets hydrothermal vents apart from all other physico-chemical settings [16]. Moreover, the release of free energy and the synthesis of reduced carbon compounds at vents are united in a common reaction sequence that operates in the laboratory without enzymes [15] and that is simultaneously the core of carbon and energy metabolism in real bacteria and archaea—acetogens and methanogens. Vents are unique among settings for the origin of *metabolism* (as opposed to the origin of *life*), because no other site for life's origin harbours chemical reactions that resemble real microbial carbon and energy metabolism.

The far-from-equilibrium conditions at alkaline hydrothermal vents entail steep redox gradients owing to a constant flux of H_2-rich effluent over geological timescales [17]. The main redox reaction they harbour is the H_2–CO_2 system,

in which the equilibrium lies far on the side of organic compounds [18], such that the reaction can proceed spontaneously as long as suitable catalysts are available and strictly reducing conditions are maintained [10,15,19,20]. In the presence of activated nitrogen species, hydrothermal vents can synthesize the building blocks of life [12,13]. Because of their abundance of chemical energy, and despite the absence of light, modern alkaline hydrothermal vents are teeming with microbial life [21,22], life that is ultimately fuelled by the reaction of H_2 with CO_2.

The H_2–CO_2 redox reaction is an attractive source of energy for the first chemical reactions en route to life, because it provides direct links between a known geochemical process (serpentinization) and known biochemical processes. These are most notably the reactions of core carbon and energy metabolism in acetogens and methanogens, anaerobic autotrophs that live from the reduction of CO_2 with H_2. Acetogenesis and methanogenesis represent the most primordial forms of metabolism in bacteria and archaea [23,24], rooting life's chemistry to reactions of gasses, rocks and water.

The continuity between exergonic geochemical and biochemical reactions can be seen as a virtue of hydrothermal origin theories, because it generates concrete mechanistic links between processes catalysed by minerals in the Earth's crust (exergonic CO_2 reduction) [25] and processes catalysed by enzymes in the metabolism of prokaryotic lineages [26]. At hydrothermal vents, life as we know it connects to geochemistry as we know it.

2. Activation of CO_2 and H_2: the door to CO_2 fixation

In biology, acetogens and methanogens fix CO_2 via the H_2-dependent reduction of CO_2 to a methyl group and CO, followed by condensation of the methyl moiety and CO to a nickel bound acetyl group that is thiolytically cleaved from nickel to generate the thioester acetyl-CoA. The acetyl-CoA pathway is unique in microbial physiology, because it is carbon and energy metabolism in one. Carbon metabolism involves the H_2-dependent reduction of CO_2 to acetyl-CoA. Under standard physiological conditions, the synthesis of the thioester is exergonic by about –59 kJ mol^{-1} [27], while there is not enough energy to generate thioesters and synthesize ATP via substrate level phosphorylation [28]. Thus, for energy metabolism, acetogens that lack cytochromes and quinones couple methyl synthesis to the generation of ion gradients via electron bifurcation and ferredoxin oxidation at the membrane-bound Rnf complex [29], while methanogens that lack cytochromes generate their ion gradient by coupling the transfer of the methyl group from a nitrogen atom in methyl-tetrahydromethanopterin to a sulfur atom in coenzyme M [30].

If the acetyl-CoA pathway is the most ancient carbon fixation pathway, and various lines of evidence indicate that to be the case [14,15,23,24,27,31], there are still some dots that need to be connected. For H_2 to have played a role in early chemical evolution, it required activation—it required catalysis. It is noteworthy that H_2 never interacts directly with any organic oxidant (substrate) in metabolism, it always releases electrons into metabolism via a catalyst: hydrogenase. There are only three classes of hydrogenases known. All three harbour Fe atoms at their active site

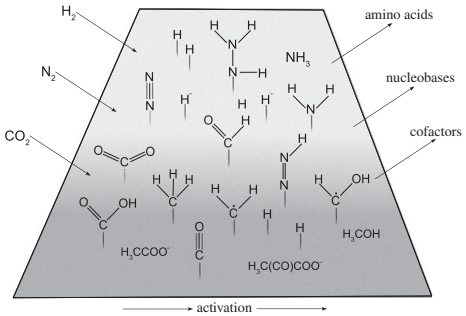

Figure 1. Simultaneous activation of H_2, CO_2 and N_2 on mineral surfaces leading to the formation of a variety of biologically relevant molecules, such as amino acids, nucleic acid bases and cofactors. Molecules, such as pyruvate, acetate, methanol and ammonia, are known to form on transition metal containing surfaces [15,45]. Little is known about the products obtained when the separation of N and C fixation is revoked. Heterogeneous catalysis may have been the key for early processes of protometabolism. Dashed lines indicate physisorption, non-dashed lines indicate chemisorption on the surface.

[32,33], all three harbour carbon metal bonds at their active site [26]. The central enzyme of the acetyl-CoA pathway, the only exergonic CO_2 fixation pathway known [34,35], is bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS), which also harbours carbon metal bonds. These two activities, hydrogenase and CODH/ACS, trace to the last universal ancestor, LUCA [26]. Organisms that use the acetyl-CoA pathway employ flavin-based electron bifurcation to generate ferredoxins with a lower reducing potential than H_2 [36–38]. Flavin-based electron bifurcation thus accounts for the thermodynamics of H_2 oxidation, but what about the kinetics? In kinetically controlled reactions, catalysts can have an important influence on the nature of the products that accumulate—and the same is true for geochemical CO_2 fixation with H_2.

The H_2-dependent reaction from the most oxidized form of carbon, CO_2, to its most reduced form, methane (CH_4), is thermodynamically favourable under reducing conditions. However, in serpentinizing, alkaline hydrothermal systems [39] the direct transfer of electrons from H_2 to CO_2 has a large activation energy and requires either high temperatures and high pressures [40] or, at milder conditions, chemical activation and catalysis [41,42]. The requirement for catalysis stems from kinetic barriers in the sequence of reactions from CO_2 to CH_4. Catalysts decrease the activation energy and thus the kinetic barrier, allowing intermediate products such as formate, acetate, methanol and pyruvate to accumulate after a short time under mild conditions [15] rather than the thermodynamically favoured end product CH_4. While high temperatures, high pressures and long reaction times lead to the accumulation of CH_4, the most stable product [40,43], catalysts influence the product distribution in the short term. In biology, enzymes effect such shifts from thermodynamically controlled reactions to kinetically controlled reactions [44]. In purely geological settings, however, heterogeneous catalysis can occur on mineral surfaces (figure 1)—which are not unlike the catalysts used in industry to produce hydrocarbons [15,25]. The activation of molecules on mineral surfaces is likely to have preceded

the chemical activation that enzymes provide in modern organisms [46,47].

3. Adding nitrogen

In order to synthesize amino acids and nucleic acid bases, living cells have to incorporate dinitrogen (N_2) into biosynthetic pathways. From a chemical point of view, N_2 as a starting material is not the easiest choice in comparison to more oxidized or reduced nitrogen compounds [48]. Nevertheless, looking at early Earth's conditions, an atmosphere filled with N_2 would have led to an ocean with dissolved N_2 and thus—via sequestration through the Earth's crust—to a nitrogen source in serpentinizing systems [49,50]. Looking at biology, N_2 fixation is considered ancient [50,51]. There is only one way for N_2 to enter metabolism: via the nitrogenase complex. Nitrogenase consists of two proteins, dinitrogenase reductase, which contains an FeS-based active centre and the dinitrogenase protein, harbouring an Mo (or V, or Fe) containing Fe_7S_9 centre with a carbide carbon at the active site [52,53]. Mechanistically, the complex works with dinitrogenase reductase harvesting the energy of ATP hydrolysis and transferring it via conformational changes to dinitrogenase, which then binds the N_2 molecule [53,54]. The following steps involve sequential hydrogenations of the nitrogen molecule. There, as for CO_2 fixation, hydrogenase activity is needed to deliver electrons from H_2 to N_2. This hydrogenase activity is promoted by the FeS clusters of the nitrogenase complex [53,55], which is the sole entry point of N_2 into metabolism. As CODH and hydrogenase, nitrogenase also traces back to LUCA [26,56].

Biology operates within constraints of temperature and pressure. Biological N_2 reduction follows very different kinetics from those of the industrial process [57]. For both processes, inorganic catalysts have a central role in the reduction of N_2. In industry, the reduction of N_2 might resemble prebiotic FeS-based nitrogen fixation [45]. The greatest impediment to N_2 reduction is its activation energy. N_2 is very stable at normal atmospheric temperatures and pressures. Thus, few processes are capable of activating N_2 sufficiently in order to form N-rich molecules. Industrial N_2 conversion to NH_3 via the Haber–Bosch process (H_2-dependent) requires Fe-based catalysts such as Fe_3O_4, high pressure (200 bar) and temperatures exceeding 400°C [58]. The Haber–Bosch process currently consumes about 1–2% of the World's total energy production. Biological nitrogen fixation catalysed by the nitrogenase enzyme operates at ambient pressure and room temperature. Accordingly, there is immense commercial interest in the mechanism of biological N_2 fixation [57].

Not unlike the stepwise use of Fe atoms found in the active sites of the nitrogenase complex, industrial N_2 reduction is extremely dependent on the physico-chemical state of the catalysts. Thus, the yield of ammonia is affected as a result of several factors such as particle size, purity and subsurface dissociation of nitrogen into Fe catalysts, leading to iron nitrides such as Fe_xN [59].

Can serpentinization reduce N_2? Although there is abundant evidence for abiotic CO_2 reduction in serpentinizing systems [60,61], evidence for abiotic N_2 reduction is so far lacking. Laboratory simulations suggest that N_2 can be reduced to ammonia (NH_3) with mineral catalysts under mild hydrothermal conditions [45,62]. Incorporation of N from N_2 into organic compounds under hydrothermal conditions presents a more substantial challenge for laboratory simulations. In principle, activated forms of nitrogen chemisorbed to geochemical catalysts (figure 1) might be better starting points for prebiotic synthesis of such compounds than NH_3 [25], but this remains to be shown experimentally.

There are nevertheless very curious parallels between industrial hydrogenation processes and geochemical H_2-dependent reactions. Serpentinization not only reduces H_2O to H_2 and CO_2 to formate and CH_4, it also generates inorganic catalysts within the Earth's crust [25]. These include magnetite, Fe_3O_4, which is the catalyst of choice for the industrial Haber–Bosch process (H_2-dependent N_2 reduction) and for Fischer–Tropsch (CO_2 reduction) applications [59,63] and awaruite, Ni_3Fe, which catalyses the H_2-dependent reduction of CO_2 to methane at high pressures and temperatures [40]. While H_2 and CO_2 deliver carbon and energy, for an autocatalytic network to emerge, one from which microbial metabolism could unfold, organic cofactors, bases and amino acids are required. All are nitrogenous compounds.

4. What if C, N and H are activated together?

As shown in figure 1, it is possible that mineral surfaces can activate H_2, CO_2 and N_2 simultaneously. If so, amino acids or even bases and cofactors might be obtained via such routes. It has been reported that Fe^{2+} and Fe^0 can catalyse reactions of 2-oxoacids with hydroxylamine to give aspartate, alanine, glycine and glutamine [64]. These should also be the first amino acids to appear in the evolution of metabolism, if metabolism evolved from a pyruvate-fed, incomplete citric acid cycle and if amino acids arose ancestrally as they do in metabolism, namely via reductive amination of the keto group in oxalacetate, pyruvate, glyoxylate and 2-oxoglutarate [9]. Pyruvate is new as a possible prebiotic compound [14]. Using hydrothermal iron minerals instead of enzymes, it is possible to synthesize pyruvate from H_2 and CO_2 [15]. Pyruvate now appears to be a much more readily synthesized prebiotic compound than previously assumed.

If N_2 can be activated efficiently under hydrothermal conditions, nucleic acid bases might not be far away. Recent studies show that even aromatic heterocyclic compounds such as tryptophan can be formed abiotically in serpentinizing hydrothermal systems [13]. The connection of simpler amino acids like aspartate and glycine to bases is direct, they sit in the middle of the aromatic pyrimidine (aspartate and glycine) and purine (aspartate) rings. This is shown in figure 2, modified from reference [9]. In metabolism, pyrimidines are made from aspartate and carbamoyl phosphate. Carbamoyl phosphate is made from carbamate and ATP, carbamate forms spontaneously as a colourless precipitate in hot solutions containing CO_2 (or carbonate) and ammonium. Four of the atoms in the pyrimidine ring come from aspartate. Purines are more complex, but the components are simple. Glycine comprises the centre of the rings, which are completed by inclusion of C1 units from formyl tetrahydrofolate [65] or from formyl phosphate (in methanogens) [66], by N from the amido group of glutamine, and, as with pyrimidines, by CO_2 and N from aspartate.

There is a clear record of geochemical origins preserved in metabolism [26]. This record can be resurrected in the

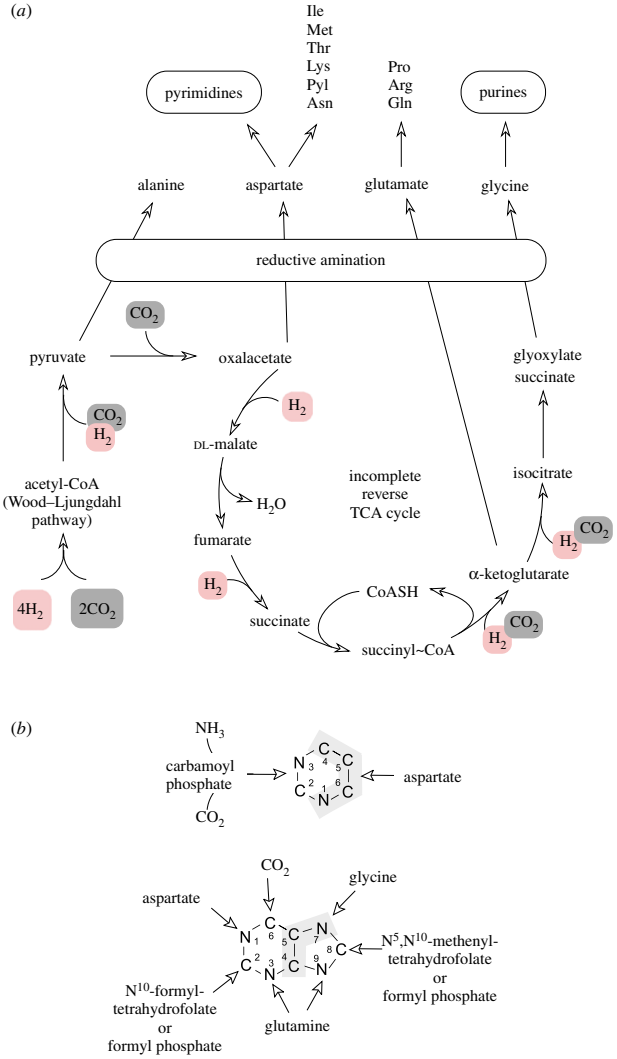

Figure 2. A path from H_2 and CO_2 to nucleic acid bases (adapted from figs. 3, 4 and 6 of [9]. (*a*) The lower portion of the panel shows the biosynthesis of carbon backbones in microbes that use the acetyl-CoA pathway and the incomplete (horseshoe) reverse citric acid cycle. Reductive steps of CO_2 fixation are indicated as H_2-dependent, though reduced ferredoxin or NAD(P)H are the reductants in metabolism. The first four 2-oxoacids to arise via the route shown, and, if reductively aminated, generate Ala, Asp, Glu and Gly (upper portion of the panel). Muchowska *et al*. [64] showed that pyruvate, oxaloacetate, 2-oxoglutarate and glyoxylate are readily reduced to the corresponding amino acids by hydroxylamine under mild conditions in the presence of native iron. Asp is the starting point for biosynthesis of five other canonical amino acids and pyrrolysine, Glu is the starting point for synthesis of Gln, Arg and Pro. (*b*) Asp and Gly are central to pyrimidine and purine biosynthesis, respectively (modified from fig. 4 of [9]). The involvement of CO_2 in purine and pyrimidine synthesis is noteworthy, as is the involvement of folate-bound C1 intermediates of the acetyl-CoA pathway in purine synthesis, which are replaced by the simpler intermediate formyl phosphate in methanogens. This suggests the possibility of a small prebiotic biochemical network linking CO_2 reduction to nucleic acid base synthesis. (Online version in colour.)

laboratory, if we find the right conditions. The four amino acids that Muchowska *et al*. [64] synthesize (Gly, Ala, Asp, Glu) even suggest (reveal, one might say) a connection to the evolution of the genetic code. These are the very same amino acids that are identified as ancient in different theories about the origin and evolution of the genetic code. In some theories, exactly these four (Gly, Ala, Asp, Glu) are the oldest [67]. In other theories, they are the most ancient as members of larger sets [68], while in yet other theories they rank well in order of antiquity, with Gly, Ala and Asp being the oldest, Glu coming in seventh [69]. A look at the biosynthetic families of amino acids reveals that the Asp and Glu families stand out as central.

5. Autocatalytic networks

If we assume that simultaneous activation of N_2, H_2 and CO_2 can lead to thermodynamically stable products that include

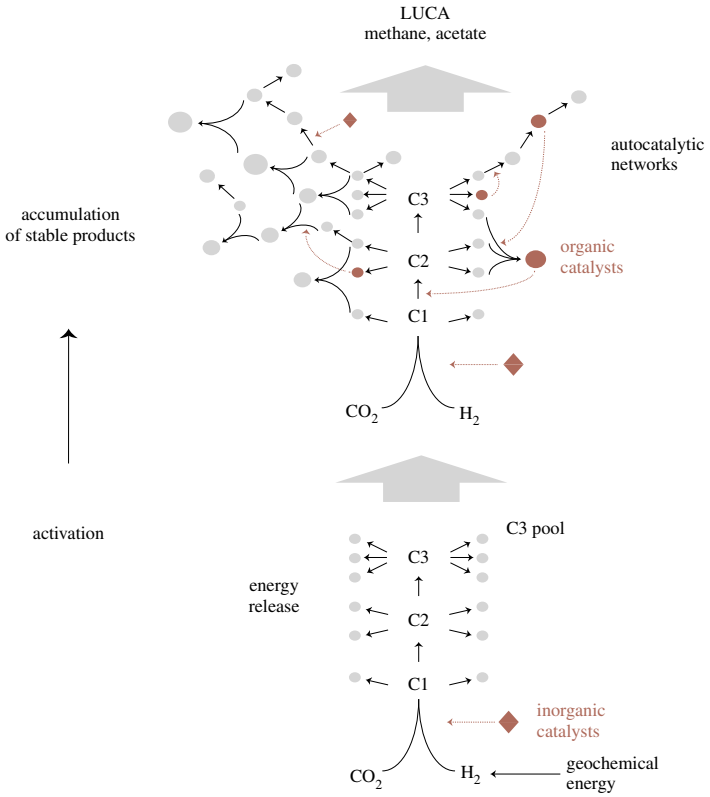

Figure 3. Purely geochemical reactions such as CO_2 fixation with H_2 can give rise to autocatalytic networks and protometabolism, as long as energy is released. Kinetically controlled reactions build up a specific set of products which interact further to form an autocatalytic network that serves as a basis for higher complexity. C1, C2, C3 represent carbon compounds with 1, 2 or 3 carbon atoms such as formyl groups, acetyl groups, and pyruvate. (Online version in colour.)

amino acids, nucleic acid bases and cofactors (that is currently a big assumption, we admit), then small chemical networks on a laboratory scale become possible. Central to various schools of thought on chemical origins are constructs called autocatalytic networks [70]. These can represent abstract mathematical constructs or they can describe interactions in real sets of molecules. As applied to molecular interactions, autocatalytic networks contain molecules that promote the synthesis of copies of themselves [71]. According to this very general definition, autocatalytic networks can provide theoretical frameworks for both the genetics first and the metabolism first approaches to prebiotic evolution. In the former, they can be sets of nucleic acids that ligate to form specific products [72], in the latter, they can be sets of metabolites that interact in such a way as to generate self-sustaining metabolic networks [24].

When describing molecular interactions, autocatalytic sets require input molecules in order to promote the synthesis of their constituent elements. This condition draws attention to a particular class of autocatalytic networks called reflexively autocatalytic food-generated networks—RAFs [73]—in which each reaction is catalysed by a molecule from within the network, and all molecules can be produced from a set of food molecules by the network itself. RAFs are particularly interesting in the context of early evolution, because they do not require a pre-existing catalyst for a reaction before it is required. The reaction can proceed uncatalysed, or rather catalysed by an unknown molecule, as long as the known catalyst is produced at some point by the network and assumes the role of catalysis in that reaction of the RAF. Moreover, when it comes to the concrete modelling of early evolution, the nature and source of the food molecules [74] that generate a given RAF or other autocatalytic set are of particular interest, because in order for the reactions in the set to take place, the overall thermodynamics of the network must be exergonic. In other words, in order for RAFs (or other autocatalytic networks) to serve as a useful model for early evolution, the set of reactants (educts) needs to release energy en route to the products (adducts), as is always the case in metabolism [18].

Of course, in cellular metabolism, the overall energetics are given by the sum of the changes in free energy for the core bioenergetic reactions [18]. For individual reactions of metabolism, the change in free energy from substrate to product is often endergonic, which is why such reactions are usually coupled to energy-releasing reactions involving exergonic electron transfer, ion gradients across the plasma membrane, or hydrolysis of high-energy bonds, such as ATP, acyl phosphates or thioesters [18,37]. Energetic coupling can also occur within RAFs, which makes them more interesting models of cellular metabolism.

It seems likely that at least a subset of the catalysts, high-energy bonds and energetic currencies that occur in modern metabolism were generally present and functional in prebiotic chemistry. Sources and transduction of modern metabolic catalysis and energy should then have analogues or homologues in geochemical settings. Regarding catalysis, there are now good indications that metals and simple organic cofactors could have promoted the emergence of cell-sized autocatalytic networks [15,64,75,76]. In physiology, the term energy metabolism generally means ATP synthesis. There are two sources of ATP in cells: chemiosmotic coupling and substrate level phosphorylation (SLP). Chemiosmotic coupling needs ion gradients as an energetic intermediate and proteins, without exception. SLP does not require ion gradients, its energy source is the Gibbs free energy of chemical reactions, and SLP reactions can take place without enzymes [77–79]. Although vents harbour natural ion gradients, ATP synthesis via chemiosmotic coupling always involves the ATPase, for which there is no known geochemical homologue or mechanistic analogue. The energy for SLP stems from the redox chemistry of carbon whereby both carbon oxidation to CO_2 and H_2-dependent CO_2 reduction can be coupled to SLP [80]. Because the H_2--dependent CO_2 reducing reaction that drives SLP in acetogens (acetate synthesis) operates in the laboratory under simulated hydrothermal vent conditions with only metals and metal ions as catalysts [15], it is currently the only candidate for a primordial (geochemical) source of energy conservation (acyl phosphates via SLP) that is mechanistically linked to naturally occurring carbon redox reactions at vents.

A set of molecules that is generated by kinetically controlled reactions (the most rapidly formed products accumulate) will contain chemical energy that permits members of the set to interact further and to form an autocatalytic network that can serve as a basis for higher complexity [76]. Such a process is sketched in figure 3. The energetic input is necessarily centralized because thermodynamically stable metabolites and end products are synthesized from the core exergonic reaction, in our example the reduction of CO_2 with H_2 via the acetyl-CoA pathway [9,15,31].

6. Conclusion

Hydrothermal vents contain catalysts and chemical disequilibria that resemble life and metabolism in many ways. However, the natural chemical environment at vents does not strongly resemble metabolism in many forms of life, because metabolism is extremely diverse. Rather, it very specifically resembles the physiology of acetogens and methanogens, even down to the catalysts involved. The connections between the origin of microbial life and the chemical elements seem more tangible than ever before. Current genomic analyses indicate that the last universal common ancestor of all life, LUCA, lived from gasses: H_2, CO_2 and N_2 [23,56]. Although our main focus is on these three gasses, it is evident that the incorporation of sulfur (S) and phosphorus (P) into early metabolism was also essential. Sulfur enters metabolism as HS^- at cysteine synthesis from *O*-acetyl serine or *O*-phospho serine [81], while phosphorus enters metabolism via thioesters as acyl phosphates [9]. Under reducing conditions, H_2S (HS^- in alkaline vents) would be the likely sulfur source, phosphorus could enter the geochemical setting as phosphate dissolved in seawater or leached from the primordial crust, but data on phosphate under early Earth conditions is scarce [82–84]. Focusing on the enzymes that channel H_2, CO_2 and N_2 into metabolism might uncover clues about the environment within which life arose and about the catalysts that activated these gasses at origins. The presence of carbon metal bonds in the active sites of hydrogenases, nitrogenase and carbon monoxide dehydrogenase suggest that these might be ancient relicts of the catalytic realm that led to the autocatalytic synthesis of the first organic compounds. We propose that the biology of methanogens and acetogens, anaerobic autotrophs that inhabit vents today, holds clues about the primordial catalysts that enzymes ultimately came to replace.

Data accessibility. This article has no additional data.

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Competing interests. We declare we have no competing interests.

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3.3. The ambivalent role of water at the origins of life

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Summary: Water is the universal solvent in cellular life. Without it, life as we know it is not viable. Although substrate-water interactions are responsible for biosynthetic reactions and the maintenance of biochemistry, water poses a threat to product concentration and stability due to its hydrolytic properties. In this manuscript, we suggest how this seemingly destructive nature of water is circumvented in hydrothermal vent systems. The explanation lies in a constant supply of reactants, thermal gradients and anastomosing mesoporous structures that together limit hydrolysis locally. Thus, allowing product concentration and enhancing molecular reactivity.

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REVIEW ARTICLE

The ambivalent role of water at the origins of life

Andrey do Nascimento Vieira[1], Karl Kleinermanns[2], William F. Martin[1] and Martina Preiner[1]

1 Institute for Molecular Evolution, University of Düsseldorf, Germany
2 Institute for Physical Chemistry, University of Düsseldorf, Germany

Correspondence
M. Preiner and W. F. Martin, Institute for Molecular Evolution, University of Düsseldorf, 40225 Düsseldorf, Germany
Tel: +49(0)211 81-12736
Email: preiner@hhu.de (MP), bill@hhu.de (WFM)

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Life as we know it would not exist without water. However, water molecules not only serve as a solvent and reactant but can also promote hydrolysis, which counteracts the formation of essential organic molecules. This conundrum constitutes one of the central issues in origin of life. Hydrolysis is an important part of energy metabolism for all living organisms but only because, inside cells, it is a controlled reaction. How could hydrolysis have been regulated under prebiotic settings? Lower water activities possibly provide an answer: geochemical sites with less free and more bound water can supply the necessary conditions for protometabolic reactions. Such conditions occur in serpentinising systems, hydrothermal sites that synthesise hydrogen gas via rock–water interactions. Here, we summarise the parallels between biotic and abiotic means of controlling hydrolysis in order to narrow the gap between biochemical and geochemical reactions and briefly outline how hydrolysis could even have played a constructive role at the origin of molecular self-organisation.

Keywords: geochemistry; hydrolysis; hydrothermal vents; mineral catalysis; molecular self-organisation; origin of life; protometabolism; serpentinising systems; water activity; water–rock interactions

Water is essential for all known forms of life [1]. As the solvent for life, it provides protons (H^+) and hydroxyl groups (OH^-) for myriad reactions but it creates a central problem when it comes to life's origin: hydrolysis. Water molecules dissociate chemical bonds and thereby break larger molecules or polymers into their monomeric components. In free solution, condensation reactions that generate water are thermodynamically unfavourable. Both protons and hydroxide ions can catalyse hydrolysis reactions, making them highly pH-dependent processes [2]. Water molecules can easily cleave ester and amide bonds and thus hydrolyse nucleic acids and proteins or they affect the half-life of reactants. In hydrolysis, OH^- usually replaces another moiety in the molecule (e.g., phosphate, amino or thiol group) by nucleophilic substitution. In *Escherichia coli* metabolism, for example, the most common reactant is H^+, followed by water, which participates as a substrate or product in over 500 reactions [3].

Cells counter hydrolysis by a number of mechanisms, including energy metabolism [4]. The main polymers of cells, proteins and nucleic acids are susceptible to hydrolysis. Their synthesis consumes about 80% of an anaerobic cell's energy budget [5]. Energy metabolism continuously supports ATP-dependent polymer synthesis, thereby ensuring that the rate of polymer synthesis is faster than the rate of hydrolysis. At the origin of life roughly 4 billion years ago, however, before polymerisation of nucleotides, amino acids or sugars could be coupled to an elaborate energy metabolism, there must have been other means to avoid the natural tendency towards hydrolysis [6,7]. In serpentinising systems such as alkaline hydrothermal vents (see Box 1 'Serpentinisation'), for example, the chemically reactive environment can provide a steady supply of monomers from simple inorganic compounds (CO_2, carbonates, hydrogen) via rock–water interactions [8–11], although the exact source of these monomers is a matter of debate [12].

Box 1. Serpentinisation

Serpentinisation is a geochemical process that occurs when ultramafic rocks in the upper mantle interact with seawater drawn from cracks in the crust [13]. The main gas-phase product of this process is molecular hydrogen (H_2), resulting from the reduction of water protons with iron minerals.

The upper part of the Earth's mantle consists mainly of peridotite, which is composed mostly of pyroxene (chain silicate forming minerals) and olivine [14]. Olivine is an iron-magnesium silicate, a solid solution between the magnesium silicate forsterite (Mg_2SiO_4) and the iron silicate fayalite (Fe_2SiO_4). Below 400 °C forsterite dissolves in water [15–18]:

$$Mg_2SiO_4 + 4\ H^+ \rightarrow 2\ Mg^{2+} + SiO_2(aq) + 2\ H_2O$$

At sufficient concentration of dissolved species, serpentine ($Mg_3Si_2O_5(OH)_4$) and brucite ($Mg(OH)_2$) nucleate and precipitate [15]

$$3\ Mg^{2+} + 2\ SiO_2(aq) + 5\ H_2O \rightarrow Mg_3Si_2O_5(OH)_4 + 6\ H^+$$
$$Mg^{2+} + 2\ H_2O \rightarrow Mg(OH)_2 + 2\ H^+$$

Both reactions hence consume water and produce H^+ which promotes the dissolution of forsterite. Fayalite reacts with hydrothermal water to magnetite (Fe_3O_4) and H_2.

$$3\ Fe_2SiO_4 + 2\ H_2O \rightarrow 2\ Fe_3O_4 + 3\ SiO_2(aq) + 2\ H_2$$

This reaction also consumes water, but magnetite is a minor component of serpentinisation, although it is the main product of Fe^{2+} oxidation. The majority of water is consumed through the reactions to serpentine and brucite. The equilibrium pH of the hydrothermal fluid is nearly neutral at temperatures near 300 °C but increases to about pH 11 at 50 °C because the solubility of brucite increases at lower temperatures, releasing dissolved Mg^{2+} and OH^- ions [18].

The mineral content in serpentinising systems strongly varies with the environment in which each system is situated. If sufficient amounts of H_2 accumulate in surroundings that bear Ni^{2+} containing compounds, native NiFe alloys such as awaruite (Ni_3Fe) can form [19,20].

$$(FeO) + 3\ (NiO) + 4\ H_2 \rightarrow Ni_3Fe + 4\ H_2O$$

Similarly, iron or nickel sulphides can form in systems with a higher proportion of H_2S instead of H_2 [19,21]. The amount of H_2 generated by serpentinisation depends upon temperature and the water:rock ratio of the reacting formation. For example, at 100 °C, serpentinisation starting with harzburgite, an ultramafic olivine-containing rock, generates about 0.9 moles of H_2 per kg of rock at a water:rock ratio of 0.2 (an excess of rock over water) but increases to about 130 moles of H_2 as the water:rock ratio increases to 10 [18].

Serpentinisation is a very widespread process and was probably more abundant on the early Earth than it is now [16].

Recent findings reveal striking parallels between the first steps of biochemical CO_2 fixation with hydrogen (H_2) and carbon fixation under geochemical conditions similar to those in serpentinising systems [22]. The organic acids formate, acetate and pyruvate are formed readily from H_2 and CO_2 in the presence of H_2O with the help of metals and mineral catalysts also found in serpentinising systems [22,23]. Metal ion-assisted reactions between pyruvate and glyoxylate lead to more complex organic acids observed in metabolism, for example fumarate, malate or α-ketoglutarate [24]. In presence of reduced nitrogen compounds such as ammonia [25] or hydroxylamine [24], pyruvate, oxaloacetate and acetate can react to the simple amino acids alanine, aspartic acid and glycine. Such reduced nitrogen compounds can be obtained under hydrothermal conditions through the hydrogenation of dinitrogen (N_2) over mineral catalysts [26–28]. Heterocyclic monomers as nucleobases can either be derived from formamide (which itself is the product of the reaction between formic acid and ammonia) [29] or via a condensation reaction of amino acids, a route closer to biological pathways [30]. Both approaches require, however, low water activity. Although the direct synthesis of amino acids or nucleobases starting from N_2 and CO_2 coupled to serpentinisation has not yet been

demonstrated [11,12], under the high pressure and high temperature conditions provided by serpentinising systems, activating N_2, CO_2 and H_2 simultaneously on mineral surfaces could, in principle, lead to complex biomolecules and monomers including amino acids, cofactors and nucleobases, which could foster the formation of protometabolic autocatalytic networks [31]. Thus, the synthesis of simple biological organics from H_2 and CO_2 using hydrothermal catalysts is facile [22], yet the synthesis of more complex biomolecules from H_2, CO_2 and N_2 has yet to be reported.

But even if biomonomers can be formed in serpentinising systems, as long as polymerisation products are hydrolysed faster than they are synthesised, no molecular complexity will ensue, because the formation of complex monomers themselves can be prevented by hydrolysis. This calls for a closer look at water activity in geochemical, biological and primordial protometabolic settings. In this paper, we address the issue of hydrolysis in an origin context at the interface of geochemistry and biochemistry, considering its mechanisms and their control under environmental and cellular conditions.

Hydrolysis and water activity in biology

The water content of the cytosol varies with cell size and metabolism but it can range from a few dozen to many thousand femtolitres (10^{-15}) [32]. It consists of bound and bulk ('free') water, water activity being the mole fraction of bulk water (see Box 2 'What is water activity?'). Cytosol is saturated with a wide range of molecules making it a crowded environment with concentrations up to hundreds of $g{\cdot}L^{-1}$ of amino acids, peptides, proteins, nucleic acids, nucleobases, monosaccharides, sugars, etc. [33,34]. For example, the concentration of amino acids in the cytosol, both free and polymerised as protein, is about 550 $g{\cdot}L^{-1}$ [5]. The number and nature of dissolved molecules in the cytosol result in very unique solvent properties of the intracellular water which ultimately also facilitate polymerisation of biomolecules [35,36]. One can differentiate between water of hydration (see Box 3 'Water of hydration'), which is strongly absorbed to proteins and other cellular compounds, and water that has the physical and chemical properties of bulk water [37]. In crystallised proteins, for example, about 40% of the crystal weight comes from water, a mixture of water of hydration and bulk water [37]. The two forms of water have very different properties. Water of hydration has a higher heat capacity, is less mobile and more ordered than bulk water, such that they differ in their abilities to dissolve different compounds [37]. For enzyme activity, water of hydration is essential as it stabilises tertiary and quaternary conformations via hydrogen bonding, charge–dipole interactions and hydrophobic (entropic) effects. In short: water of hydration keeps enzymes intact for catalysis [38,39].

Salts can also exert immense influence on water's characteristics as a solvent [45,46]. Sodium (Na^+),

Box 2. What is water activity?

Water activity is a measure of the 'effective concentration' of water in a system, also referred to as 'bulk water'. When bulk water molecules bind to ions, to surfaces, or are trapped otherwise, the overall water activity of a system decreases.

A simple means to obtain the water activity is to measure the water vapour pressure of an aqueous solution. Fundamental thermodynamic relations show:

$$\frac{p_w}{p_w^*} = a_w = f_w \cdot x_w$$

where p_w is the water vapour pressure of the aqueous solution.

p_w^* is the vapour pressure of pure water at the same external pressure and temperature as the solution

X_w is the mole fraction of water in the solution, and

f_w is the dimensionless activity coefficient.

Water activity is particularly affected by strong electrostatic interaction of the partial charges of water with ions from dissolved salts. The strong interaction leads to comparable low water activity. In case of a sufficiently diluted, 'ideal' solution, $f_w \approx 1$ and:

$$\frac{p_w}{p_w^*} = x_w = 1 - x_2$$

$$x_2 = 1 - \frac{p_w}{p_w^*}$$

$$x_2 = \frac{(p_w^* - p_w)}{p_w^*}$$

According to this relation the relative lowering of the vapour pressure of the solvent is equal to the mole fraction x_2 of the solved compound, for example the salt in an aqueous salt solution (Raoult's empirical law from 1890) [40].

Box 3. Water of hydration

The term water of hydration is used in chemistry to designate water in the crystal structure of a metal complex or salt which is not directly bound to the cation. Often the crystal properties are lost when this water is removed by heating. In a biological context, the importance of water of hydration is obvious. Proteins crystallise with up to 50% water in their lattice, much more than inorganic salts [37,41]. Often their enzymatic function is reduced and their structure changed if that water is removed. Most water in biological cells is water of hydration [37]. In this paper, we use the concept of water of hydration in a broader sense than just water of crystallisation to take effects in biological systems into account.

Water of hydration is considered here as all water that is not bound to the fluctuating network of bulk water (pure liquid water) but to other species in the aqueous phase. Often water of hydration is stronger and more orderly bound than bulk water [42] and therefore exhibits a substantially different Gibbs free energy [43]. Water of hydration can be bound in the first or second solvation shell of the cation or anion of a solved salt, bound to the charged side chain(s) of amino acids in a protein, ordered to hydrophobic molecules or chemical groups, bound to a chain of water molecules in a membrane protein channel conducting protons or bound to a solid surface [41]. All these interactions can be classified according to their Gibbs free binding energy which can be quite large. A high vacuum in a reactor, for example, can only be obtained by heating its walls well above 100 °C for many hours during pumping (a process known as bake-out) to release the tightly attached monolayers of water [44].

potassium (K^+), magnesium (Mg^{2+}), phosphate (PO_4^{3-}) and chloride (Cl^-) contribute to protein and enzyme folding, structure and specificity by creating in situ microenvironments where certain ions are more abundant than in the bulk [36,47]. These microenvironments regulate water activity via electrostatic interaction. Hydrophilic surfaces are associated with a higher concentration of water whereas hydrophobic surfaces cause water to migrate to other sites. This complex interplay helps to increase heat stability of the macromolecules in their aqueous surrounding [48–50]. The resulting intracellular water dynamics—also called biological water activity—enable polymerisation and energy conservation as enzymes control water-mediated interactions. Among these interactions are hydrogenations [51,52], condensations [53,54] and hydrolysis. The latter is central for energy metabolism [55,56], because endergonic reactions can be coupled to an energy-releasing hydrolytic reaction, ATP hydrolysis in particular, thus facilitating endergonic reactions in metabolism. Amino acid polymerisation, for example, is endergonic and does not take place spontaneously in pure aqueous (abiotic) systems [57]. Salt-induced or wet–dry cycle-driven peptide formation [58,59], hydrothermal synthesis [60] or polymerisation in the adsorbed phase, for example on mineral surfaces [61,62], have been introduced as possible mechanisms to promote peptide formation in an abiotic context [57].

In cells however, amino acid polymerisation requires the transfer of AMP from ATP to activate the amino acid for polymerisation via tRNAs [63–65]. The transfer releases pyrophosphate (PP_i) which is subsequently hydrolysed to make the activation irreversible under physiological conditions ($\Delta G_0' = -19.3$ kJ·mol^{-1}) [65]. A similar mechanism is employed in nucleotide condensation, where pyrophosphate is released when a phosphodiester bond between two nucleotides is formed [66]. Pyrophosphate is immediately hydrolysed into two free phosphate groups by pyrophosphatases [65], enzymes that use Mg^{2+} ions to promote controlled hydrolysis (shown in Fig. 1) [67]. Mg^{2+} ions also assist with the assembly of nucleic acids by positioning the nucleotides in the correct conformation [68]; another Mg^{2+} then activates the hydroxyl group at the 3′ end of the primer nucleotide, promoting the formation of the ensuing phosphodiester bond [69].

The utility of ATP in biology resides its ability to be hydrolysed at a phosphoanhydride bond, thus generating less energy-rich ADP and inorganic PO_4^{3-} (P_i). The energy released by the reaction from ATP to ADP and P_i is 30.5 kJ·mol^{-1} and 45.6 kJ·mol^{-1} for the reaction of ATP to AMP and PP_i [65]. The continuous cycle of hydrolysis and condensation reactions of ATP, ADP, AMP, PP_i, and P_i molecules allows energy harnessing from macromolecules in metabolism via energy coupling reactions [70,71]. In addition to ATP, there are many other molecules in biology with the ability to transduce chemical energy via energy coupling reactions such as NAD, acyl thioesters, aminoacyl esters and ribosyl moieties [72–75].

A number of reactions in the cell involve controlled hydrolysis during the degradation of lipids, nucleic acids and proteins. The enzymes responsible for these reactions are hydrolases, which represent, with about one third of all known enzymes, the largest enzyme

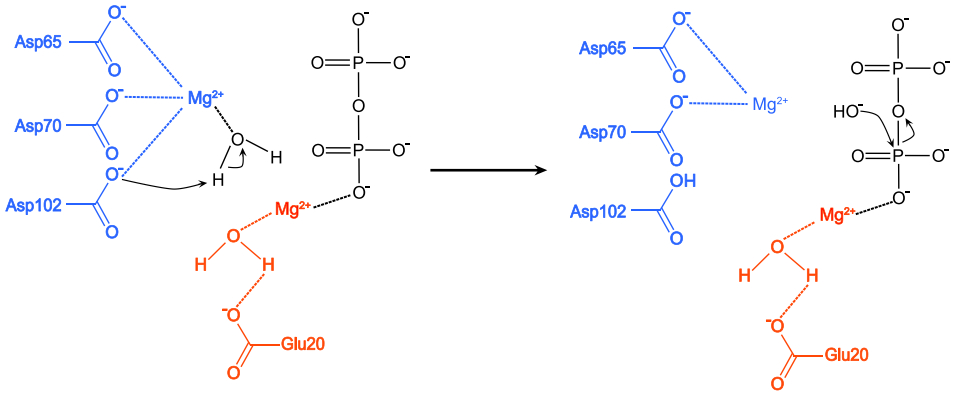

Fig. 1. Catalytic mechanism of Mg^{2+}- or Mn^{2+}-containing hydrolases (pyrophosphatase-type hydrolases). The metal complex formed with the carboxyl groups of acidic amino acids and water molecules can activate both substrate (site shown in red) and water (site forming OH^-, shown in blue). Adapted from [67].

class [67]. Subgroups of hydrolases employ divalent metal cofactors like Mg^{2+}, manganese (Mn^{2+}), cobalt (Co^{2+}) and zinc (Zn^{2+}) [67,76–78], all of which can also be relevant in a prebiotic context [79–82]. Four hydrolases are considered particularly ancient, all of them employing metal cofactors, three of them function without nucleotide-derived cofactors like ATP or NAD [83], pointing to a conserved and possibly primordial mechanism. Among the most ancient hydrolases are thioester hydrolases (e.g. acetyl-CoA hydrolase) which operate with Mg^{2+} or Mn^{2+} in their active centres [78,83].

The molecular mechanisms of catalysis in hydrolases are quite well understood [67], although not known in detail for every subgroup of the enzyme family yet. Fig. 1 depicts the mechanism of a hydrolase active site containing Mg^{2+} or Mn^{2+}, showing that acidic amino acids are crucial for positioning the ions such that they produce OH^- from water to perform targeted hydrolysis. As described earlier, the hydrolysis of pyrophosphate enables the polymerisation of amino acids and nucleotides. Notably, Mg^{2+} can also catalyse hydrolysis on its own, without the protective environment of the enzymatic active site [84]. In abiotic systems, where there is no enzymatic activity, the overall availability of salts and other charged molecules can regulate rates of hydrolysis and condensation.

Hydrolysis is also a central aspect of carbon metabolism [85–87]. One example is the participation of water in all known CO_2 fixation pathways, including the acetyl-CoA pathway, which allows acetogens and methanogens to grow from H_2 and CO_2 [88]. Genomic reconstructions indicate that the last universal common ancestor (LUCA) followed a similar route, using the acetyl-CoA pathway for carbon and energy metabolism [89]. As already stated, recent studies show that formate, acetate and pyruvate form overnight from H_2 and CO_2 in water using mineral catalysts alone under hydrothermal conditions [22]. Similar abiotic routes have recently been shown for intermediates and products of the reverse citric acid cycle [24,90]. The gap between such hydrothermal conditions and the chemistry of real life as it is manifested in modern autotrophs is narrowing in this respect. Thus, there might also be connection between the biochemistry in autotrophs and the geochemistry in serpentinising systems when it comes to hydrolysis.

In hydrogenotrophic methanogenesis, the first step towards the production of methane is the reduction of CO_2 to formyl-methanofuran (formyl-MFR) using ferredoxin as the electron donor. This reaction is catalysed by formyl-MFR dehydrogenase. In the enzymatic mechanism, the CO_2 molecule is funnelled through a hydrophobic channel towards a catalytic chamber with a tungsten active centre, into which electrons are drawn via a long [4Fe-4S] cluster chain [91]. In the catalytic chamber, CO_2 is reduced to formate, a hydrophilic molecule. Formate (or formic acid) diffuses from the active site via a hydrophilic tunnel to a zinc active centre where it is conjugated with MFR as a carbamate [91]. This is a redox reaction involving electrons from ferredoxin; water is generated as secondary

product during carbamate formation. That is, an interplay of hydrophobic and hydrophilic surfaces in cooperation with metallic catalysts promotes controlled water–substrate interactions at the initial CO_2 fixation step. The carbamate is reduced to a MFR-bound formyl-group. A stepwise reduction to a MFR-bound methyl-group follows, before the group is transferred to coenzyme M. After a last reduction step, the methyl-group is released as CH_4 [88].

It is common knowledge that enzymes tend to exclude water from the active site and can readily catalyse reactions against the water activity of the cytosol. The question of what environmental conditions could have promoted a prebiotic route from CO_2 towards organics and life is more challenging.

The hydrolysis conundrum in origin of life research

Water is indispensable for life as we know it to emerge. But at the same time, water elimination (condensation, polymerisation) is one of the most common reactions in metabolism underpinning the synthesis of cells [53,54]. The reverse reaction, water addition, is hydrolysis and can be chemically destructive to many essential biomolecules. Through hydrolysis, water literally works against the synthesis and accumulation of polymers at life's emergence.

Although enzymatically mediated hydrolysis can break any type of known carbon bond [92], modern biomolecules are protected from random hydrolysis by their structured spatial arrangement of hydrophobic/hydrophilic surfaces and by interactions with inorganic ions and other organic molecules [37]. They contribute to an environment in which water participates in the biochemical processes in a very controlled and targeted manner. Of course, modern biomolecules have undergone permanent selection during evolution to remove those that are unstable, insoluble or toxic in the cellular environment. At life's origins, however, during the phase of prebiotic chemistry before there were genes and encoded proteins, there were no enzyme pockets, no complex proteins, no ordered membranes or enzymatically elaborated energy metabolism to manage the constant chemical pressure of reactions involving water. Hence, the primordial polymerisation of simple molecules into the precursors of proteins and nucleic acids via the removal of water, a prerequisite for life, took place without the help of the biological mechanisms that modulate water activity in a living cell. Nevertheless, we will see below that serpentinisation itself generates inorganic mechanisms that modulate water homeostasis.

At face value, the origin of life and fully aqueous chemistry do not really work together well, especially if high temperatures are required for chemical reactions to take place [93,94]. But at the same time, the cytosol of a cell is also not an environment of fully aqueous chemistry. So, is there something wrong with the basic concept of hydrothermal origins, or is there something wrong with the premise that hydrothermal settings generally involve fully aqueous chemistry? There are specific physicochemical sites within serpentinising systems that harbour and even maintain low water activity (see Box 2) at rather moderate temperatures (100–200 °C). Such systems provide a spectrum of conditions as are required to get from C_1 compounds to complex organics [93,94]. As in cells, free water in the right geochemical environment could be bound as water of hydration with the help of salts and other polar molecules, decreasing the water activity and thus promoting the synthesis of larger molecules [37,93]. Another possible way to promote hydrolysis-sensitive reactions are mineral surfaces [22,27,95–104]. Mineral surfaces provide diversity of catalytic environments, enabling reactions at the solid–liquid phase boundary under aqueous conditions [22,23,101,104–106]. A combination of highly catalytically active minerals and low water activities (provided by high salt conditions or other means) could enable a variety of possible chemical pathways.

Modern high salt and high temperature environments present extreme settings for life. As such harsh conditions were likely prevalent on the early Earth—especially inside its crust—at the time when life emerged [15,107], biologists have long suspected that modern extremophiles might hold clues about the biology of the first cells [108,109]. Found in Earth's most life-defying places, extremophiles could provide valuable insights into the transition from nonlife to life. Haloarchaea, for example, can survive in salt crystals for very long periods of time, possibly over geological time periods, although how long exactly is open to speculation [40,110]. In order to counteract the osmotic pressure of the saline environment, some halophiles transfer K^+ ions into the cell [40]. Most other halophiles, however, choose a more energy-intensive route to deal with high salt concentrations by synthesising osmolytes like sugars, glycerol or amino acid derivatives. With this strategy, only organisms with very effective metabolic rates (high levels of ATP synthesis) can survive under very saline conditions [111].

Salt can have interesting effects on the three-dimensional structure of proteins under abiotic conditions. It has been shown, for example, that homochiral leucine-lysine (Leu-Lys) polypeptides fold as random coils in

pure water [112]. But in salt they form thermostable and hydrolysis stable bilayers of beta-sheets with the hydrophilic side chains of Lys (positively charged amino group) pointing outwards into the salt solution and the hydrophobic side chains of Leu (isobutyl group) inwards [112]. The structure of larger proteins (140 amino acids) is also influenced by high salt concentrations (up to 2 M). Acidic amino acid chains are stabilised by the salt cations, and beta-sheets fold so as to point outwards. With simple hydrophobic amino acids as Leu, Ile (isoleucine) and Val (valine) pointing inside, a hydrophobic core/pocket can be formed, where possible polymerisation reactions would be protected from the surroundings [113,114]. Such studies show how high salt settings—and thus environments with low water activity—can provide dynamic physicochemical conditions that naturally modulate structures for prebiotic chemistry while keeping the risk of hydrolysis lower than in purely aqueous environments. These conditions arise via the operation of simple ionic forces.

Various mechanisms have been proposed to solve or circumvent the hydrolysis problem at the origin of life. The most commonly encountered of these evokes the existence of wet–dry cycles [59,97,106,115,116], which entail alternating periods of hydrolysis (high water activity) and condensation (no water activity) in order to achieve polymerisation. Although such cycles deliver promising results for nucleotide synthesis under laboratory conditions [115], it is important to not only constrain an environment for origins by water avoidance, but also by considerations of carbon or energy availability, the main prerequisites for microbial life. An underexplored alternative to wet–dry cycles might be solid phase–aqueous phase interfaces in geochemical systems where water activity is constantly low. This was proposed recently for hydrothermal sedimentary layers where pore spaces between volcanic particles can be filled with silica gels [117], which leads to less free water in the geochemical system. Of course, water activity is a prominent variable in efforts to detect life beyond the confines of Earth, as such there are many implications of water activity for astrobiology (discussed in Box 4).

In environments with very low water activity, hydrolysis might even require catalysis to occur. As mentioned above, divalent ions such as Mn^{2+} and Mg^{2+} can promote hydrolysis both in the active centre of hydrolases and under abiotic conditions (without enzymes). So it is possible that such ions, which are quite abundant in hydrothermal vent/serpentinising settings, could provide exactly the rate of hydrolysis needed if the average water activity in a system is small enough. At the same time, various salt ions can actually help decrease the water activity by binding water (hydration). Thus, a complex interplay of salts, ions, minerals, gels, clays and water, not to mention organic compounds themselves should these be

Box 4. Implications of water activity for astrobiology

Water activity bears directly upon theories and experiments dealing with the origin of life. The search for life on other planets or moons is guided by the search for water [118]. When it comes to finding evidence for ongoing rock–water interactions in appreciable magnitude, the moons of Saturn Enceladus and Titan have been in the focus of research recently. The Cassini mission has delivered spectrometric evidence for the existence of serpentinisation on Enceladus [119]. Its rocky interior is covered by several kilometres of liquid water that are in turn covered by several kilometres of ice that form the surface [120,121]. The water is kept in the liquid state because of the gravitation of Saturn, which constantly kneads the small moon of roughly 500 km diameter so as to generate heat [120,121]. Enceladus has geysers at its South pole that spew liquid water hundreds of kilometres into space [120,121].

During the Cassini mission, H_2 was measured in the geyser plumes of Enceladus [122]. The presence of H_2, the detection of silicate nanoparticles and models suggesting an alkaline pH of Enceladus' ocean is interpreted as evidence for ongoing serpentinisation under the moon's ice crust [120,122,123]. The plumes also contain organic compounds. These could be either fragments of polyaromatic hydrocarbons, that is, breakdown products of carbon brought to Enceladus by meteorites [124,125] or products of *de novo* organic synthesis fuelled by serpentinisation [126]. Although the exact source of the organic compounds in the plumes is still not clear, rock–water interactions seem to be taking place on Enceladus, meaning that there is nothing special about the process that would limit its occurrence to Earth. If we entertain the possibility that life might have evolved in serpentinising systems, rather than exclude the possibility *a priori* [127], the implications for astrobiology are far reaching. Sunlight would have little to no role in origins and that, in turn, would expand the habitable zone in our solar system and in newly characterised solar systems, to regions where sunlight provides no energy. The chemical energy of serpentinisation would require only water-reducing rocks and CO_2 to unfold.

present, figure into the water conundrum at the origin of life.

Serpentinising systems and water activity

Natural environments of low water activity need not to be restricted to terrestrial surfaces, they can and do exist in serpentinising hydrothermal systems and thus may have been germane to the geochemical setting in which life arose. Of course, low water activity is not the only requirement that such a setting has to meet. Nonequilibrium conditions, carbon (cells are roughly 50% carbon by dry weight) [128], nitrogen (cells are 10% nitrogen by dry weight) [129], as well as sulfur and phosphorus, but perhaps most importantly a continuous source of energy was required at origins. Serpentinising systems combine carbon, energy and electrons in the form of CO_2 and H_2 in environments replete with low water activity [130–133]. Serpentinisation is a widespread phenomenon in today's oceanic crust [134,135]. Ultramafic, iron silicate containing rocks react with seawater, release H_2, and are transformed into serpentine group minerals ('serpentinite', $Mg_3Si_2O_5(OH)_4$) in the process—hence the name serpentinisation. One of the best investigated serpentinisation sites is the Lost City hydrothermal field, discovered near the mid-Atlantic ridge in 2001 [8]. At this geological site, Earth mantle rocks containing an iron-magnesium silicate mineral group called olivine are, due to magma upwelling, exposed at the seafloor where they react with seawater to produce H_2 [136].

Modern serpentinising systems harbour temperature, pH and redox gradients [94] and provide carbon (mostly in the form of CO_2/carbonates), sulphur (H_2S and sulphide minerals), and in lesser or trace amounts also nitrogen and phosphate [137]. A unique feature of serpentinising systems is the continuous production of hydrogen (H_2) from water, providing the surroundings with a constant supply of electrons, a very low midpoint potential [138], and a chemical energy source [139–141]. H_2 production entails also a constant renewal of iron-containing minerals that can function as catalysts for prebiotic reactions [98,99,142,143], among them magnetite (Fe_3O_4), a direct product of serpentinisation, iron sulphides like pyrite (FeS_2) or greigite (Fe_3S_4) [19,21,144], and even native metal compounds such as awaruite (Ni_3Fe) [19]. Prebiotic reactions catalysed by such minerals supposedly started with the energy-releasing reaction of CO_2 and H_2 [22] that today is still utilised by acetogens and methanogens for growth [11,88,133,145–149].

What about water activity in serpentinising systems? It decreases as vast amounts of sea water are consumed in the interactions between seawater and olivine and salinity in the rock pores increases [150]. The ultramafic minerals participating in the process sequester seawater, resulting in mineral hydration (hydroxide formation) [151]. A recent *in situ* serpentinisation experiment (at 280 °C and 500 bar) provided important mechanistic insights into the interactions between rock and water and into the influence of salinity on free water in mineral pores and thus on water activity [15]. Via Raman spectroscopy and microscopy, Lamadrid et al. [15] monitored the concentration of salts and minerals in the micropores of olivine. The formation of serpentine minerals, brucite ($Mg(OH)_2$), magnetite (Fe_3O_4) and H_2, consumes water. As the water content in the pores decreases, the concentrations of salts and minerals increase during serpentinisation. Ultimately, the pores are filled with a highly concentrated, 'crowded' hydrothermal fluid with low water activity. What Lamadrid et al. [15] describe is a situation that comes surprisingly close to what is observed in intracellular fluids. In the geochemical microsites they observe [15], serpentinisation stops as soon as the water activity gets too low (meaning the salinity gets too high) and new seawater with lower salinity has to diffuse into the system to restart serpentinisation. The rock volume increases during serpentinisation [139], leading to open fractures and seawater migration into the (micro)cracks which allows serpentinisation to continue. This scenario of fluctuating water activity resembles wet–dry cycles described above. Lamadrid et al. [15] report a kind of self-regulating system that hones in on low water activity with the exergonic reaction (H_2 production) ceasing when water activity becomes too low, resuming only when water is added. Chemically, water availability during serpentinisation at very small scales of micron sized inorganic compartments resembles ion homeostasis in modern cells at several levels.

Within inorganic pores of ultramafic rocks, a combination of mineral surface catalysis, low water activity and continuous supply of energy and carbon (in addition to nitrogen and other nutrients), could, in principle, lead to complex biomolecules. Such a sustained source of specific 'food' and energy is required for the emergence of autocatalytic networks, metabolism-like chemical reaction systems that are simpler than metabolism in modern cells as they support themselves without enzymes [78]. Such reaction systems are thought to be intermediates in the transition from nonliving to living systems [152,153]. From the geochemical standpoint, there is every reason to think that

serpentinisation was a very common reaction on early Earth [11,16,154,155]. The necessary ingredients are seawater and ultramafic rocks which the early Earth had in virtually unlimited supply [156]**.** Radioactive isotope dating of the carbonate structures and sediments in Lost City show an age of at least 30 000 years for that serpentinising system. There is enough mineral in the massif below Lost City to drive serpentinisation for further hundreds of thousands, if not millions, of years [139]. Rocks from former serpentinising systems preserved at depth beneath the Mid-Atlantic Ridge, where Lost City resides, revealed abiotically synthesised organic compounds including amino acids and more complex molecules just recently [157–159]. Such findings emphasise the potential of prebiotic synthesis serpentinising systems offer. This ultimately means that serpentinising systems such as alkaline hydrothermal vents can provide low water activity. Many misconceptions about vents are seated in water activity and hydrolysis, misconceptions that presume chemistry in free aqueous solution at hydrothermal vents. [127,160,161].

To date, the catalytic and organic synthetic potential of serpentinising systems, in combination with low water activity, has not been exhausted in sufficient detail in laboratory experiments. The effluents of active sites such as the Lost City hydrothermal field are regularly monitored, mainly showing simple carbonic acids like formate, methane and possibly acetate as abiotic products of the redox reactions occurring inside the porous crust [137,141,148,162]. Direct *in situ* observation of the reactions within serpentinising systems in submarine crust poses immense technical challenges. But the rewards of direct observations could be equally great, as current findings suggest that serpentinising systems catalyse biomimetic reactions [157–159]. A problem is, however, that vents are densely inhabited by microbes and possibly bear sources of biologically derived organic matter, such that controlled laboratory experiments in sterile systems [22–24] are needed to complement *in situ* chemical sampling studies.

Conclusions and perspectives

Avoiding constant hydrolysis while retaining access to hydrolysing chemical steps is pivotal for the emergence of prebiotic chemical networks [163]. So far, most prebiotic CO_2 fixation experiments are performed in aqueous solution which can block heterogeneous mineral catalysts. From industrial processes, it is known that water poisons mineral catalysts through hydroxyl formation on their surfaces, blocking the prospective catalysis sites [164]. This would also explain why the yields of all aqueous CO_2/H_2 experiments are usually very low in comparison with those of industrial gas-phase chemical processes, such as Fischer–Tropsch and Haber–Bosch synthesis [22,23,151,164,165]. On the other hand, studies with water vapour in hydrogenation processes have shown that H_2O, although lowering the output of industrially relevant products including methane and larger hydrocarbons, increases the percentage of C_1–C_4 'oxygenates'—oxidised carbon compounds—possibly including organic acids central in metabolism [166]. Thus, limited water poisoning might be a manageable problem and possibly beneficial as long as water activity is generally low.

Hydrolysis is often viewed as a destructive force in prebiotic chemistry [93]. But in the context of the mechanistic details of small-scale geochemical reactions within serpentinising systems, hydrolysis becomes a surprising and potentially powerful force that could foster self-organisation in prebiotic chemistry (Fig. 2). There are two possible roles. First, in an environment with a constantly low water activity, hydrolysis is impaired, transforming it from an omnipresent problem into an essential reaction that can be mediated by divalent ions as found in modern hydrolases [67,84]. Second, under medium (not high) water activity conditions, hydrolysis can be a selective driving force towards complex molecules. Under simulated hydrothermal (high water activity) conditions, mineral catalysts, reductants and CO_2 can generate reduced carbon compounds including 2-oxoacids [22,23] overnight, in the presence of activated nitrogen amino acids also readily form [24,25]. In metabolism, amino acids are the precursors of nitrogenous heterocyclic compounds (cofactors and nucleobases), the starting material and end products of protometabolic autocatalytic networks [31,78,153]. With high salt concentrations and low water activity inside the pores of serpentinising rocks (medium to low water activity), peptides could form from amino acids, even at higher temperatures [114]. Peptides then become even more resilient against hydrolysis once they reach lengths that permit the formation of alpha-helices and beta-sheets [167]. Under conditions of sustained peptide synthesis and sustained peptide hydrolysis, a selection process sets in through which hydrolysis-resistant peptides can accumulate [168,169], but not by virtue of faster synthesis, rather by virtue of their slower hydrolysis ('survival of the sturdy').

In this way, low water activity could foster synthesis of random peptides, while hydrolytic removal of the most labile among them would enrich for nonrandom structures within the realm of randomly synthesised

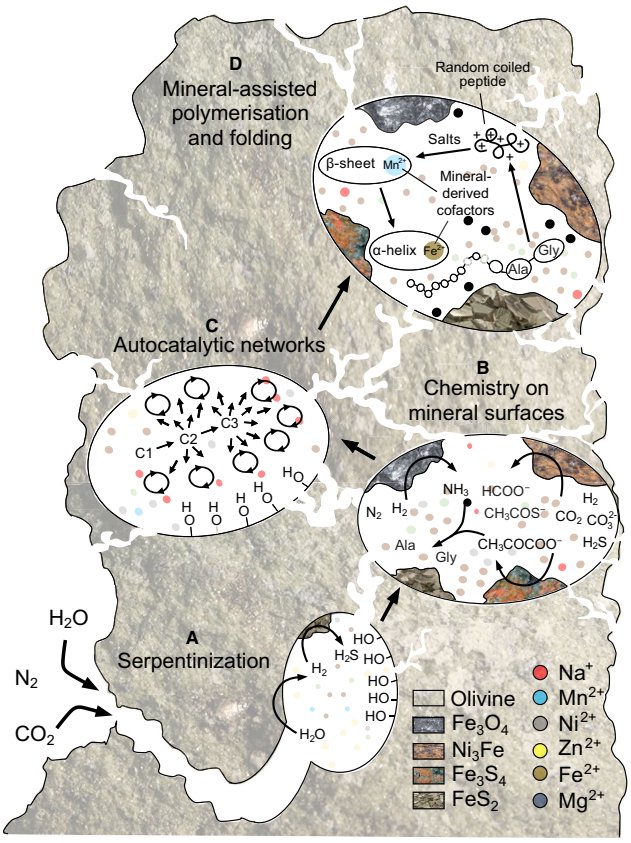

Fig. 2. Serpentinising systems, water activities and origins. The figure schematically depicts hydrated pores (rock-bound water, hydroxyl groups), embedded in the olivine matrix of serpentinising systems. (A) *Serpentinisation*. H_2 is synthesised via interactions between water and olivine (for details see). Catalytically active minerals including magnetite (Fe_3O_4), iron sulphides (FeS_2, Fe_3S_4) and Ni, Fe-alloys (Ni_3Fe) are constantly produced, whereas Fe_3O_4 arise from serpentinisation, and sulphides and alloys as reaction products of H_2, H_2S and Fe^{2+} or Ni^{2+} ions. (B) *Chemistry on mineral surfaces*. With the help of such minerals as catalysts, N_2 can be hydrogenated to ammonia [26–28], and CO_2 reduced to carbon compounds like α-ketocarboxylic acids [22–24]. The latter can react with activated ammonia to amino acids [24,25]. Theoretically, also thioesters could be synthesised at this stage, although this is debatable [172]. (C) *Autocatalytic networks*. The reduction products could react to a variety of other N-containing carbon compounds like cofactors or nucleobases, especially in low water activity (high salt) conditions [29,30]. Such complex monomers would fuel autocatalytic protometabolic networks [31,78,153]. (D) *Mineral-assisted polymerisation and folding*. Due to high salt concentrations and low water activity inside the pores, polymers such as polypeptides can form from amino acids [113,114]. Most folded proteins could achieve the necessary structure precision for their catalytic function without nucleic acids as templates, merely directed by water activity and salt concentration [112]. They could concentrate substrates in their protected interior. Here, controlled hydrolysis (e.g. via trapped Mg^{2+} or Mn^{2+} ions as shown in Fig. 1) and condensation reactions through mineral-derived cofactors could occur. Also targeted CO_2 fixation would be possible in such protein pockets, using amino acids with nucleophilic side chains and incorporated transition metals such as Fe^{2+} or Ni^{2+} [22,23,88,173]. The micropores in ancient serpentinising hydrothermal fields could be the earliest precursors of biological cells. All reactions described in this figure could subsequently happen in the same micropore, but pores at different physicochemical conditions may be needed for some of the stages to evolve. So chromatographic effects (separation of products while migrating between two different pores) should be considered.

variants, possibly without genetic instruction. Such peptides would necessarily harbour specific structural surfaces, hence the equivalent of multiple active sites [170], and could in turn favour some kinetically controlled reactions over others, thereby accelerating molecular self-organisation. Prebiotic peptide synthesis requires a sustained source of amino acids and energy [171], in addition to suitable surfaces, to catalyse polymerisation [57,97]. Serpentinising systems could provide both over geological time scales, with survival of the sturdiest enriching for nonrandom structures with catalytic properties of their own, as catalytically active peptides that currently serve as elements of autocatalytic networks preserved in microbial metabolism [78].

In summary, we have described how highly saline, serpentinising, porous rock environments could circumvent and perhaps even modulate uncontrolled hydrolysis in a manner analogous—similar but unrelated—to modern metabolism (Fig. 2). Whether these processes could also be homologous, that is, similar by virtue of common ancestry, is an open question for further study.

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Author contributions

MP wrote the manuscript with contributions from AV on hydrolysis in biology, contributions from KK on hydrolysis in a chemical context and serpentinisation and contribution from WFM on the origin of life context. KK researched and drafted Boxes 1–3 and formulated, together with MP and WFM, the scheme depicted in Fig. 2. MP adapted Fig. 1, AV has drawn Fig. 2 with input from KK, WFM and MP. AV and WFM drafted Box 4. WFM edited and contributed throughout the manuscript.

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3.4. The autotrophic core: An ancient network of 404 reactions converts H_2, CO_2, and NH_3 into amino acids, bases, and cofactors

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Authors: Jessica L. E. Wimmer, Andrey do N. Vieira, Joana C. Xavier, Karl Kleinermanns, William F. Martin, and Martina Preiner

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Summary: Autocatalytic networks are motors for molecular complexity in alkaline hydrothermal vent systems. In this manuscript, we identified 404 reactions starting from CO_2, NH_3, H_2, and the cofactors involved in the synthesis of amino acids and nucleic acids. These reactions form the metabolic core that encompasses the simplest biosynthetic activities performed by ancient prokaryotes. The core reactions indicate the environment in which metabolism arose. The presence of reduced substrates indicates that the environment where metabolism likely emerged was highly reducing. In such a system, we identified water as the universal solvent in these reactions, meaning that life likely started in an aqueous, reducing environment such as those found in alkaline hydrothermal vents.

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The Autotrophic Core: An Ancient Network of 404 Reactions Converts H_2, CO_2, and NH_3 Into Amino Acids, Bases, and Cofactors

Jessica L. E. Wimmer [1,*], Andrey do Nascimento Vieira [1], Joana C. Xavier [1], Karl Kleinermanns [2], William F. Martin [1] and Martina Preiner [1]

[1] Department of Biology, Institute for Molecular Evolution, Heinrich-Heine-University of Düsseldorf, 40225 Düsseldorf, Germany; nascima@uni-duesseldorf.de (A.d.N.V.); xavier@hhu.de (J.C.X.); bill@hhu.de (W.F.M.); preiner@hhu.de (M.P.)

[2] Department of Chemistry, Institute for Physical Chemistry, Heinrich-Heine-University of Düsseldorf, 40225 Düsseldorf, Germany; kkleinermanns@yahoo.de

* Correspondence: jessica.wimmer@hhu.de

Abstract: The metabolism of cells contains evidence reflecting the process by which they arose. Here, we have identified the ancient core of autotrophic metabolism encompassing 404 reactions that comprise the reaction network from H_2, CO_2, and ammonia (NH_3) to amino acids, nucleic acid monomers, and the 19 cofactors required for their synthesis. Water is the most common reactant in the autotrophic core, indicating that the core arose in an aqueous environment. Seventy-seven core reactions involve the hydrolysis of high-energy phosphate bonds, furthermore suggesting the presence of a non-enzymatic and highly exergonic chemical reaction capable of continuously synthesizing activated phosphate bonds. CO_2 is the most common carbon-containing compound in the core. An abundance of NADH and NADPH-dependent redox reactions in the autotrophic core, the central role of CO_2, and the circumstance that the core's main products are far more reduced than CO_2 indicate that the core arose in a highly reducing environment. The chemical reactions of the autotrophic core suggest that it arose from H_2, inorganic carbon, and NH_3 in an aqueous environment marked by highly reducing and continuously far from equilibrium conditions. Such conditions are very similar to those found in serpentinizing hydrothermal systems.

Keywords: chemolithoautotrophy; early metabolism; serpentinizing systems; hydrothermal vents; origins of life

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1. Introduction

Biologists have traditionally linked the topic of C1 metabolism to thoughts about life's origins. Haeckel (1902) posited that the first cells probably lived from CO_2 [1], perhaps in a manner similar to organisms discovered by Winogradsky (1888), growing from CO_2 with the help of electrons from inorganic donors [2]. The chemolithoautotrophic lifestyle—converting inorganic carbon into cell mass with inorganic electron donors using chemical energy instead of light—is common among modern microbes that inhabit environments similar to those on the early Earth [3]. Although microbiologists have traditionally favored the view that the first cells were anaerobic autotrophs [4–7], the electric spark experiments of Miller shifted the focus in origins literature from microbiology to nucleic acid chemistry [8]. The facile synthesis of nucleobases from cyanide condensations [9], Spiegelman's in vitro RNA replication experiments using Qβ replicase [10], and the demonstration that RNA has catalytic activity [11] led to the concept of an RNA world [12] in which RNA molecules became synthesized by abiotic chemical means and then competed with one another for resources (activated ribonucle-

oside triphosphate monomers) via replication [13]. This line of thinking diverted attention away from the more challenging problem concerning the origin of living cells and toward the more tractable problem concerning the origin of nucleic acid monomers [14,15]. However, for critics, the allure of the RNA world concept has caveats, as RNA synthesis, regardless how good, does not alone solve the problem of how living cells arose [16]; *Escherichia coli* is clearly alive; RNA is clearly not.

1.1. Metabolism vs. Genetics?

As an alternative to the concept of an RNA world, Wächtershäuser's theory of surface metabolism rekindled the idea of a chemolithoautotrophic start of life and brought energy into the origins debate, positing that the exergonic conversion of iron-sulfur (FeS) minerals to pyrite provided the thermodynamic drive to fuel the origin of biochemical pathways and the first autotrophic cells [17]. Wächtershäuser's theory ignited a "genetics first vs. metabolism first" debate [18–21] that Lipmann had presciently perceived decades in advance [22]. While the theory of surface metabolism interfaced well with catalytic mechanisms in autotrophic cells, it did not interface well with energy conservation in the currency of high-energy phosphate bonds [23] nor did it offer inroads to accounting for the fundamental property of life that the living cell maintains itself in a thermodynamic state that is far from equilibrium. The discovery of deep-sea hydrothermal vents [24] and alkaline hydrothermal vents of serpentinizing systems [25] impacted the origins issue in that they harbor geochemically continuous far from equilibrium conditions that help to define the living state of cells [5].

The idea that life started from CO_2 is appealing, but it only solves half the problem because both for life and for organic synthesis, CO_2 requires a reductant. This is why serpentinizing hydrothermal systems are so interesting in the origins context. Serpentinization synthesizes H_2, the main energy and electron source of chemolithoautotrophs, from protons and electrons within the Earth's crust through the reduction of H_2O by Fe(II) minerals. The amount of H_2 generated by serpentinization is substantial, on the order of 16 mmol/kg in some modern systems [26], which is orders of magnitude more H_2 than modern chemolithoautotrophs require for growth [27]. The synthesis of H_2 during serpentinization is a continuous process that has been going on since there was water on Earth [28]. The further researchers explored the properties of serpentinizing systems, the more similarities they revealed to the life process [29], with compartmentation, energy harnessing, catalysis, and the chemical reactions of C1 compounds at vents converging on processes that comprise the core of carbon and energy metabolism in primitive autotrophic cells [30].

From a biological perspective, the genetics first vs. metabolism first debate misses the point because neither by itself is sufficient for life. Countering the genetics first view, cells are made of far more than RNA. Cells consist by dry weight of about 50% protein and 20% RNA, with DNA, lipids, cell wall, reserves, and metabolites making up the rest [31]. Most of the RNA resides in the ribosome, which synthesizes proteins in a process that consumes about 75% of the cell's ATP investment in biosynthetic processes [32], whereby a large portion of the ATP that a cell synthesizes is not used for the synthesis of cell mass—it goes to what is called ATP spilling and maintenance energy [33,34].

Countering the metabolism first view, a handful of small molecules reacting with each other do not qualify as metabolism. A cell is a very complicated chemical system composed of more than 1000 individual partial reactions that harness energy and synthesize building blocks as well as polymers. A decisive property of metabolism is redox balance: the number of electrons that enter the cell in substrates has to be equal to the number of electrons that leave the cell in waste products plus those that remain sequestered in compounds of cell mass; otherwise, metabolism and life come to a halt [35]. Although most reactions in cells are catalyzed by enzymes, enzymes do not perform feats of magic; they just accelerate reactions that tend to occur anyway. Many core metabolic reactions of cells readily take place without enzymes [36–38]. The sum of the chemical re-

actions in the cell (metabolism) runs both the synthesis and the operation of the cellular machinery that produces progeny, harboring a new copy of instructions in DNA, hence heredity, which over generations forms the process called genetics.

Yet the main thing that cells do is neither genetics nor metabolism but energy harnessing because without energy, neither metabolism nor genetics can take place. Metabolism and genetics are merely manifestations of the actions of sustained sets of exergonic chemical reactions over generations. There is a third option in the genetics first vs. metabolism first debate, namely "energy first", because it is hands-down obvious that energy has to come first [39], for without favorable energetics and energy release, neither genetics, metabolism, nor anything at all will take place, so says the 2nd law of thermodynamics. Cells themselves underscore that view, because the amount of ATP that a cell synthesizes always exceeds the amount of energy required for the synthesis of new cells during growth, often by about a factor of 3 [40] (the converse would violate the 2nd law), underscoring the point that life is an energy-releasing process. For a cell, staying alive means staying far from equilibrium, which it achieves by merely running the exergonic reactions that synthesize ATP: maintenance energy or ATP spilling [33,34]. In low-energy environments, where survival becomes more important than growth [41], maintenance energy becomes the main process of life.

1.2. Autotrophic Origins and Energy First Link C1 Metabolism to Vents

Is there an origins option that starts with energy first? Yes, and it is seated firmly in C1 metabolism and autotrophic origins. In 2021, serpentinizing systems have gone a long way to closing the gap between CO_2 and cells. Convergent lines of evidence indicate that reactions of C1 compounds were not only the source of carbon for the first cells but also the source of energy at the origin of the first metabolic reactions. This is because in the reaction of H_2 with CO_2, the equilibrium lies on the side of the simple reduced carbon compounds that comprise the backbone of carbon metabolism in organisms that use the acetyl Coenzyme A (CoA) pathway of CO_2 fixation—formate, acetate, and pyruvate. The synthesis of these acids from H_2 and CO_2 is exergonic under standard conditions [39], in cells that use the acetyl CoA pathway [6] and under conditions of simulated hydrothermal vents [30]. Hydrothermal vents are generally of interest in modern theories for origins [3] because they present continuously far from equilibrium conditions, with geochemically catalyzed redox reactions and gradients that could be tapped by the first cells for energy harnessing [29].

In hydrothermal systems, both modern and on the early Earth, the key to redox reactions, catalyst synthesis, and the formation of ion gradients, is molecular hydrogen, H_2, which is generated by the spontaneous geochemical process of serpentinization [28,42,43]. During serpentinization, mineral catalysts awaruite (Ni_3Fe) and magnetite (Fe_3O_4) are formed in situ in serpentinizing hydrothermal vents [44]. These minerals catalyze the synthesis of formate, acetate, and pyruvate as well as methane [30] in the laboratory from H_2 and CO_2 in the presence of only water and the mineral catalyst overnight at 100 °C and only 24 bar. It is likely, but not directly demonstrated, that hydrothermally formed awaruite and magnetite catalyze the synthesis of formate and methane found in the effluent of modern serpentinizing systems [45–48]. Serpentinization also renders the effluent of hydrothermal systems alkaline [48], generating the ion gradients that form at hydrothermal vents.

The synthesis of simple organics from C1 precursors in hydrothermal systems is the only known geochemical process that follows the same chemical route as a modern core pathway of carbon and energy metabolism [30,49]. In addition, modern organisms that use the acetyl CoA pathway for carbon and energy metabolism, acetogens and methanogens, exhibit a physiology that, among known life forms, is most similar to that inferred from genomic reconstructions for the last universal common ancestor of all cells, LUCA [50]. This implicates acetogens and methanogens that lack cytochromes and quinones as very primitive microbial lineages, in line with early predictions from physiolo-

gy [4] and with predictions based on similarities between geochemical and biochemical reactions [36]. It is also consistent with the identification of overlapping autocatalytic networks in the metabolism of acetogens and methanogens that implicate a role for small molecule reaction systems prior to the advent of both protein and RNA [51].

2. Methods

2.1. Reaction Data Collection

Metabolic reactions were gathered and curated from the Kyoto Encyclopedia of Genes and Genomes (KEGG) reaction database [52] (version December 2020) manually. Synthesis pathways for 46 target compounds (Table S1) were obtained and curated by hand with the help of KEGG pathways [53] and KEGG modules. The 46 target compounds comprise 20 amino acids, four ribonucleoside triphosphates, four deoxyribonucleoside triphosphates, and 18 cofactors shown in Figure 1. KEGG lacked biosynthetic pathway information on iron–sulfur clusters, so these were not included. Although depicted in Figure 1, polymers and the genetic code are also not part of the target set. The reductive acetyl CoA pathway as well as the reverse tricarboxylic acid cycle (rTCA) cycle were added to the reaction set, covering the basal CO_2 fixation along with the gluconeogenesis and pentose phosphate cycle, allowing for the synthesis of key intermediates needed to produce amino acids, nucleic acids, and cofactors from α-ketoacids, sugars, and aldehydes. Nitrogen fixation pathways were not included, since NH_3 gets incorporated via amino acid synthesis. If a pathway was unavailable in KEGG, it was manually reconstructed based on KEGG pathway maps. The collection of reactions unfolds in a short example: For methanofuran biosynthesis, KEGG module M00935 was used to add reactions R10935, R11038, R11039, R00736, R10902, and R11040. The very last step of producing methanofuran is missing in the module. This reaction from APMF-Glu is depicted in pathway map00680; thus, it was added manually.

In all pathways collected, oxygen-dependent reactions were either replaced with an anaerobic alternative if possible or omitted if not. This was the case for the synthesis of dimethylbenzimidazole, which is a precursor for cobamide. Although an anaerobic synthesis pathway for this precursor is known, starting from 5-aminoimidazole ribotide (short AIR) [54], several other intermediaries are not implemented in KEGG yet. Neither was there an anaerobic alternative for the production of 2-phospholactate as a precursor in the F_{420} synthesis pathway available. For both precursors, dimethylbenzimidazole and 2-phospholactate, as well as reduced ferredoxin (involved in the reductive acetyl CoA pathway) and reduced flavodoxin (in the rTCA cycle), we assume them to be producible in an unknown way in early metabolism. Assuming the reactions in question arose before the genetic code, the according proteins were presumably replaced by an alternative at that early period. Three reactions were constructed manually, because they appear as a dashed line in KEGG pathways with no corresponding reaction identification number. The reactions named *RMAN1-3* are presumed to be incomplete, since only the key compounds were listed. Two reactions are affected within tetrahydromethanopterin synthesis and the very last step was within methanofuran synthesis. Involved chemical elements such as molybdenum, sulfur donors, cobalt, and nickel were assumed to be present in the environment. During curation of the final reaction set, redundant reactions occurring in multiple syntheses were reduced to a single occurrence, such as the reaction chorismate <=> prephenate that occurs in both tyrosine and phenylalanine syntheses.

For the detection of autocatalytic cycles within cofactor biosynthetic pathways, catalysis rules (indicating which cofactors are used as catalysts in each reaction) were gathered from [51] (Supplemental Dataset S1A) [51]. Autocatalysis is assumed if a target is needed as a catalyst within its own biosynthetic pathway.

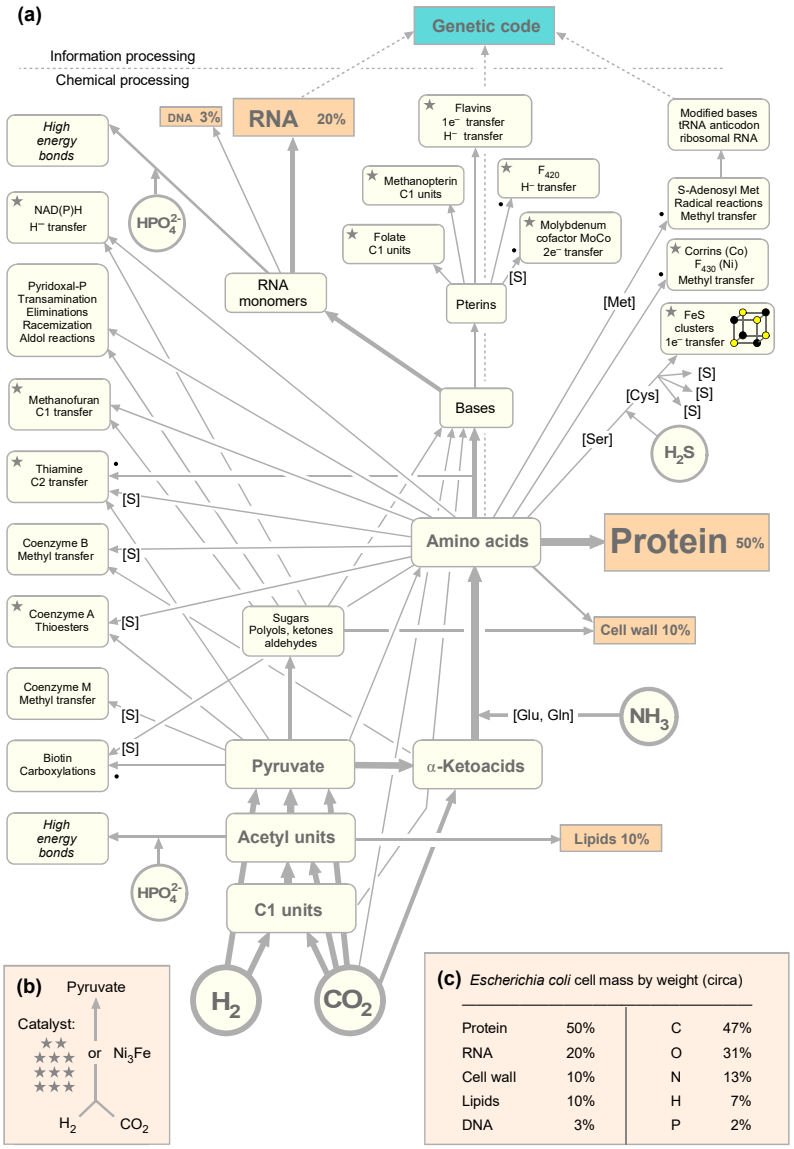

Figure 1. (**a**) A general map of core metabolism. The arrows in the map do not cover every atom in every cofactor, amino acid, or base, showing main mass contributions instead. A dot indicates that radical S-adenosyl methionine (SAM) enzymes are involved in the biosynthetic pathway leading to the product. [S] indicates that sulfur is incorporated in the biosynthetic pathway. (**b**) Cofactors indicated by a star are required in the pathway from H_2 and CO_2 to pyruvate in either acetogens or methanogens or both. (**c**) The composition of cells in terms of its main components and elemental contributions to dry weight (from [31]).

2.2. Visualization of the Autotrophic Core Network

An undirected metabolic network showing the autotrophic core was generated in simple interaction format (sif) using a custom Python script. The resulting network consists of the given 404 metabolic reactions and 380 involved compounds. The bipartite network was visualized using CytoScape [55] v. 3.8.0. One partition class corresponds to reaction nodes (diamonds), the other one corresponds to compound nodes (circle-shaped). The latter were sized according to their node degree. Target compounds were colored in blue, whereas reaction nodes are depicted smaller and in gray.

2.3. Different Core Reaction Sets Based on Distinct Identification Approaches

Two additional reaction datasets were used to determine their intersection with the 404 reactions of the autotrophic core. The LUCA set, containing 355 genes, was identified via the phylogenetic approach [50], translating to 163 metabolic reactions and the ancient 'reflexively autocatalytic food-generated' (RAF) set with 172 reactions (from [51] Figure 4). The intersection between the autotrophic core, LUCA, and the ancient RAF was determined by examining which reactions overlap in the respective analyzed datasets. In addition, the overlap of reactions between all three datasets was determined. The intersection for each comparison (Figure S1) is available in Supplemental Table S3A and the initial reaction lists are in Table S3B.

2.4. Statistical Analysis

A contingency table for each highly connected compound (Table 1) was built, comparing the compound frequency in the autotrophic core with the frequency in the global prokaryote anaerobic network consisting of 5994 reactions (from [51] S1A). A significant enrichment of compound frequency in the autotrophic core compared to the global prokaryotic set was observed for *p*-values smaller than 0.05. One-tailed Fisher tests were performed using the package scipy.stats in Python 3.6 (Table S4).

Table 1. Highly connected nodes.

Compound	Frequency
H_2O	125
ATP	77
H^+	76
P_i	66
ADP	55
CO_2	49
Glutamate	44
PP_i	37
NAD^+	37
$NADP^+$	35
NADPH	34
NADH	33
2-Oxoglutarate	24
Pyruvate	22
NH_3	21

3. Results

3.1. The Autotrophic Core of Biosynthesis Requires 19 Cofactors

For the purpose of this paper, let us assume for the sake of argument that life really did start from exergonic reactions of H_2 and CO_2 along the acetyl CoA pathway. Why do we assume the acetyl CoA pathway as the starting point of CO_2 fixation? Among the six known pathways of CO_2 fixation [6,56,57], it is the only one that occurs in both bacteria and archaea, the only one that traces in part to LUCA [50], and it is the only one that has

been shown in the laboratory to produce acetate and pyruvate from H_2 and CO_2 without enzymes, using only hydrothermal minerals as catalysts [30]. In that sense, it is the obvious choice as the starting point for metabolic evolution investigations based upon current laboratory evidence. The horseshoe (incomplete) rTCA cycle follows in Figure 1 because it is the pathway that autotrophs using the acetyl CoA pathway employ to generate C4 and C5 precursors for amino acid and other syntheses [6,36,58]. Although the incomplete horseshoe the rTCA cycle occurs in bacteria and archaea, it is fed by the acetyl CoA pathway, which is the only pathway of CO_2 fixation that is known to occur in bacteria and archaea. The other five are known to operate in only one domain [6,56]. The rTCA cycle is also an ancient pathway [59,60], and most of its reactions also operate in the laboratory in the absence of enzymes provided that pyruvate and glyoxylate are supplied as starting material, but the non-enzymatic reaction sequence operates in the oxidative direction, that is, in the absence of H_2 and CO_2 [37]. The acetyl CoA pathway, the rTCA cycle, and the dicarboxylate/4-hydroxybutyrate cycle, which is a derivative of the rTCA cycle and occurs only in archaea, employ O_2 sensitive enzymes, an ancient trait [6]. The other three CO_2 fixation pathways have no O_2-sensitive enzymes and are typically found in aerobes, occur in only one domain each, and they arose more recently in evolution, using enzymes co-opted from preexisting pathways [6].

We also assume that the first living things on the path to cells we recognize today required the universal amino acids and bases of life, the modern synthesis of which requires in turn cofactors as catalysts. We asked: How big, exactly, is the set of reactions required for the synthesis of the building blocks of cells and the cofactors needed to make them? This gives us an impression of how challenging it would be to generate the main compounds of life at origins, with or without enzymes. We started by sketching out Figure 1a, in which the main pathways of biosynthesis in anaerobes and the amounts of main biosynthetic end products are summarized. Cofactors are usually not present in amounts that would contribute appreciably to cell mass, but they are required as catalysts. Along the acetyl CoA pathway, there are differences in the methanogenic and acetogenic versions [61].

If we look at the cofactors required to get from H_2 and CO_2 to pyruvate in acetogens and methanogens [6,62], we find that methanofuran, NAD(P)H, corrins, coenzyme A, thiamine, flavins, F_{420}, three pterins—folate, methanopterin, and the molybdenum cofactor MoCo—as well as FeS clusters, a prosthetic group of proteins that we count as a cofactor here, are required. That is a substantial cofactor requirement, not to mention the enzymes that hold those cofactors in place for function in the pathway. The mere requirement for those 11 complicated organic cofactors would appear to make the proposition that C1 metabolism from CO_2 to pyruvate could be the first pathway [36] seem downright absurd, were it not for the recent observation that a bit of metal, awaruite (Ni_3Fe), or a piece of iron oxide magnetite (Fe_3O_4), can also catalyze the synthesis of pyruvate from H_2 and CO_2 [30] overnight at 100 °C in water. As a set of chemical reactions, the acetyl CoA pathway is older than the genes that encode its enzymes [58], and it is also older than the cofactors required by those enzymes.

By the foregoing count, it takes 11 cofactors to synthesize pyruvate in the modern pathway, whereby we have not counted the two steps requiring pyruvoyl enzymes at decarboxylation steps in the CoA (pantothenate) synthesis pathway; the pyruvoyl cofactor is synthesized from serine residues in the polypeptide chain of the enzyme [63]. Very surprisingly, only three additional cofactors (biotin, pyridoxal phosphate, and SAM) are required for the synthesis of the 11 other cofactors plus the main nucleosides of nucleic acids and the 20 amino acids, whereby only two more (coenzyme M and coenzyme B) are required specifically in the methanogenic pathway of energy conservation. That makes a total of 19 cofactors (counting NAD and NADP separately as well as the flavins flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) along with two corrins F_{420} and cobamide) to support their own synthesis plus the synthesis of four ribonucleoside triphosphates, four deoxyribonucleoside triphosphates, and 20 amino ac-

ids. The genetic code and polymers are not included in the autotrophic core. In total, that makes a list of 47 target compounds (19 cofactors, 8 nucleotides, and 20 amino acids; Table S1) that would be required to synthesize the substance of cells, as summarized in Figure 1.

The starting point of Figure 1a is H_2 and CO_2. Critics of autotrophic origins will be quick to point out that cyanide chemistry can readily give rise to amino acids and bases under laboratory conditions [14,64], such that we need not worry about Figure 1. However, in reply, we would be equally quick to point out that there are 415 distinct reactions in microbial metabolism involving CO_2 as a substrate in either the forward or reverse direction [65], but there are no reactions known to us in which cyanide serves as a main source of carbon in core anabolic metabolism. Some bacteria can convert CN^- to CO_2 and NH_3 or formate and NH_3 for growth [66,67], because CO_2, formate, and NH_3 readily enter metabolism, whereas cyanide does not. In other words, CO_2 directly enters and exits the organic chemistry of the cell substance at 415 reactions, where cyanide puts up a zero. We interpret the fact that cyanide has nothing to do with modern metabolism as a clear metabolic fossil: no role for cyanide in modern metabolism indicates that cyanide had nothing to do with primordial metabolism either, or was at best <1/415th as important as CO_2. In that sense, the main message of Figure 1 is the overall scheme, the metabolism of cells, not that it contains amino acids and bases as products.

That brings us to nitrogen. If carbon did not enter metabolism via cyanide, then the same must be true for nitrogen. If not via cyanide, how did N enter metabolism? All the amino acids, bases, and cofactors contain nitrogen (except coenzyme M). Nitrogen enters metabolism as NH_3 (NH_4^+ is very unreactive) with N atoms replacing O atoms in amino acids, either via an acyl phosphate intermediate in the glutamine synthase reaction or via reductive aminations of 2-oxoacids [36,68]. An exception is the carbamoyl phosphate synthase reaction, in which NH_3 reacts with carboxyphosphate to form carbamate in pyrimidine and arginine biosynthesis. Of course, NH_3 is synthesized from N_2 by nitrogenase to make it available for incorporation into organic compounds. However, N does not enter metabolism as N_2; it enters metabolism as NH_3, which is why we selected NH_3 as the source of nitrogen in Figure 1. Similarly, sulfur enters metabolism as H_2S in cysteine synthesis from serine, either via serine activation as *O*-acetylserine or *O*-phosphoserine [69]. N and S enter metabolism as dissolved gasses (NH_3 and H_2S) via amino acid synthesis [36]. In cells that live from H_2 and CO_2, C, N, S, and electrons (H_2) enter metabolism as gasses.

3.2. Enzymatic Reactions in the Autotrophic Core

Figure 1a depicts the relationships among reactions that underpin the core synthesis of cells from H_2, CO_2, and NH_3, but it does not depict the reactions themselves. To find out which, what kind of, and how many reactions are required to synthesize 18 cofactors, 8 nucleotides, and 20 amino acids from H_2, CO_2, NH_3, and H_2S, we turned to KEGG pathways using Figure 1 as a framework to identify the reactions and enzymes that catalyze them. The metabolic network for the 404 reactions (Table S2) that comprise the autotrophic core is shown in Figure 2.

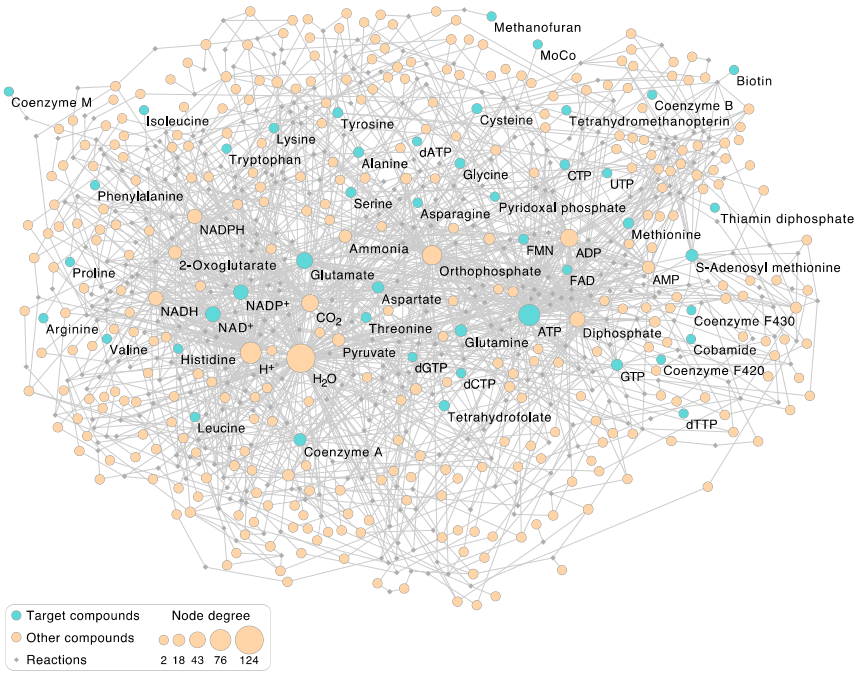

Figure 2. The autotrophic core network of 404 reactions underlying Figure 1. The undirected bipartite graph comprises 404 reaction nodes (displayed as gray diamonds) and 380 compound nodes (circles). The 46 target compounds are colored blue; other compounds involved in the reactions appear orange. Target compounds correspond to the core compounds in Figure 1. Each compound participating in a reaction is connected to the respective reaction node with an edge. Compounds are sized according to node degree (number of reactions the compound takes place in). For example, H_2O appears either as reactant or product in 125 reactions and is the most frequent compound in the 404 reactions (see also Table 1). In primordial metabolic processes, before the existence of enzymes, a more limited spectrum of compounds than those in Figure 1 was provided by the environment. Compound nodes are labeled if they are targets or if the node degree is ≥20. Note that FeS clusters are not included in this figure since their synthesis cannot be reconstructed using KEGG. The network contains only L-amino acids.

Other than supplying a greater level of detail than Figure 1, and showing the relative size of nodes, the network itself in Figure 2 is not hugely informative, but some of its properties are. Keeping in mind that Figure 2 comprises the marrow of modern metabolism, hence reactions that were present in life's common ancestor, we first asked what the most highly connected metabolites are. The fifteen most common metabolites are given in Table 1. The most common compound in the autotrophic core is by far H_2O. As stated above, water is the solvent of life's chemistry and its most common reaction partner. Proponents of the RNA world generally view water as a poison for origins, because it promotes the hydrolysis of RNA [70]. However, the host rocks of serpentinizing hydrothermal systems are replete with environments of low water activity, mainly because water is consumed by rock in the serpentinization process [71,72]. Furthermore, fluctuating water activities that occur during serpentinization can be conducive to polymerization reactions [71]. Life counteracts the hydrolysis problem by coupling nucleic acid and protein polymerization reactions to exergonic reactions via ATP synthesis and hydrolysis such that polymer synthesis vastly outpaces hydrolysis [58]. Accordingly, ATP is the second most common reactant in the autotrophic core (Table 1), right before protons. Protons are of course normally bound to water as H_3O^+, although they are not counted as water here. Protons arise in hydride transfer reactions involving NADH and NADPH

which yield NAD^+ and $NADP^+$, respectively. The frequency of protons in the network mainly reflects the frequency of NAD(P)H-dependent redox reactions in the autotrophic core (Table 1).

Among reactions that involve the formation or alteration of bonds with carbon atoms, the most common carbon-containing compound in the autotrophic core is, fittingly, CO_2, which underscores the CO_2-dependent nature of core metabolism. Among the 404 reactions in the core, 49, or every eighth reaction, involves CO_2. This can be seen as physiological evidence in favor of autotrophic origins. The next most common carbon backbone in the core is glutamate, which is the main workhorse of nitrogen metabolism. Glutamate arises as a product in amidotransferase reactions involving glutamine as an amino donor and in transamination reactions that produce 2-oxoglutarate, which is also among the top 15 reactants in the core. ATP hydrolysis products P_i and PP_i round out the list as well as pyruvate, which links the acetyl CoA pathway to sugar synthesis and the reverse citric acid cycle [73] and is a common starting point for cofactor synthesis in the autotrophic core (Figure 2). Last among the top fifteen is NH_3, which is often donated to biosynthetic reactions from glutamine via an amidotransferase [74] during the enzymatic reaction, without being released as free NH_3 in the cytosol.

We identified five autocatalytic cycles in the network, that is, cofactors that are required for their own biosynthesis: pyridoxal phosphate and thiamine, whose biosyntheses were previously identified as autocatalytic cycles [36], plus ATP, NAD, and NADP. Though not contained within our set, Davidson recently reported that coenzyme A is required for activation of the complex that synthesizes the active moiety of decarboxylating pyruvoyl enzymes, which are involved in CoA biosynthesis [63]. That would make a sixth autocatalytic cycle.

3.3. Comparison of the Autotrophic Core With LUCA's Genes and Ancient Autocatalytic Sets

Other recent papers have addressed the nature of ancient metabolism by looking at phosphate-independent reactions among all KEGG reactions [75], the properties of thioester-dependent reactions [76] or chemical investigations of metabolic reactions without enzymes [30,37,49,77]. A different approach has been to focus on evidence for the nature of ancient microbial metabolism that is recorded in the genomes and metabolism of bacteria and archaea. A phylogenetic approach to ancient microbial metabolism uncovered 355 genes present in bacteria and archaea trace to LUCA on the basis of vertical intradomain inheritance as opposed to archaeal–bacteria transfer [50]. Autocatalytic networks called RAFs, for reflexively autocatalytic food-generated networks, have been identified in the metabolism of anaerobic autotrophs, with an ancient RAF of 172 genes that overlaps in the metabolism of H_2-dependent acetogens and methanogens [51]. Do these sets overlap with the autotrophic core, and if so, how?

A comparison of these three sets (Table S3; Figure S1) reveals that among the 404 reactions of the autotrophic core, only 24 are represented among the 355 genes (6%) that trace to LUCA. That low degree of overlap is not surprising for two reasons. First, only a fraction of genes that trace to LUCA by phylogenetic criteria were involved in amino acid or cofactor biosynthesis, most being involved in ribosomal biogenesis or other categories. Second, only 3% of all genes shared by bacteria and archaea were not subjected to bacterial–archaeal transfers by the measure of phylogenetic trees [50], which is a criterion that played no role in the construction of Figure 1. However, it is very noteworthy that all of the cofactors shown in Figure 1, with the exception of the archaeal-specific cofactors CoM and CoB, do trace to LUCA via phylogeny, because enzymes that trace to LUCA possessed those cofactor requirements for activity [50]. In that sense, there is excellent agreement between the physiology of LUCA as inferred from phylogeny and the present autotrophic core, their commonality being cofactors, organic catalysts that are smaller and involved in a greater number of reactions than any individual enzyme.

Among the 172 reactions present in the ancient autocatalytic network shared by acetogen and methanogen RAFs [51], 81 (47%) are present in the autotrophic core. This

substantial overlap also makes sense, because all cells use the same amino acids and because both this study and Xavier et al. [51] focused on bacteria and archaea that use the acetyl CoA pathway, which by itself involves almost all of the cofactors shown in Figure 1 as it operates in bacteria and archaea. That is again noteworthy, because even though pyruvate, the central C3 product of the acetyl CoA pathway [6], can be obtained from H_2 and CO_2 using only simple minerals as catalysts [30], the biological pathway requires about a dozen enzymes and cofactors. These cofactors trace to LUCA [50], are well represented in RAFs [51], and comprise the basal foundation of the ancient autotrophic core (Figure 1b). Clearly, in early metabolism, cofactors and the catalytic minerals that were their inorganic precursors were very important [78]. Although self-evident, this indicates that there existed a vectorial progression in metabolic evolution that centered around the nature of catalysts: from transition metal minerals to organic cofactors to enzymes, each adding specificity and rate enhancement to exergonic reactions that tend to occur anyway. The retention of transition metal centers in some enzymes, such as carbon monoxide dehydrogenase, acetyl CoA synthetase, hydrogenases, or nitrogenase, suggests that microbes have been unable to invent catalysts that can perform the same reactions without the help of electrons in the *d*-orbitals of transition metals.

The comparison with 5994 anaerobic prokaryotic reactions (see S1A in [51]) tells us which compounds are enriched in the autotrophic core. Table S4 shows that this is true for ATP (and ADP plus P_i), CO_2, glutamate, pyruvate, and 2-oxoglutarate. This suggests a more crucial role of these compounds in the origin of the core subsequent to later evolution in anaerobes, reflecting a process of carbon backbone elongation from CO_2 at the heart of the core as a supply of precursors for cofactor and amino acid biosynthesis, the latter being the starting point for nucleotide biosynthesis [78].

4. Conclusions

It is human nature to wonder about the origin of life, which is an issue that is among the most debated of all scientific questions. However, in comparison to questions concerning the existence of dark matter or how consciousness works, the origins process lies in the ancient past, and its events are only accessible through inference. Debates within the origins field can be fierce and have a long history. They hinge upon definitions about what qualifies as being alive, what one assumes to be the habitat that brought forth the first biochemical reactions, what came first, small molecule metabolism and proteins or nucleic acids and genetics, what the nature of first energy source(s) was that the early life forms harnessed in order to grow, and what kinds of chemical compounds existed before the first energy-releasing reactions germane to modern metabolism started taking place [21]. The literature harboring those debates is generally exhausting, because the same arguments resurface over and over again. The more broadly one reads the literature on early evolution, the more one gets the impression that scientists not only do not agree about origins and the nature of the first forms of life, but worse, that scientists know little about early evolution, leaving the topic open to unconstrained speculation and argument. That puts the origins field at risk of defining scientific progress in the units of debate preparation and presentation skills rather than units of empirical findings that are linked to the explanandum (real life); it also risks vulnerability to criticisms about the role of dogma in science.

Biologists tend to hold that there are traces of early evolution preserved in metabolism itself [4,6]. While there is no obvious proof for that conjecture, the nature of basic building blocks of life is dramatically well conserved across all cells [79]. All life forms we know use proteins made of amino acids, nucleic acids made of purines, pyrimidines, sugars, and phosphate. That means that the first forms of life from which all modern forms descend had that core chemistry in place, in addition to the universal genetic code to transfer information from nucleic acids to protein at the ribosome. This adds direly needed constraints to the origins problem. By looking at metabolism from a comparative standpoint, one can distill insights into the nature of early cells.

Here, we have identified 404 reactions that comprise the autotrophic core. It contains five small autocatalytic cycles in which cofactors participate in their own synthesis. The core represents a collection of reactions that underpin the synthesis of RNA and proteins. It was present in the first cells, but it can hardly have arisen all at once. The aqueous synthesis of pyruvate from H_2 and CO_2 using only solid-state metal or metal oxides as a catalyst [30] indicates that the core itself likely started from H_2 and CO_2 and grew outwards from pyruvate while incorporating nitrogen from NH_3. How complex the core could have become prior to the origin of enzymes is a question for future study. However, let us keep in mind that enzymes just accelerate reactions that tend to occur anyway. It is well known that many enzymatic reactions take place without enzymes [36], although sometimes, the non-enzymatic reaction rates can be so slow as to be irrelevant [80]. However, it was also demonstrated that citric acid cycle reactions [49,81] and a number of reactions involving sugars in central metabolism [77,82] can be catalyzed non-enzymatically. This suggests that a fairly complex system of reactions, yet with far less specificity than that in the core, could have arisen before the advent of genes and proteins.

H_2O is the most common reactant in the autotrophic core, indicating an aqueous environment during its formation. That environment was not only aqueous but also reducing, as revealed by the abundance of redox reactions in the autotrophic core, the central role of CO_2, and the circumstance that the core's main products (amino acids and nucleic acids) are far more reduced than CO_2. Furthermore, the number of central reactions depending upon the hydrolysis of high-energy phosphate bonds indicates that the core arose in the presence of a continuous and highly exergonic chemical reaction capable of continuously synthesizing high-energy phosphate bonds, both before and after the origin of enzymes; here, an H_2-dependent CO_2 reduction to acetate [30] forming acyl phosphate bonds [58] is the proposition.

Thus, the chemical reactions of the autotrophic core suggest that it formed in an aqueous environment that supplied H_2, CO_2, and NH_3, was highly reducing, and harboring continuously far from equilibrium conditions. Those conditions are very similar to those found in serpentinizing hydrothermal systems [44,77], and furthermore, they are very similar to those inferred from the functions of enzymes that vertically trace to the last universal common ancestor [50,83].

Notwithstanding pyrrolysine [84], selenocysteine [85], and a number of modified bases [86], the lack of fundamental deviation among modern life forms from the core building blocks of life, core information processing, and the core repertoire of cofactors [87] indicates that whatever chemical processes occurred at origin did not give rise to alternative cores with enough staying power to persist to the present. "Still, other cores could have existed" the critic might interject, which is true. "But even if they existed, they are irrelevant", we would counter, because they are disjunct from the biologist's explanandum: the autotrophic core that we can observe in modern life forms.

Supplementary Materials: The following are available online at www.mdpi.com/2076-2607/9/2/458/s1, Table S1: List of 47 target compounds of the autotrophic core metabolism, Table S2: Autotrophic core consisting of 404 metabolic reactions needed to synthesize amino acids, nucleic acids, cofactors and intermediate precursors, Table S3: (A) Reaction lists for 404 autotrophic core reactions, 163 LUCA reactions, 172 Core RAF reactions, (B) their respective intersections and (C) intersection of all three reaction sets with functional KEGG annotation, Table S4: Frequency of highly connected nodes among the autotrophic core and the global prokaryote anaerobic network and results of statistical tests for significant compound enrichment using Fisher's exact test, Figure S1: Venn diagram showing the proportion of intersecting reactions of three different core datasets.

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3.5. Energy at origins: Favorable thermodynamics of biosynthetic reactions in the last universal common ancestor (LUCA)

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Authors: Jessica L. E. Wimmer, Joana C. Xavier, Andrey do N. Vieira, Delfina P. H. Pereira, Jacqueline Leidner, Filipa L. Sousa, Karl Kleinermanns, Martina Preiner, and William F. Martin.

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Summary: If a biochemical core can be identified, the question of what source of energy fueled it emerges. In this manuscript, we calculated the Gibbs free energy of 402 individual reactions in the metabolic core synthesizing amino acids, nucleobases and cofactors from CO_2, NH_3, H_2, H_2S and phosphate. We tested the natural tendency of these reactions to unfold at different temperature and pH conditions. We found that 95 to 97% of the core reactions are exergonic when at temperatures of 80 to 100 °C and pH ranges of 7 to 10. These results not only point toward the biochemistry involved in LUCA's metabolism but also give us a hint on the environment it likely emerged.

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Energy at Origins: Favorable Thermodynamics of Biosynthetic Reactions in the Last Universal Common Ancestor (LUCA)

Jessica L. E. Wimmer[1], Joana C. Xavier[1], Andrey d. N. Vieira[1], Delfina P. H. Pereira[1], Jacqueline Leidner[1], Filipa L. Sousa[2], Karl Kleinermanns[3], Martina Preiner[1] and William F. Martin[1]**

[1] Department of Biology, Institute of Molecular Evolution, Heinrich Heine University Düsseldorf, Düsseldorf, Germany, [2] Department of Functional and Evolutionary Ecology, University of Vienna, Vienna, Austria, [3] Department of Chemistry, Institute of Physical Chemistry, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

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***Correspondence:**
Jessica L. E. Wimmer
jessica.wimmer@hhu.de
William F. Martin
bill@hhu.de

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Though all theories for the origin of life require a source of energy to promote primordial chemical reactions, the nature of energy that drove the emergence of metabolism at origins is still debated. We reasoned that evidence for the nature of energy at origins should be preserved in the biochemical reactions of life itself, whereby changes in free energy, ΔG, which determine whether a reaction can go forward or not, should help specify the source. By calculating values of ΔG across the conserved and universal core of 402 individual reactions that synthesize amino acids, nucleotides and cofactors from H_2, CO_2, NH_3, H_2S and phosphate in modern cells, we find that 95–97% of these reactions are exergonic ($\Delta G \leq 0$ kJ·mol^{-1}) at pH 7-10 and 80-100°C under nonequilibrium conditions with H_2 replacing biochemical reductants. While 23% of the core's reactions involve ATP hydrolysis, 77% are ATP-independent, thermodynamically driven by ΔG of reactions involving carbon bonds. We identified 174 reactions that are exergonic by –20 to –300 kJ·mol^{-1} at pH 9 and 80°C and that fall into ten reaction types: six pterin dependent alkyl or acyl transfers, ten S-adenosylmethionine dependent alkyl transfers, four acyl phosphate hydrolyses, 14 thioester hydrolyses, 30 decarboxylations, 35 ring closure reactions, 31 aromatic ring formations, and 44 carbon reductions by reduced nicotinamide, flavins, ferredoxin, or formate. The 402 reactions of the biosynthetic core trace to the last universal common ancestor (LUCA), and reveal that synthesis of LUCA's chemical constituents required no external energy inputs such as electric discharge, UV-light or phosphide minerals. The biosynthetic reactions of LUCA uncover a natural thermodynamic tendency of metabolism to unfold from energy released by reactions of H_2, CO_2, NH_3, H_2S, and phosphate.

Keywords: origin of life, energetics, bioenergetics, metabolism, early evolution, biosynthesis, thermodynamics, last universal common ancestor

INTRODUCTION

Between the first appearance of liquid water on the Earth roughly 4.3 billion years ago (Mojzsis et al., 2001) and the appearance of the first signs of life roughly 3.8 billion years ago (Rosing, 1999), simple spontaneous geochemical reactions gave rise to the enzymatically catalyzed reaction network of microbial metabolism: a highly organized set of specific organic reactions that provides the amino acids, nucleotides and cofactors to sustain ribosomal protein synthesis and growth. How metabolism arose is a keystone issue for understanding how the first microbes arose from the elements. It is a complex problem with many facets, several approaches to investigate the issue are current.

From the standpoint of theory, autocatalytic networks provide a useful framework for the study of metabolic origin (Kauffman, 1986; Hordijk and Steel, 2004). In autocatalytic sets, elements of the set can catalyze the synthesis of other elements of the set, potentially giving rise to molecular self-organization provided that a food source is supplied to drive the network forward (Hordijk et al., 2010). Autocatalytic sets are not purely theoretical objects because they can be identified in the metabolism of both modern cells and their inferred ancestors (Sousa et al., 2015; Xavier et al., 2020).

From the standpoint of individual reactions, inorganic catalysts have long been known to catalyze many metabolic reactions under laboratory conditions (Wächtershäuser, 1992; Huber and Wächtershäuser, 1997; Martin and Russell, 2007; Sousa et al., 2018). More recently, complex reaction sets approximating biochemical pathways (Muchowska et al., 2019, 2020) and in some cases even exactly retracing biochemical pathways (Preiner et al., 2020) have been reported, uncovering a natural tendency of numerous metabolic reactions to unfold in the presence of transition metal catalysis. From the computational standpoint, simulations have been widely employed to study metabolic origin, particularly network expansion algorithms. These have been shown to generate small molecule networks consisting of up to hundreds of compounds with properties that resemble metabolism, with the caveat that networks so generated are not manifest as natural pathways in modern cells (Goldford et al., 2017, 2019; Tian et al., 2019).

Independent of the methodological approach, current investigations of metabolic origin tend to start from the acetyl-CoA pathway of CO_2 fixation (Fuchs and Stupperich, 1985; Fuchs, 2011) for a number of reasons. It is the only pathway of CO_2 fixation (i) that is both linear and exergonic (Berg et al., 2010), (ii) that occurs in both bacteria and archaea (Berg et al., 2010; Fuchs, 2011), and (iii) that traces to the last universal common ancestor (LUCA) (Weiss et al., 2016). Its exergonic nature allows coupling of H_2-dependent CO_2 reduction to ion pumping and ATP synthesis, as in acetogens (Schuchmann and Müller, 2014) and methanogens (Thauer et al., 2008), strict anaerobes that obtain both their carbon and energy from the reduction of CO_2 with H_2. Organisms that use the acetyl-CoA pathway still inhabit H_2-producing geochemical systems (Magnabosco et al., 2018; Smith et al., 2019), habitats that existed on the early Earth (Sleep et al., 2011). The first intermediate of the acetyl-CoA pathway, formate, is synthesized geochemically via abiotic reactions in modern hydrothermal systems (Lang et al., 2010; Schrenk et al., 2013), as are the endproducts of energy metabolism via the pathway in acetogens (acetate; Sherwood Lollar et al., 2021) and in methanogens (methane; Proskurowski et al., 2008). In carbon metabolism, the acetyl-CoA pathway generates pyruvate as the main product (Fuchs, 2011) via reactions that require 10 enzymes and cofactors, yet those enzymes can be replaced by simple hydrothermal minerals such as awaruite (Ni_3Fe), which convert H_2 and CO_2 into formate, acetate and pyruvate overnight at 100°C in water (Preiner et al., 2020). Such findings connect the carbon and energy metabolism of acetogens and methanogens to spontaneous geochemical processes in H_2-producing hydrothermal vents via the chemical reactions of the acetyl-CoA pathway (Martin, 2020).

Thermodynamic studies in geochemical systems also point to an origin of metabolism from H_2 and CO_2 in a hydrothermal setting, as the synthesis of amino acids (Amend and Shock, 1998) and even prokaryotic cell mass (Amend and McCollom, 2009) from H_2, CO_2 and NH_3 is exergonic under the chemical conditions germane to H_2-producing hydrothermal vents. However, calculating ΔG for a one-step geochemical reaction that converts H_2, CO_2 and NH_3 into amino acids (Amend and Shock, 1998; Amend et al., 2013) does not begin to capture the thermodynamic landscape of metabolism, either modern or ancient, because the biosynthesis of amino acids and all other cell constituents involve the entry of H_2, CO_2, and NH_3 at a very small number of very specific enzymatic reactions, followed by their distribution in activated form as hydride, organic carbon or amino moieties in highly connected networks of intermediate conversions. For example, over 20 distinct reactions are involved in the synthesis of either tryptophan or purines from H_2, CO_2, and NH_3 (Kanehisa and Goto, 2000). Studies of thermodynamics at metabolic origin ideally need to address the thermodynamics of individual metabolic reactions as they are organized in modern cells or in the inferred ancestors thereof.

Our present investigation into metabolic origin is based on comparative physiology. Wimmer et al. (2021a) identified roughly 400 reactions that are used by bacteria and archaea to synthesize the amino acids, nucleotides and cofactors required for growth. Because these reactions are universal, they represent core biosynthetic metabolism in the last universal common ancestor (LUCA). As such, they can be seen as the endpoint of metabolic origin on the one hand and the starting point of physiological diversification on the other. Here we have updated this set of reactions, which we designate as the metabolic core, to include the two-enzyme reaction sequence of substrate level phosphorylation used by acetogens and some methanogens (Rother and Metcalf, 2004) as an acetyl-CoA dependent source of cytoplasmic (membrane independent) ATP synthesis. Although the acetyl-CoA pathway is not universal, having been replaced by many other autotrophic (Berg et al., 2010; Fuchs, 2011; Hügler and Sievert, 2011; Steffens et al., 2021) and heterotrophic (Schönheit et al., 2016) carbon assimilation pathways during

evolution, it traces to LUCA (Weiss et al., 2016) and, like many of LUCA's biochemical reactions (Sousa et al., 2018), is older than the enzymes that catalyze its reactions (Martin, 2020). Though the remaining chemical reactions of the core do not occur in all genomes, as auxotrophies arise recurrently in evolution, they are universal at the level of primary production, the process that has fueled all ecosystems from origins to today (Hamilton et al., 2016; Martin et al., 2018). However, the enzymes that catalyze the reactions of the core are not universal, such that the core cannot be identified through purely genomic comparisons because (i) reactions that arose post LUCA, in particular O_2-dependent reactions (Dailey et al., 2017; Jabłońska and Tawfik, 2021), need to be filtered out (Wimmer et al., 2021a), (ii) because lateral gene transfers of recently arisen pathways have to be filtered out (Weiss et al., 2016), and (iii) because the enzymes that catalyze these reactions are often unrelated across the archaeal-bacterial divide (Sousa et al., 2013), suggesting independent origins of enzymatic pathways from LUCA en route to the last common ancestors of archaea (Williams et al., 2017) and bacteria (Xavier et al., 2021), respectively.

Despite many unknowns concerning the process of metabolic origin, one factor provides stringent constraint: The chemical reactions that comprised LUCA's metabolism, and those from which it arose, were perforce exergonic, for without energy release, no reactions will take place. It has long been recognized that energy was required to promote reactions at metabolic origin, but the nature of that energy has been debated. Many possible environmental sources of energy at origins have been suggested, including pyrophosphate (PP_i; Schramm et al., 1962), cyclic polyphosphates (Ozawa et al., 2004), reduced phosphorous minerals (Pasek, 2020), ultraviolet light (Patel et al., 2015), radioactive decay (Ebisuzaki and Maruyama, 2017), lightning (Ducluzeau et al., 2009), geochemical pyrite synthesis (Wächtershäuser, 1992), geochemical ion gradients (Russell and Cook, 1995), geoelectrical potential (Kitadai et al., 2021), bolide impacts (Ferus et al., 2015), and heat (Muller, 1995). Modern cells in nature, however, harness none of those environmental energy sources, they harness redox reactions instead (Mitchell, 1961; Thauer et al., 1977; Müller et al., 2018), and conserve energy for metabolic use in the chemically accessible currency of ATP (Decker et al., 1970) or reduced ferredoxin (Herrmann et al., 2008; Buckel and Thauer, 2013; Müller et al., 2018). The fact that only a fraction of core biosynthetic reactions entail ATP hydrolysis (Wimmer et al., 2021a) leads to a seldom formulated question: What drove the majority of LUCA's metabolic reactions forward? We reasoned that ATP-independent biosynthetic reactions might themselves be a possible primordial energy source, one that would be particularly conducive to the formation of autocatalytic networks (Xavier et al., 2020). To investigate further, we polarized the core biosynthetic network of LUCA in the direction of cell synthesis and estimated the changes of Gibbs energy for each individual reaction using the component contribution method (Flamholz et al., 2012; Noor et al., 2013; Beber et al., 2021) to identify the nature of ATP-independent exergonic reactions endogenous to LUCA's biosynthetic metabolism.

MATERIALS AND METHODS

Biosynthetic Network

The 402 metabolic reactions comprising the core were manually polarized in the direction of cell synthesis (Wimmer et al., 2021a; **Supplementary Table 1**). Reactions of the acetyl-CoA pathway in the CO_2 fixing reductive direction (Fuchs, 2011) [the archaeal pathway is missing in The Kyoto Encyclopedia of Genes and Genomes (KEGG)], gluconeogenesis (Say and Fuchs, 2010), the reverse citric acid cycle (Steffens et al., 2021) and the pentose phosphate pathway generate most key intermediates. No anaerobic synthesis was available in KEGG (the standard database for microbial metabolic pathways; Kanehisa and Goto, 2000) for dimethylbenzimidazole, 2-phospholactate and flavins. Three cofactors (CoB, CoM, and F_{430}) that are not required in biosynthesis but are essential for ATP synthesis in methanogenic archaea (Thauer et al., 2008) are included in the core. The rare amino acids selenocysteine and pyrrolysine were not included, nor were modified amino acids in proteins as cofactors, including pyruvoyl enzymes. Reactions were obtained from KEGG (Kanehisa and Goto, 2000), version December 2020, excluding degradation reactions and oxygen-dependent reactions (Wimmer et al., 2021a), including H_2-dependent substrate level phosphorylation (Martin and Thauer, 2017), ferredoxin:NAD(P)H interconversion, and H_2-dependent CO_2 reductase (Schuchmann and Müller, 2014). Of the 18 cofactors in **Figure 1**, 10 are required by the acetyl-CoA pathway in archaea and bacteria from H_2 and CO_2 to pyruvate (Fuchs and Stupperich, 1985; Martin, 2020). The biosynthetic pathway to iron-guanylylpridinol, required for H_2-dependent methenyl H_4MPT reduction in methanogenesis under nickel limitation (Huang et al., 2020), is not represented in KEGG and missing in the network, leaving only two entry points of H_2 into metabolism via ferredoxin-reducing hydrogenases (Huang et al., 2020) and H_2-dependent CO_2 reductase (Schuchmann and Müller, 2014). Except biotin, the compounds clockwise from Trp to methanofuran in **Figure 1** contain at least one aromatic ring. Aromatic ring forming reactions in the core entail five rings in amino acids, six in nucleoside bases, seven in pterins (two shared and five specific), eight in tetrapyrroles (four in pyrrole formation and four leading to F_{430} and cobalamin), two for methanofuran, one each for thiamine, pyridoxal, and pyridine dinucleotides. Each aromatic compound requires a ring formation reaction plus two non-aromatic rings in biotin and one each in ribose and proline. Modern chemolithoautotrophs live from the components shown on the left in **Figure 1** plus trace elements (Magnabosco et al., 2018; Smith et al., 2019), growing on biotic H_2 from fermentations (Wolfenden, 2011) or abiotic H_2 from hydrothermal systems (Schrenk et al., 2013; Dick, 2019; Lang and Brazelton, 2020).

Estimation of Gibbs Energy for Individual Reactions

A few words are needed concerning the component contribution (or group contribution) method. Traditionally, biochemists determine the change of Gibbs energy, ΔG, in a physiological

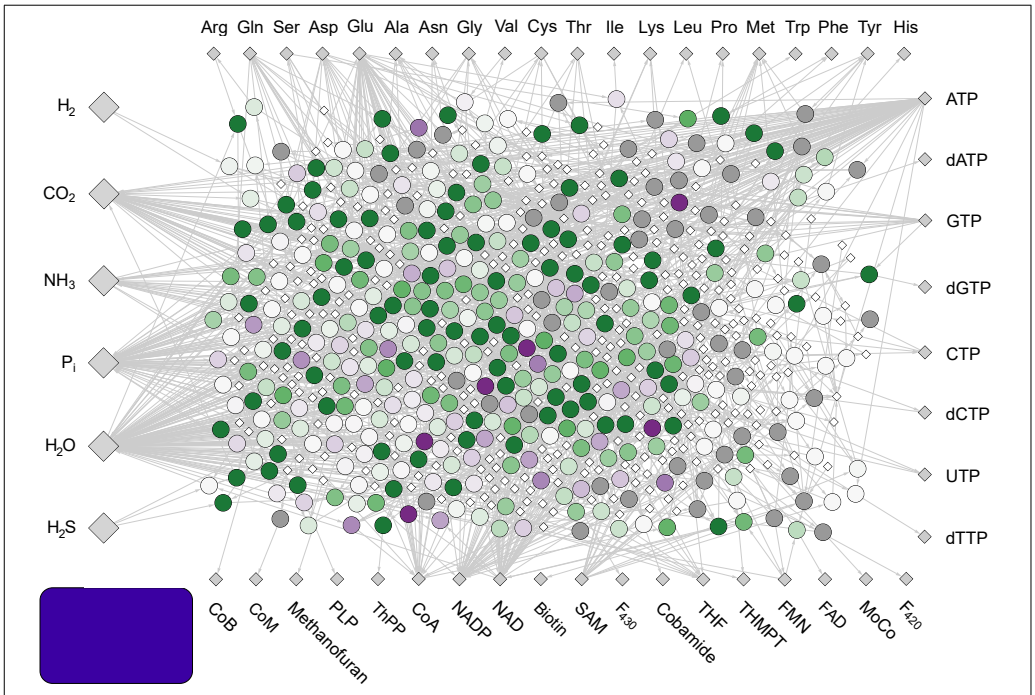

FIGURE 1 | The biosynthetic core. Large gray diamonds: starting compounds (excluding trace elements). Small gray diamonds: products. White diamonds: intermediates. Large circles within the network: Reactions, values of ΔG calculated for 351 reactions at 25°C, pH 7 and 1 mM equal reactant and product concentration are indicated by large circles and colored according to color scale at lower left. The 51 reactions for which no value of ΔG could be calculated are indicated with dark gray shading. Reactions and values of ΔG given in **Supplementary Table 3**.

reaction by measuring the concentrations of reactants and products in the presence of the enzyme. The change in Gibbs energy ΔG for the reaction A + B ⇌ C + D is obtained from the equation:

$$\Delta G = \Delta G^{\circ\prime} + \mathrm{RT} \ln \frac{[C]\,[D]}{[A]\,[B]} \tag{1}$$

Where R is the gas constant, T is the temperature in Kelvin and [A], [B], [C], and [D] are the molar concentrations (more precisely activities) of reactants and products forming the reaction quotient. ΔG' is the change of free standard enthalpies during reaction in water at physiological pH 7, 25°C, 1 M molar concentrations and 1 atm gas pressure. If H^+ is involved in the reaction, its activity is 1 in eq. (1) at pH 7. If water is involved in the reaction, its activity in eq. (1) is also 1 because ΔG' is obtained from measurements in water and the water concentration in water as the solvent does not change appreciably by reaction water.

At equilibrium, $\Delta G = 0$ (no net driving force and therefore no change of reactant and product concentrations anymore), resulting in:

$$0 = \Delta G^{\circ\prime} + \mathrm{RT} \ln \frac{[C]_{eq}\,[D]_{eq}}{[A]_{eq}\,[B]_{eq}} \tag{2}$$

$$\Delta G^{\circ\prime} = -\,\mathrm{RT} \ln \frac{[C]_{eq}\,[D]_{eq}}{[A]_{eq}\,[B]_{eq}} = -\,\mathrm{RT} \ln K' \tag{3}$$

Therefore, ΔG°' can be obtained from the reactant and product concentrations measured at reaction equilibrium in water at pH 7. K' is the equilibrium constant at pH 7. The increments used in the component contribution method to obtain ΔG°' derive their values from measurements of K' in water, hence the activity of water is already taken into account in ΔG°' and can be set to 1 in the reaction quotient. At physiological conditions, concentrations are generally different from 1 M and eq. (1) with the reaction quotient term is used to calculate the Gibbs energy ΔG'. For clarity, we manually polarized the reactions toward synthesis by writing the KEGG reactions from left to right such that the flux of carbon and nitrogen starts from CO_2 and NH_3 and proceeds within the KEGG pathways in the direction of amino acid, nucleotide and cofactor synthesis. To estimate ΔG

under nonequilibrium conditions, unequal reactant to product concentration ratios were inserted into in the reaction quotient for the polarized reaction, see below.

For many reactions catalogued in large biochemical databases such as KEGG (Kanehisa and Goto, 2000) the equilibrium concentrations are not known or not readily obtained, but the value of $\Delta G'$ can still be estimated using the component contribution method, which is based on the group contribution method originally developed by Benson (1968) to study the equilibria of chemical reactions in the gas phase and later adapted by Alberty (1998) and others to the study of aqueous reactions. It is an indirect method for estimating the position of the equilibrium in a reaction based on the thermodynamic contributions of the moieties in the compounds in question (Jankowski et al., 2008). In this paper we will use $\Delta G'$ to indicate 1 M reactant and product concentrations and 1 bar pressure for gasses at 25°C, in the strict sense. When we refer to conditions that deviate from $\Delta G'$, for example different temperatures or different reactant and/or product concentrations, we use the generic term ΔG, whereby its parameters are then unambiguous by context.

Gibbs energies were calculated using eQuilibrator API (Flamholz et al., 2012; Beber et al., 2021) version 0.4.1 under Python v. 3.6.7 which bases its estimates on the component

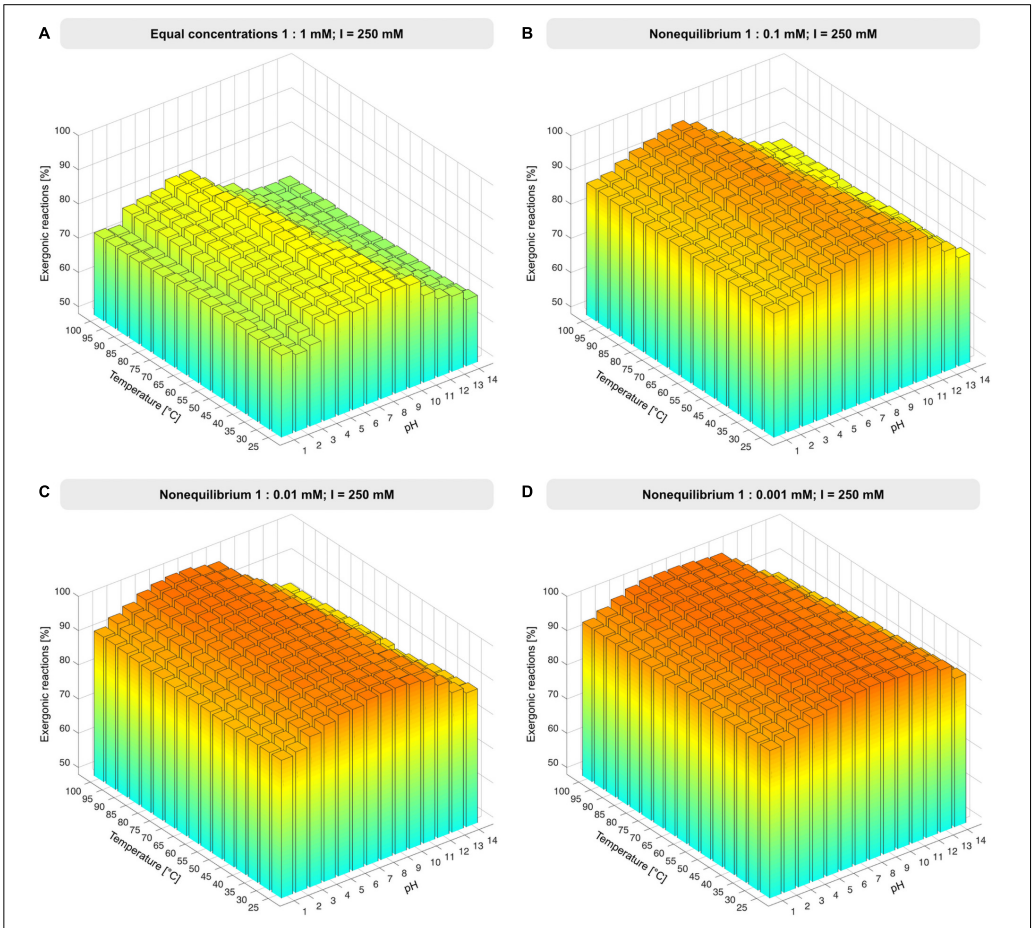

FIGURE 2 | Proportion of exergonic reactions under nonequilibrium conditions. **(A)** At constant ionic strength, I, and reactant concentration of 1 mM, the concentration of products was decreased from 1 mM to 1 μM **(B–D)**. ΔG was calculated for the 351 reactions that deliver a value of ΔG across the pH and temperature range and the proportion of exergonic reactions was plotted for each condition set (see also **Supplementary Figure 1** and **Supplementary Table 3**).

contribution method (Noor et al., 2013). eQuilibrator is widely used in biochemical and genome-based investigations, inter alia because it is capable of operating with reactions and compounds in the KEGG database. To cross check the current set, we compared values obtained using eQuilibrator to those determined by the traditional biochemical method for core carbon metabolism (**Supplementary Table 2**; Fuchs, 2011). As in earlier studies (Alberty, 1998; Jankowski et al., 2008; Flamholz et al., 2012), the agreement was good, usually within a few $kJ \cdot mol^{-1}$, indicating that the method delivers useful estimates.

Unless otherwise specified, environmental conditions were simulated by varying the pH from 1 to 14 in increments of 1 and temperature from 25 to 100°C in increments of 5°C at constant ionic strength of 250 mM, Mg^{2+} concentration fixed to 3 mM, and reactant concentrations set to 1 mM. Nonequilibrium conditions were simulated by altering the reactant to product ratio from 1:1 to 1:0.1 mM, 1:0.01 mM, 1:0.001 mM, 1:0.0001 mM and 1:10 mM (**Figure 2**, **Supplementary Figure 1**, and **Supplementary Table 3**). Atomic balancing was checked prior to calculation, such that ΔG was only calculated for balanced reactions, excluding partial reactions. For 351 reactions Gibbs energy was calculable, for the remaining 51 reactions ΔG calculation failed due to involvement of KEGG compounds undefined in the eQuilibrator database, compounds having ambiguous structures, or unbalanced reactions.

Even though the reactions of biosynthetic metabolism are interconnected, we can consider each reaction individually with regard to its change in free energy in the biosynthetic direction, because the value for change of free energy for a given enzymatic reaction results from the physicochemical properties of its reactants and products under the specified conditions as in eq. (2). A directed metabolic network representing the 402 reactions was created in simple interaction format (sif). The bipartite graph was drawn with CytoScape (Shannon et al., 2003) v. 3.8.0. Reaction nodes and compound nodes were labeled as indicated in **Figure 1**.

Substitution of Biochemical Reductants With Hydrogen

To investigate the influence of environmental H_2 in the 73 reactions involving biochemical reductants, NAD(P)H, reduced ferredoxin and reduced flavodoxin were replaced with H_2, generating a reduced product and protons in the balanced equation (reaction equations are given in **Supplementary Table 4**), simulating H_2 as a reductant present in an environmental setting. Ferredoxin:NADH oxidoreductase and ferredoxin reducing hydrogenase reactions were excluded from H_2 substitution because H_2 would have appeared on both sides of the reaction. Gibbs energies were calculated as for the altered set. In the substituted set, two additional reactions (353 total) yielded a value for ΔG, 49 did not. The compound concentration ratio was set to nonequilibrium 1:0.01 mM with fixed H_2 reactant and product concentrations 1 μM, 10 μM, 100 μM, 1 mM, 10 mM, and 100 mM (**Figure 3**, **Supplementary Figure 2**, and **Supplementary Table 4**). The influence of ionic strength, I, was probed by altering I from standard 250 mM to 2.5 mM, 25 mM, 2.5 M, and 0 M under a nonequilibrium concentration ratio of 1:0.01 mM and with H_2 fixed to 1 μM (see **Supplementary Figure 3** and **Supplementary Table 5**). For all calculations, reactions are classified as exergonic if $\Delta G \leq 0$.

Metal Catalyzed NAD^+ Reduction With H_2

NAD^+ solution (3 mM) was prepared in a phosphate buffer at pH 8.5. Both glass reaction vials containing 4 ml NAD^+ solution (no catalyst) and vials containing 4 ml NAD^+ solution and nickel (Alfa Aesar) and iron powder (Alfa Aesar) as solid phase catalysts, added as 26 mg Fe plus 28 mg Ni powder per ml solution, were placed in a stainless-steel reactor (Berghof). The vials were closed with PTFE septum lids which were penetrated with syringe needles (Sterican) to ensure the reaction gas could enter the vials. The closed reactor was pressurized with 5 bar of hydrogen gas and heated up to 40°C for a total of 4 h. After depressurizing the reactor, samples were transferred to 2 ml Eppendorf tubes, centrifuged for 15 min at 13,000 rpm (Biofuge fresco, Heraeus) and the supernatant was collected to spectrophotometrically observe NADH synthesis (characteristic maximum absorbance at 339 nm; Cary 3500 UV-Vis, Agilent) (see **Supplementary Figure 4**). For convenience, conversion tables relating H_2 partial pressures and H_2 concentrations in water at different temperatures are given in **Supplementary Table 6**.

Energetics of Amino Acid Synthesis

Energetics of synthesis pathways for the 20 canonical amino acids consisting of KEGG reactions starting from key intermediates pyruvate, oxalacetate, 2-oxoglutarate, phosphoenolpyruvate, 3-phosphoglycerate, and C5 sugars (Martin, 2020; **Supplementary Table 7** and **Figure 4**) were analyzed. The pathways, when expressed as linear sets of reactions, are detached from the biosynthetic core network by the removal of edges. Alternative pathway branches and reactions are indicated by numbers, for example 2.1 corresponds to the first reaction in the second pathway alternative. Gibbs energies for 1 mM reactant and product concentrations, pH 7 and 25°C are given in **Supplementary Table 3** and for vent-like conditions (nonequilibrium 1:0.01 mM, pH 9 and 80°C) in **Supplementary Table 4**.

RESULTS

Thermodynamics in the Metabolism of the Last Universal Common Ancestor

Theories of autotrophic origin posit that the first free living cells grew from CO_2 and inorganic compounds without the help of light (Mereschkowsky, 1910; Fuchs and Stupperich, 1985; Wächtershäuser, 1992; Fuchs, 2011). For such chemolithoautotrophic cells to arise at a specific environmental site, the reactions underpinning their origin, that is, the overall

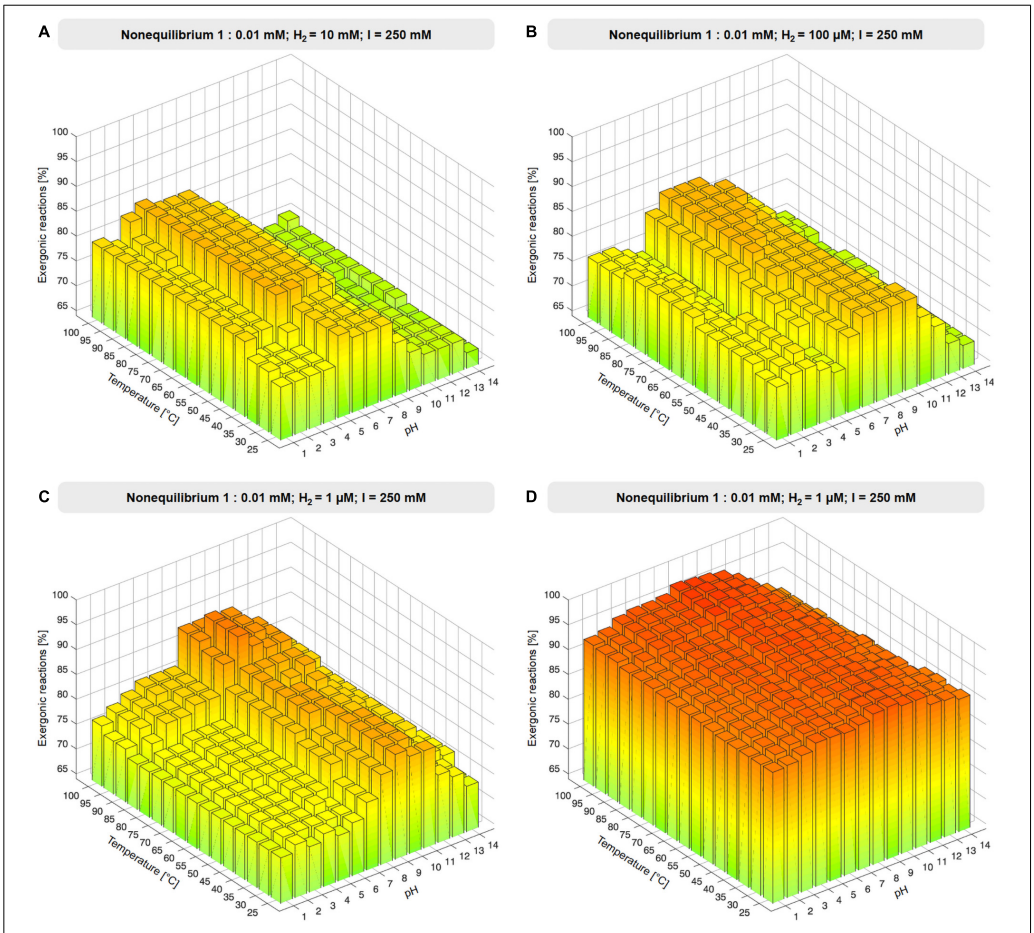

FIGURE 3 | Effect of environmental H_2. **(A–C)** Proportion of exergonic reactions at 1 µM, 100 µM, 10 mM H_2 and 1:0.01 nonequilibrium at constant ionic strength I expressed as % of the 67 redox reactions of the core that yield values of ΔG. **(D)** Proportion of exergonic reactions at 1 µM H_2 and 1:0.01 nonequilibrium expressed as % of 353 reactions for which values of ΔG were obtained (see also **Supplementary Figure 3** and **Supplementary Table 4**).

set of reactions that synthesize the cell needs to be exergonic and no individual reaction should be so endergonic as to block the reaction network under physiological conditions. The source of energy that allows those reactions to go forward is of interest here. The synthesis of the amino acids, nucleotides and cofactors germane to life from H_2, CO_2, NH_3, H_2S, and P_i requires only 402 reactions (Wimmer et al., 2021a; **Supplementary Table 1**) which are listed in KEGG (Kanehisa and Goto, 2000). We polarized those reactions so that carbon flux through each reaction proceeds from H_2 and CO_2 in the direction of monomer synthesis. We then employed the component contribution method (Noor et al., 2013) to estimate the change in Gibbs energy, ΔG, for the 402 reactions in the biosynthetic direction (see section "Materials and Methods").

The set of 402 polarized reactions in KEGG format contained 51 entries that yielded no value of ΔG because one or more reactants are poorly defined or have ambiguous structures, that is, they were not among the underlying data with which eQuilibrator works (see section "Materials and Methods"). The remaining 351 reactions yield thermodynamic estimates, providing a very broad sample for changes in ΔG, covering 87% of reactions in the core (see **Supplementary Table 3**). We started with the simple case of all reactants (compounds on the left side of reactions) and products (right side) at 1 mM concentration, a value well

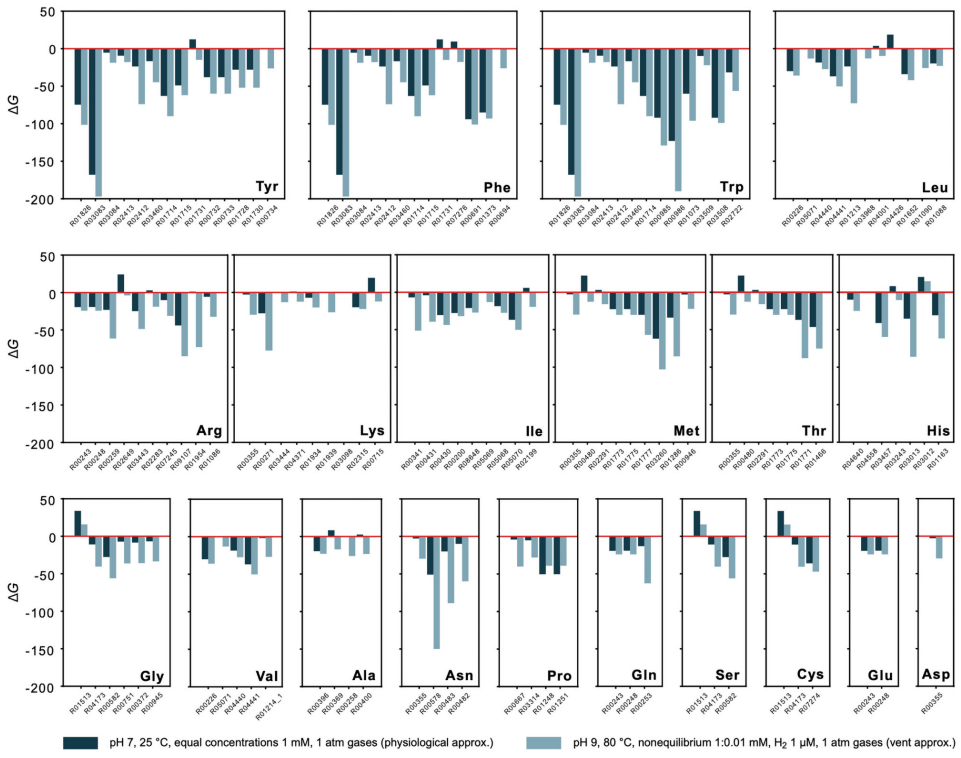

FIGURE 4 | Energetics of amino acid synthesis. Gibbs energies ΔG were calculated for reactions involved in the synthesis of the 20 canonical amino acids. Only reactions yielding a value for ΔG are plotted. Values of ΔG are indicated for physiological (dark blue) and vent conditions (light blue) as defined in the figure. Underlying synthesis pathways are listed in **Supplementary Table 7**. Only pathways starting from key intermediates pyruvate, oxalacetate, 2-oxoglutarate, phosphoenolpyruvate, 3-phosphoglycerate, and C5 sugars (as proposed in Martin, 2020) are examined. Additional synthesis alternatives from KEGG are not taken into consideration.

within the 1 μM to 10 mM range of metabolite concentrations in *Escherichia coli* during exponential growth (Bennett et al., 2009) to examine the effect of pH and temperature regarding metabolic origins under hot (Stetter, 2006) vs. cold (Miyakawa et al., 2002) or acidic (Wächtershäuser, 1988) vs. alkaline (Martin and Russell, 2007) conditions. Roughly 77% of core reactions are exergonic at pH 6-7, with temperature exerting little effect (**Figure 2A**). Note that the component contribution method does not obtain values for ΔG as a function of temperature, and that temperature effects are considered by the reaction quotient (see eq. (1) in section "Materials and Methods").

Nonequilibrium Conditions

Metabolism in cells is a connected series of far from equilibrium reactions in which reactants continuously react to products at every step (Decker et al., 1970; Battley, 1987; Dai and Locasale, 2018), whereby the products of one reaction become the reactants of the next in succession. As it concerns calculations of thermodynamic values, this presents a stark difference to geochemical thermodynamics, where one step reactions are the rule, for example balanced single step reactions for the synthesis of amino acids from H_2, CO_2, and NH_3 (Amend and Shock, 1998, 2001). In the context of metabolic origin, the process to model concerns a situation in which compounds supplied by the environment (H_2, CO_2, and NH_3 for example) react to generate products that do not initially exist (Martin and Russell, 2007), such as formate and pyruvate (Preiner et al., 2020) or amino acids. In a hydrothermal vent context, such compounds can either react further, or be eluted from their site of synthesis via hydrothermal effluent by convection and/or thermal or concentration diffusion. In cells, the products can either react further, or be excreted as an end product, generating steady state equilibrium (German: *Fließgleichgewicht*), or they can be converted to biological polymers—proteins, sugars, nucleic

acids—exiting the metabolic network as cell mass. In acetogens, for example, roughly 24 molecules of CO_2 are converted to acetate as an end product for every atom of carbon that is incorporated into cell mass (Daniel et al., 1990). We designate the situation of higher reactant concentrations relative to product concentrations as nonequilibrium conditions.

When examined using the component contribution method, the effect of nonequilibrium conditions is large. Increasing the product concentration 10-fold relative to reactant concentrations renders most reactions of the core endergonic (**Supplementary Figure 1B**). This is because many reactions in metabolism are close to equilibrium in terms of ΔG, with every 10-fold reduction in product concentration relative to reactant concentration corresponding to a change in ΔG of −5.7 kJ·mol^{-1} at 25°C (Walsh et al., 2018) for reactions having equal stoichiometric coefficients of reactants and products. Increasing product concentrations shows that the reactions of the core have little tendency to run backward (**Supplementary Figure 1B**), which is in line with the concept of autotrophic origins (Fuchs, 2011).

Lowering the concentration of products relative to reactants approximates the situation in an environmental setting in which H_2, CO_2, H_2S, NH_3, and phosphate (**Figure 1**) are continuously supplied in roughly constant amounts, while the products of reactions are allowed to react further or removed by flow processes. To model nonequilibrium conditions, we reduced the product concentrations in steps of 10-fold change relative to reactants (**Figures 2B-D** and **Supplementary Figure 1**). At 100-fold less product than reactant, 98% of core reactions become exergonic (**Figure 2C**), with marginal increase at higher ratios and no marked effect of temperature except at very high pH.

Regardless of the specific environment within which LUCA arose, the reactions fueling the synthesis of its building blocks underwent a transition during the origin of metabolism: Reactions that were initially either uncatalyzed or catalyzed by substances in the environment eventually came to be catalyzed by cofactors and enzymes encoded by genes. During that transition, it is possible, and cannot be excluded, that some or many of the chemical reactions themselves might have changed. But it is also possible, and cannot be excluded, that the reaction set remained essentially the same, as in the example of the acetyl-CoA pathway (Preiner et al., 2020) and reverse citric acid cycle (Muchowska et al., 2020). In that case, only the nature of the catalysts changed from inorganic to organic, adding specificity and rate to preexisting reactions that tend to occur anyway.

Because the core constitutes a minimal set of enzymatic reactions required for the synthesis of amino acids, nucleotides and cofactors, it contains neither a rotor stator ATPase, nor cytochromes, quinones, or even membrane-associated reactions. Although the rotor stator ATPase is as universal in cells as the ribosome itself, and was present in LUCA (Weiss et al., 2016), is not essential for the biosynthetic core to operate. Net ATP synthesis can be derived within the core from substrate level phosphorylation via acetate synthesis from H_2 and CO_2 in soluble reactions, similar to the situation of *Methanosarcina mazei* growing on CO (Rother and Metcalf, 2004). Also note that we are considering each reaction individually, not as a system of interconnected reactions set in series, in which case reactant concentrations would approach zero under nonequilibrium conditions. We are not querying the extent to which the overall balanced one-step reactions from H_2, CO_2, and NH_3 to the individual amino acids, bases and cofactors are energy releasing, which for amino acids and nucleotides is known to be the case under the conditions of H_2-producing hydrothermal vents interfacing with ocean water (Amend and McCollom, 2009). Instead, we are investigating the exergonic nature of the individual reactions in LUCA's biosynthetic pathways, as they are manifest in modern enzymatic reactions, which are intensely interconnected in a metabolic network (**Figure 1**), applying the same concentration gradient to each, so that the individual chemical reactions underlying energy release within the network, as opposed to energy release for the network as a whole as in the energetics of growth (Battley, 1987; Hansen et al., 2009), can be identified.

The finding that 98% of the reactions in the core that deliver a value of ΔG using the component contribution method are exergonic under nonequilibrium conditions starting from H_2 and CO_2, with 100-fold less product than substrate, is noteworthy. It also reminds us that the reactions of metabolism as they operate in modern cells are generally exergonic, otherwise metabolism would not run. Yet even with equal substrate and product concentrations, on average 78% of the reactions in the core are exergonic under the conditions sampled here (**Figure 5**). As an caveat, many enzymatic reactions in the core might not go forward under prebiotic conditions for lack of suitable catalysts, for reasons of inhibitory inorganic compounds, due to substrate sequestration on surfaces, or for other reasons. Favorable thermodynamics are thus a necessary but not sufficient condition for the emergence of metabolism. We also note that our study addresses only monomer synthesis, not polymerization reactions. Notwithstanding, the present findings indicate that there is a natural thermodynamic tendency for the reactions of LUCA's biosynthetic network to unfold from H_2, CO_2, NH_3, H_2O, and P_i. This is not self-evident, because it introduces the possibility that the energy needed at the origin of metabolism simply stemmed from within metabolism itself, as opposed to some external source.

The Effect of Environmental H_2

At the very onset of the process that gave rise to LUCA's metabolism, it is reasonable to assume there were no preformed organic redox cofactors in supply in the environment, as these are products of organic synthesis. Microbiologists have, however, long held that reduced low potential FeS centers such as those in ferredoxin were the source of reducing power in the early stages of biochemical evolution (Eck and Dayhoff, 1966; Hall et al., 1971). In line with that view, all hydrogenases in modern chemolithoautotrophs that use H_2 as a reductant reduce FeS clusters, with only one known exception, the Fe hydrogenase of methanogens that transfers electrons from the active site of the iron-guanylylpyridinol (FeGP) cofactor directly to F_{420}, generating $F_{420}H_2$ without the involvement of FeS or other intermediate electron carriers (Huang et al., 2020).

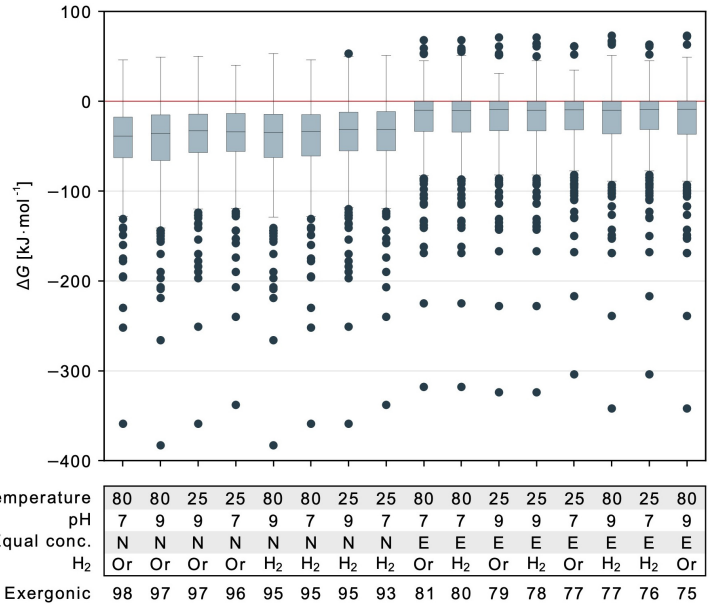

FIGURE 5 | Energetic effect of temperature, pH, reductants and reactant to product concentration ratios. Gibbs energy is indicated for 351 calculable reactions that deliver a value of ΔG for different parameter combinations. Values for each parameter of each calculation are specified in the table below the figure and varied with respect to physiological and vent conditions. Temperature is described in degree Celsius. Equal conc.: *E* indicates all concentrations set to 1 mM, *N* indicates nonequilibrium 1:0.01 mM reactant to product ratio. Retention of organic reductants (NADH, NADPH, flavodoxin$_{red}$, ferredoxin$_{red}$) is indicated as *Or* while the replacement of these organic reductants with hydrogen is marked by H_2. Note that in this case, two additional reactions yield a value for ΔG (353 reactions). Proportions of exergonic reactions ($\Delta G \leq 0$ kJ·mol^{-1}) across the biosynthetic core are listed below the table. In each boxplot, the horizontal line indicates the median ΔG among calculable reactions. The colored boxes represent the interquartile range (IQR) with ΔG within quartile 1 (Q1, median of lower half of the data) and quartile 3 (Q3, median of upper half of the data). The range bars mark the minimum (Q1 – 1.5·IQR) and maximum (Q3 + 1.5·IQR) value of the data excluding any outliers. Outliers are indicated by individual dots and do not fall into the defined range between minimum and maximum.

Hydrogen gas is also the source of electrons for chemolithoautotrophic archaea and bacteria that synthesize ATP by reducing CO_2 (Thauer et al., 1977; Fuchs, 2011; Schuchmann and Müller, 2014; Preiner et al., 2020). In modern geological environments that generate abiotic hydrogen (Schrenk et al., 2013), H_2 is synthesized in amounts that generate midpoint potentials on the order of –700 to –900 mV (Boyd et al., 2020), more than sufficient to substitute for known biochemical reductants such as NAD(P)H or reduced ferredoxins (**Supplementary Table 6**). The very low midpoint potentials come from an interplay of two factors: serpentinization generates H_2 in a geochemical process that also generates metal hydroxides such as $Mg(OH)_2$, which in turn generate alkalinity. Alkaline solutions foster the release of protons from H_2 via heterolytic cleavage, leading to the release of electrons onto suitable acceptors. Some modern microbes that inhabit such H_2-rich alkaline environments even appear to lack known hydrogenase enzymes (Suzuki et al., 2018), suggesting that there might be alternative or bypass entry points for H_2 into their metabolism. To investigate the effect of environmental redox potential on the thermodynamics of the biosynthetic core, we replaced biological reductants by the environmental source of electrons in CO_2-reducing autotrophs, H_2, in all reactions of the core. This captures the thermodynamic effect of an environmental redox buffer, but entails the premise that mineral catalysts naturally occurring in hydrothermal vents (Fontecilla-Camps, 2019) can readily replace hydrogenases and ferredoxin to reduce the main biochemical hydride carrier, $NAD(P)^+$, with H_2. To that end, we tested H_2-dependent $NAD(P)^+$ reduction in the laboratory using simple transition mineral catalysts. The reaction is facile under hydrothermal conditions (**Supplementary Figure 4**).

As an environmental parameter, H_2 reactant and product concentrations must be equal. This impacts redox reactions of the core under nonequilibrium conditions. The core encompasses 73 redox reactions involving NAD(P)H, flavins, or ferredoxin. Reduced cofactors occur on the left in 48 reactions and on the right in 27 (**Table 1**). We replaced biochemical reductants on both sides of the reactions with H_2 at concentrations corresponding to an E_0 of –600 to –800 mV at pH 10 around

TABLE 1 | Most frequent reactants and products in the core.

Compound	Reactant						Product			
	Left	Right	Physiological[a]		Vent[b]		Physiological[a]		Vent[b]	
			$\Delta G \leq 0$	$\Delta G > 0$	$\Delta G \leq 0$	$\Delta G > 0$	$\Delta G \leq 0$	$\Delta G > 0$	$\Delta G \leq 0$	$\Delta G > 0$
H_2O	71	49	51	15	64	2	35	10	43	2
H^+	48	31	33	7	37	3	18	10	26	2
ATP	77	2	55	14	69	0	2	0	2	0
P_i	2	64	0	2	2	0	54	5	59	0
ADP	3	54	3	0	3	0	36	12	48	0
CO_2	12	37	3	8	10	1	34	0	34	0
Glutamate	26	18	14	10	23	1	16	1	17	0
NAD^+	21	16	12	9	20	1	16	0	15	1
$NADP^+$	6	30	5	1	5	1	20	3	22	1
PP_i	0	36	0	0	0	0	30	3	33	0
NADPH	29	6	19	3	21	1	5	1	5	1
NADH	14	19	14	0	13	1	10	9	18	1
2-Oxoglutarate	6	19	4	2	6	0	7	11	17	1
Pyruvate	12	10	9	3	12	0	8	1	9	0
AMP	2	19	1	0	1	0	13	2	15	0
NH_3	13	7	12	1	13	0	6	0	6	0
CoA	4	17	4	0	4	0	8	7	15	0
SAM	16	1	9	0	9	0	1	0	1	0
Acetyl-CoA	13	3	8	4	12	0	3	0	3	0
Glutamine	14	1	13	0	13	0	1	0	1	0

Reactant and product frequency for each compound of the 402 core reactions is given and further classified into participation in exergonic/endergonic reactions for physiological and vent conditions. Number of occurrences on the left or right side of reactions can exceed numbers of reactions for which values of ΔG are obtained.
[a]Physiological condition is defined as pH 7 at 25°C and concentrations at 1 mM.
[b]Vent condition is defined as pH 9 at 80°C and nonequilibrium 1:0.01 mM reactant to product concentrations.

100°C (**Supplementary Table 6**). For the reactant:product ratio of 1:0.01 mM under nonequilibrium conditions, we adjusted the H_2 concentration on both sides of the reaction from 1 μM to 100 mM (**Figure 3**, **Supplementary Figure 2**, and **Supplementary Table 4**). For reactions in which H_2 is a reactant, large H_2 concentrations are favorable, whereas for reactions where H_2 is product, low H_2 concentrations are favorable. *Vice versa*, for reactions where H_2 is reactant and H^+ is product, high pH is favorable.

The effect of H_2 across the core is substantial, with 337–342 out of 353 (95–97%) of core reactions that deliver a value for ΔG being exergonic ($\Delta G \leq 0$ kJ·mol^{-1}) under nonequilibrium conditions with H_2 at 1 μM, 80–100°C, and pH 7–10. Under these conditions, only 12 out of 353 core reactions are endergonic by $\geq$5 kJ·mol^{-1} (**Supplementary Table 8**). It is noteworthy that alkalinity impacts the thermodynamics of metabolic origin because it strongly affects the electron donating potential of H_2 (**Supplementary Table 6**). Modern geochemical systems synthesize formate (Lang et al., 2018) and acetate (Sherwood Lollar et al., 2021) in abiotic reactions that blueprint the CO_2-fixing reactions of microbes.

Are the conditions that we are investigating realistic in a primordial geochemical context? We have investigated the temperature range 25 to 100°C, the pH range 1–14, and H_2 concentrations from 1 μM to 100 mM. Those ranges span conditions existing today at the serpentinizing Lost City hydrothermal field, where the temperature range is 40–90°C, the pH is 9–11, and H_2 concentrations range from 1 to 15 mM (Kelley et al., 2005). Are such conditions primordial? Serpentinizing systems have existed since there was liquid water on earth (Sleep et al., 2011). We observed a tendency for the largest proportion of reactions to be exergonic around pH 9, 80°C and at low H_2 concentrations, very much in line with, but not constrained by, modern conditions at Lost City, which provide a window into conditions on the early Earth (Sleep et al., 2011; Schrenk et al., 2013). We allowed the concentration of CO_2 to vary freely across analyses, having a substrate concentration of 1 mM under nonequilibrium conditions. In natural environments, CO_2 and bicarbonate concentrations vary across extremes. While Lost City itself has very low inorganic carbon and CO_2, Kelley et al. (2002) report CO_2 concentrations in vent fluids from 3 to 215 mM, while modern sea water contains roughly 11 μM CO_2 and 2 mM HCO_3^-, some modern hydrothermal systems emit pure CO_2 gas (Steffens et al., 2021) and other submarine hydrothermal vents emit pure, supercritical CO_2 as bubbles at 1.4 km depth and high pressure (Zhang et al., 2020). On the early earth, global CO_2 levels were generally very high (Zahnle et al., 2007; Sossi et al., 2020), but local CO_2 concentrations might have varied as much as they do in modern environments. In general, submarine hydrothermal systems exist under very high pressure and therefore allow gasses to dissolve up to very high concentrations, today and on the early earth.

In laboratory scale model vents (Preiner et al., 2020), a 10 bar partial pressure of H_2 at pH 9 and 100°C corresponding to 5 mM H_2 in solution (**Supplementary Table 6**) and within the range of 1–15 mM H_2 concentrations observed at Lost City (Kelley et al., 2005), will reduce CO_2 to formate, acetate and pyruvate, although much lower H_2 partial pressures will also suffice for the same reaction (Preiner et al., 2020). That is, geochemical H_2 and CO_2 spontaneously generate central compounds of autotrophic metabolism in the acetyl-CoA pathway in the presence of metal catalysts (Preiner et al., 2020). This is noteworthy not only because of the congruence between the products of the abiotic and enzymatic products from H_2 and CO_2 but also because earlier studies of H_2-dependent CO_2 reduction under higher pressures and temperatures, but performed in inert titanium reactors in the absence of catalysts, did not detect the synthesis of either acetate or pyruvate among the products (McCollom and Seewald, 2003), whereas inclusion of iron or nickel, either as native metal or as oxide or sulfide minerals effectively replace the pathway to pyruvate, yielding physiologically relevant concentrations of pyruvate (∼40 μM) overnight. From the outset of the first abiotic reactions to the origin of an enzymatically catalyzed metabolism in LUCA, redox reactions were integral to metabolic origin, whereby H_2 provided an ample and biochemically accessible supply of electrons throughout that process, particularly under the alkaline conditions of serpentinization (Preiner et al., 2019).

Water

Views concerning the role of water at origins differ widely. One view has it that water is inhibitory at the origin of life because reactions that generate water, in particular polymerization reactions, proceed against the pushback of a 55 M product (Marshall, 2020). The other view is that water is essential to origins because it is both the solvent of all molecules of life and the most common reactant in microbial metabolic networks (do Nascimento Vieira et al., 2020). An underappreciated aspect of hydrothermal systems is that they harbor abundant local microenvironments of low water activity (Lamadrid et al., 2017). The serpentinization process that underpins the formation of H_2 for CO_2 reduction at metabolic origin entails rock-water interactions that consume about 20 molecules of H_2O per molecule of H_2 formed and about 100 molecules of H_2O per molecule of abiotic methane formed from CO_2 (Preiner et al., 2018). In the present calculations, water concentration is fixed at 55 M and cannot be changed in these calculations (Alberty, 1998; Flamholz et al., 2012). Water is furthermore the most common compound in the reactions of the core, appearing in 120 reactions, 97% of which are exergonic regardless of whether water is consumed or produced against the 55 M gradient (**Table 1**). From the thermodynamic perspective H_2O exerts no inhibitory effect upon the reactions of core biosynthesis. That, and the frequency of water as a reactant (**Table 1**) suggest that the reactions that gave rise to LUCA's metabolism arose in an aqueous environment, a premise preferable to the proposition that the chemistry of life began in non-aqueous environments, and only later transformed *en masse* into the aqueous reactions of the cytosol.

Salt

Salt concentrations differ in marine vs. freshwater origin environments, and some origin of life theories posit that life arose in freshwater environments based on arguments relating to concentrations of K^+ (Korolev, 2021) as opposed to arguments based upon reactions of carbon (Preiner et al., 2020). Seawater has an ionic strength of ca. 700 mM, while cytosol has a variable ionic strength on the order of 20–900 mM in *E. coli* (Richey et al., 1987) but exceeding 2,000 mM in some archaea (Ginzburg et al., 1970). Hydrothermal effluent has an ionic strength on the order of 20-800 mM (Kelley et al., 2002). Across the range from 0 to 2.5 M, ionic strength has very little impact on ΔG of core reactions as estimated by implementation of the component contribution method employed here (see **Supplementary Figure 3** and **Supplementary Table 5**).

Nonequilibrium Conditions Have a Pronounced but Not a Dominant Effect

Using eQuilibrator (Noor et al., 2013), water activity cannot be perturbed but is already taken into account in ΔG. The effect of ionic strength was small (**Supplementary Figure 3** and **Supplementary Table 5**). To compare the effects for parameters investigated here that did show effects, we plotted the mean and range of values of ΔG for comparison of temperature (25°C vs. 80°C), pH (7 vs. 9), nonequilibrium vs. equal reactant and product concentrations, and organic reductants vs. H_2. Nonequilibrium conditions have the most pronounced effect across reactions of the core (**Figure 5**). But even for conditions of 1 mM reactant and product concentrations, the mean of the 351 reactions that deliver an estimate of ΔG is still negative. For reactions that are only slightly endergonic, the effect of nonequilibrium conditions can render the value of ΔG negative (**Figure 5**).

Though nonequilibrium conditions have a pronounced effect, they do not fundamentally distort the picture for individual reactions. This is shown in **Figure 4**, where the estimate of ΔG for amino acid synthesis is compared for physiological conditions (with 1 mM reactant and product concentrations, pH 7, 25°C, gasses at 1 atm) vs. conditions more similar to those in serpentinizing hydrothermal systems (nonequilibrium with 1:0.01 mM concentrations, 1 μM of H_2 instead of organic reductants, other gasses at 1 atm) for 111 reactions of amino acid metabolism starting from the key intermediates for the biosynthesis of the families of amino acids: pyruvate, oxalacetate, 2-oxoglutarate, phosphoenolpyruvate, 3 phosphoglycerate and C5 sugars (**Supplementary Table 7**). The main effect is observed for reactions that are close to equilibrium ($\Delta G \approx 0$) to begin with. This indicates that there is a natural tendency for the individual reactions of amino acid metabolism from H_2, CO_2 and NH_3 in the core to go forward both in physiological and vent conditions, a finding that does not follow from calculations of one-step amino acid syntheses from the same reactants (Amend and Shock, 1998; Amend et al., 2013). It is also important because amino acids are essential sources of C and N for the biosynthesis of bases and cofactors (Wimmer et al., 2021a). Note that the reactions in **Figure 4** correspond to KEGG reactions

and are detached from the overall metabolic network, such that the products of an upstream reaction do not necessarily generate all of the reactants required for the subsequent reaction. Despite that caveat, the general exergonic nature of the individual reactions is evident.

Phosphate

Phosphate is a component of many of the metabolic energy currencies. It forms high energy bonds which are cleaved in exergonic reactions that, when enzymatically coupled to endergonic reactions of metabolism, allow the latter to go forward. The entry of phosphate into metabolism is a heavily debated topic. One view has it that high energy phosphorous minerals reacted with inert carbon compounds (Pasek, 2020), another view has it that inert phosphate reacted with highly reactive carbonyl groups (Martin, 2020), yet another view, based on computer simulations, is that simple protometabolic networks might have been possible without phosphate (Goldford et al., 2017, 2019), though subsequent work identified contrary effects (Tian et al., 2019). In the conserved core of microbial metabolism, LUCA's metabolism, phosphate is indispensable. Of 402 core reactions, 260 (65%) involve phosphate or phosphorylated compounds. For comparison, 83% of the core reactions contain nitrogen. Moreover, 80 reactions (20%) involve ATP hydrolysis or phosphoanhydride hydrolysis of other nucleoside triphosphates in the biosynthetic direction. Among those NTP hydrolyzing reactions, 26 generate ADP and P_i, 10 generate AMP and PP_i, while 33 generate phosphorylated products (**Supplementary Table 9**). Furthermore, all of the cofactors that generate amino acids, bases and cofactors themselves, except biotin, contain phosphate. There can be no question that the biosynthetic core as it existed in LUCA had phosphate inextricably hard wired into its fabric.

That phosphate was part of the core and LUCA's metabolism seems difficult to debate, but how did it enter the core? Net ATP synthesis in the core is afforded by substrate level phosphorylation involving acetyl phosphate via acetyl-CoA (Ferry and House, 2006; Martin and Russell, 2007). ATP is synthesized there by energy conserving reactions that, like thioester synthesis (Huber and Wächtershäuser, 1997; Kitadai et al., 2021), can proceed without enzymes (Sousa et al., 2018; Whicher et al., 2018). Under nonequilibrium vent conditions, the reaction of acetyl-CoA with P_i—the reaction of phosphate with carbonyl groups—to yield acetyl phosphate is exergonic by –18.6 kJ·mol^{-1}, the subsequent reaction of acetyl phosphate with ADP to yield ATP and acetate is exergonic by –40.1 kJ·mol^{-1} (**Supplementary Table 8**). The energetics of acetyl-CoA synthesis from H_2, CO_2, and coenzyme A are, however, strongly dependent upon the H_2 partial pressure (Fuchs, 2011). Under nonequilibrium conditions, the reaction is endergonic by +37 kJ·mol^{-1} at 1 μM H_2 and pH 9 but at 1 mM H_2 it becomes exergonic by –44 kJ·mol^{-1}. This crucial CO_2 activating reaction requires H_2 partial pressures corresponding to potentials on the order of –660 mV at metabolic origin, which abound in natural H_2-producing vents (Boyd et al., 2020). At pH 9 and 100°C, –660 mV corresponds to ca. 1 atm H_2 or 10^5 Pa H_2 or 560 μM H_2 (**Supplementary Table 6**), less H_2 than is found in serpentinizing systems, which contain typically 1 mM H_2 or more, with 1–15 mmol H_2 per kg aqueous effluent observed in the case of Lost City (Kelley et al., 2005).

Of the 351 core reactions that deliver a value of ΔG, 80 involve hydrolysis of anhydride bonds in ATP or other triphosphates as an energy currency (**Supplementary Table 9**). None of the reactions in the core utilize pyrophosphate (PP_i) as an energy source, but 36 reactions generate PP_i from nucleoside triphosphates (**Table 1**). In contrast to many traditional views, PP_i was not a source of energy in early metabolism (Wimmer et al., 2021b). If we subtract the contribution of phosphoanhydride hydrolysis from those 80 reactions, 63 become endergonic by more than 20 kJ·mol^{-1} (**Supplementary Table 9**), a very steep energetic barrier, even under nonequilibrium vent conditions. High energy phosphate bonds are thus essential integral components of the core, apparently as old as metabolism itself and likely the result of inert phosphate reacting with carbonyl groups generated as intermediates of CO_2 reduction. The pressing question remains, however: What is the driving force behind ∼75% of the core reactions that are exergonic independent of ATP?

The Dark Energy at Origins Resides in Carbon

Because our starting compounds are H_2, CO_2, NH_3, H_2S, H_2O, and P_i (**Figure 1** and **Supplementary Table 1**), because no other sources of energy are introduced into the system, and because no N–N or O–O bonds are formed in the core, reactions of carbon are the only candidate for the source of free energy change in core reactions without ATP. We identified 10 organic reaction types that together account for half of ATP-independent exergonic reactions (**Table 2**). Among the 351 reactions that deliver values of ΔG, 10 involve S-adenosylmethionine dependent alkyl transfers ($\Delta G'$ = −24 kJ·mol^{-1}; Lewis and Wolfenden, 2018). Six reactions involve folate dependent alkyl transfers ($\Delta G'$ = −30 kJ·mol^{-1}; Thauer et al., 1977) or acyl transfers ($\Delta G'$ = −26 kJ·mol^{-1}; Decker et al., 1970). Acyl thiol ester (thioester) hydrolysis ($\Delta G'$ = −32 kJ·mol^{-1}; Buckel and Eggerer, 1965) drives 14 reactions and acyl phosphate hydrolysis ($\Delta G'$ = −45 kJ·mol^{-1}; Decker et al., 1970) drives four reactions (**Supplementary Table 10**).

The only input compound that is reduced in reactions of the core is CO_2 (**Figure 1**). For the 44 reactions involving reductions of carbon with reduced nicotinamide, flavin, ferredoxin or formate, reactions that are exergonic under physiological conditions (Decker et al., 1970; Thauer et al., 1977), the average ΔG in the core is –28 kJ·mol^{-1} under nonequilibrium 1:0.01 mM conditions at 80°C and pH 9 (**Table 2**). Decarboxylations, with a $\Delta G°'$ on the order of –20 kJ·mol^{-1} (Dimroth and Schink, 1998) occur in 30 reactions, 10 of which are oxidative decarboxylations (**Supplementary Table 10**). In addition, many reactions of the core generate aromatics from non-aromatic substrates. Aromaticity entails very large changes in ΔG, on the order of –60 to –150 kJ·mol^{-1} or more (Morrison and Boyd, 1977). The amino acids, bases and cofactors produced by the core involve the synthesis of 31 aromatic rings and 35 ring closure reactions (Goldberg and Tewari, 1989) that are involved in their formation.

TABLE 2 | Energy release in the core.

Gibbs energy changes for exergonic carbon based reactions in the core			
Reaction	***N***	**ΔG^a**	**References**
Pyruvate formation from H_2+CO_2	1	−57	Preiner et al. (2020)
Ring formations	35	−10 to −25	Goldberg and Tewari (1989)
Decarboxylations	30	−20	Dimroth and Schink (1998)
SAM dependent alkyl transfers	10	−24	Lewis and Wolfenden (2018)
Folate dependent acyl transfers	2	−26	Decker et al. (1970)[b]
Reductions	44	−28	
Folate dependent alkyl transfers	4	−30	Thauer et al. (2008)
Acyl thiol ester hydrolyses	14	−32	Buckel and Eggerer (1965)
Acyl phosphate hydrolyses	4	−45	Decker et al. (1970)
Aromatic formation	31	−60 to −150	Morrison and Boyd (1977)

Estimated ΔG for different reaction types. N is the number of reactions among 351 reactions in the core for which values of ΔG are obtained.
[a] ΔG [kJ·mol^{-1}] as given in references.
*[b] Average ΔG for 44 NAD(P) H-, ferredoxin-, and formate-dependent reductions in the core under conditions specified in **Supplementary Table 10**.*

Including the exergonic synthesis of pyruvate from H_2 and carbon dioxide (Preiner et al., 2020), these sources of carbon-based energy (**Table 2**) contribute to favorable thermodynamics in 50% of core reactions (175/351), more than twice the number of reactions (80/351) driven by ATP hydrolysis, though sometimes with a smaller contribution to ΔG per reaction. The core's remaining 84 exergonic conversions (24%) are driven by other energy releasing reactions of carbon that do not fall into the 10 categories listed in **Table 2**. At the energetic extremes, only 12 reactions in the core (3%) are endergonic by >5 kJ·mol^{-1} under nonequilibrium conditions at 80°C and pH 9 (**Supplementary Table 8**). The most highly exergonic reaction in the core is catalyzed by pyridoxal phosphate synthase, the mechanism of which (Laber et al., 1999) requires no ATP and eliminates 3 H_2O against a 55 M gradient but with a ΔG of −383 kJ·mol^{-1} (**Supplementary Table 8**) because of the reaction product's aromaticity relative to its reactants. In the simplest interpretation, the carbon-based sources of energy shown in **Table 2** are identical to the sources of energy that gave rise to metabolism, which in turn gave rise to LUCA. The overall flow of energy through the core from high energy substrate H_2 plus low energy CO_2 to reactive carbon compounds and its thermodynamically more stable products is schematically summarized in **Supplementary Figure 5**.

CONCLUSION

The individual biochemical reactions underpinning the synthesis of amino acids, nucleotides and cofactors in modern cells trace to LUCA because of their universality. These reactions are exergonic under the conditions of H_2-producing geochemical systems, where formate (Lang et al., 2010), acetate (Sherwood Lollar et al., 2021) and methane (Proskurowski et al., 2008) are synthesized in abiotic reactions today. In the present work, we have not investigated the role of high hydrostatic pressure exerted by the water column in deep water. This is because the tool we employed to estimate values of ΔG through the component contribution method is designed for studies of microbial metabolism at ambient pressures. At higher hydrostatic pressures, as are found in hydrothermal vents (Kelley et al., 2002), a shift in equilibria toward the formation of more products for reactions of the type A + B → C might be expected according to Le Chatelier's principle. However, it is noteworthy that autotrophic microbes isolated from hydrothermal vents at depths of 2.4 km (ca. 240 bar hydrostatic pressure) grow well under ambient pressure (Beatty et al., 2005), such that in the presence of excellent catalysts (enzymes), high pressure might not be a decisive factor whereby in the presence of only mineral catalysts, hydrostatic pressure might play an important role. Indeed, gasses are compressed considerably at 240 bar and dissolve better in water so that reactant concentrations of dissolved gasses are higher than at ambient pressure. On the contrary, liquid water is compressed only very little (<1% at 240 bar) so that microbes without gas inclusions stay essentially untouched. Methanogens that lack cytochromes require only 10^{-4} to 10^{-5} atm of H_2 for growth (Thauer et al., 2008). Like acetogens, their main energy harnessing reaction results in the conversion of about 20 molecules of CO_2 into waste product (methane for methanogens and acetate for acetogens) for every molecule of CO_2 that is incorporated into cell mass (Martin, 2020). That is, cell mass, the product of metabolism, is just a byproduct of the main energy releasing reaction of the cell. The environment where metabolism arose must therefore have harbored a constantly out of equilibrium supply of carbon, electrons, and transition metal catalysts to promote energy releasing reactions. Reactions of H_2 and CO_2 in serpentinizing hydrothermal systems fulfill those criteria (Schrenk et al., 2013) in a manner that directly connects to the metabolism of modern cells (Preiner et al., 2018; Xavier et al., 2020).

The present data uncover a hitherto unique thermodynamic link between core biochemistry as a whole and the conditions of a geochemical environment known to have existed on the early Earth. The reactions of the core require neither membrane proteins, cytochromes, quinones, nor light. Their thermodynamics indicate that the core biosynthetic reactions of microbial metabolism could have arisen from soluble (Martin and Russell, 2007; Muchowska et al., 2019) and surface-catalyzed (Wächtershäuser, 1988; Preiner et al., 2020) reactions in the dark, under hot, aqueous, H_2-bearing geochemical environments, independent of exposed land masses (light) or the existence of water with a low ionic strength. Though ATP provides energy for roughly one fourth of the core's reactions, a three fourth's majority of reactions derive their energy release from reactions of carbon compounds germane to metabolism itself, sources of chemical energy that, with the exception of thioesters (Semenov et al., 2016) and acyl phosphates (Martin and Russell, 2007; Whicher et al., 2018), have escaped the focus of previous investigations into early metabolic evolution. While estimates of ΔG are, of course, silent on reaction rates, activation

energy, and catalysts (Wolfenden, 2011), the crucial energetic role of hydrogen (Thauer et al., 2008; Fuchs, 2011; Amend et al., 2013; Boyd et al., 2020; Preiner et al., 2020) and the exergonic biochemical reactions of carbon reported here uncover a natural thermodynamic tendency for the individual reactions of metabolism to arise from H_2, CO_2, NH_3, and H_2S in the presence of phosphate.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

WM and JW: conceptualization and visualization. JW, WM, FS, KK, and JX: methodology. JW, JX, and WM: data curation. JW, WM, AV, DP, JL, MP, and KK: formal analysis. WM: writing—original draft, supervision, and funding acquisition. WM, JW, JX, AV, DP, JL, FS, KK, and MP: writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.793664/full#supplementary-material

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3.6. Role of geochemical protoenzymes (geozymes) in primordial metabolism: specific abiotic hydride transfer by metals to the biological redox cofactor NAD^+

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Authors: Delfina P. Henriques Pereira, Jana Leethaus, Tugce Beyazay, Andrey do N. Vieira, Karl Kleinermanns, Harun Tüysüz, William F. Martin, and Martina Preiner

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Summary: Alkaline hydrothermal vents provide a constant supply of molecular hydrogen via serpentinization. At some point before the emergence of LUCA, hydride transfers could be performed both inorganically with metallic surfaces and organically with cofactors. The latter are important biomolecules that function as energy transducers in various redox reactions. In this manuscript, we tested the ability of NAD, one of the most important cofactors in biology, to be reduced to NADH under alkaline hydrothermal vent simulations. Our study has shown that under mild temperatures and exposure to hydrogen gas, metals, and water, conversions of up to 100% are achieved in a matter of hours, thus bridging the gap between geology and biology.

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Role of geochemical protoenzymes (geozymes) in primordial metabolism: specific abiotic hydride transfer by metals to the biological redox cofactor NAD^+

Delfina P. Henriques Pereira[1], Jana Leethaus[1], Tugce Beyazay[2], Andrey do Nascimento Vieira[1], Karl Kleinermanns[3], Harun Tüysüz[2], William F. Martin[1] and Martina Preiner[4,5]

1 Institute for Molecular Evolution, Heinrich Heine University, Düsseldorf, Germany
2 Max-Planck-Institut für Kohlenforschung, Mülheim an der Ruhr, Germany
3 Institute for Physical Chemistry, Heinrich Heine University, Düsseldorf, Germany
4 Department of Ocean Systems, Royal Netherlands Institute for Sea Research, Den Burg, The Netherlands
5 Department of Earth Sciences, Utrecht University, The Netherlands

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Correspondence
M. Preiner, Department of Ocean Systems, Royal Netherlands Institute for Sea Research, 1790 AB Den Burg and Department of Earth Sciences, Utrecht University, 3584 CD Utrecht, The Netherlands
Tel: +31 30 253 1409
E-mail: martina.preiner@nioz.nl

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Hydrogen gas, H_2, is generated in serpentinizing hydrothermal systems, where it has supplied electrons and energy for microbial communities since there was liquid water on Earth. In modern metabolism, H_2 is converted by hydrogenases into organically bound hydrides (H^-), for example, the cofactor NADH. It transfers hydrides among molecules, serving as an activated and biologically harnessed form of H_2. In serpentinizing systems, minerals can also bind hydrides and could, in principle, have acted as inorganic hydride donors—possibly as a geochemical protoenzyme, a 'geozyme'— at the origin of metabolism. To test this idea, we investigated the ability of H_2 to reduce NAD^+ in the presence of iron (Fe), cobalt (Co) and nickel (Ni), metals that occur in serpentinizing systems. In the presence of H_2, all three metals specifically reduce NAD^+ to the biologically relevant form, 1,4-NADH, with up to 100% conversion rates within a few hours under alkaline aqueous conditions at 40 °C. Using Henry's law, the partial pressure of H_2 in our reactions corresponds to 3.6 mM, a concentration observed in many modern serpentinizing systems. While the reduction of NAD^+ by Ni is strictly H_2-dependent, experiments in heavy water (^2H_2O) indicate that native Fe can reduce NAD^+ both with and without H_2. The results establish a mechanistic connection between abiotic and biotic hydride donors, indicating that geochemically catalysed, H_2-dependent NAD^+ reduction could have preceded the hydrogenase-dependent reaction in evolution.

Introduction

Hydrogen (H_2) is the main source of electrons for chemoautotrophic, industrial, and geochemical CO_2 fixation [1–3]. There are two main sources of naturally occurring H_2: abiotic geochemical production (serpentinization) and biotic biochemical production via hydrogenases in fermentations. Today, anaerobic autotrophs

Abbreviations
^{1}H-NMR, proton nuclear magnetic resonance, an analytical method to characterise and quantify hydrogen-containing molecules; Co, cobalt; Fd_{ox}/Fd_{red}, oxidised/reduced ferredoxins; Fe, iron; LUCA, the last universal common ancestor, a theoretical cell based on phylogenetic reconstructions of the most conserved genetic setup between bacteria and archaea; NAD^+/NADH, oxidised and reduced form of nicotinamide adenine dinucleotide; Ni, nickel.

such as methanogens grow mainly from H_2 of biotic origin, approximately 150 million tons of H_2 per year are produced by microorganisms and consumed by methanogens [4,5]. Only a small fraction of primary production is attributable to chemolithoautotrophy from H_2 geochemically generated during serpentinization in hydrothermal systems [5]. When the first microbial lineages evolved, however, abiotic H_2 was probably the major source of electrons for primary production in ancient ecosystems and metabolism [5–8].

Making the electrons of H_2 accessible

In the absence of effective catalysts, hydrogen is a surprisingly unreactive gas. It can only become chemically or biochemically useful when it is activated, that is, when its covalent bond is broken and the two H-atoms are separated [9]. The homolytic cleavage of gas-phase H_2 that breaks the H−H bond into two H atoms (H·) is endergonic by +436 $kJ{\cdot}mol^{-1}$ [5,10]. The heterolytic cleavage into a hydride ion (H^-) and a proton (H^+) is less endergonic [5,10] but still requires +200 kJ mol^{-1}.

Metal and mineral surfaces can adsorb H_2 both as a molecule by physisorption and as H-atoms by dissociative chemisorption [11–14]. Physisorption of H_2 usually requires very little energy (3–5 $kJ{\cdot}mol^{-1}$) so it is most easily observed at low temperature in the range of liquid helium [14]. For chemisorption, hydrogen has to overcome the activation barrier and thus higher temperatures are usually required to form the metal-bound hydride, depending on the material. If the kinetic energy of the H_2 molecule is high enough, it can be dissociated directly on the surface of a suitable catalyst. But indirect chemisorption starting from the transient physisorbed state is also possible; physisorbed H_2 molecules can diffuse quickly on catalyst surfaces and then dissociate when the right catalytic site is met [3,14–16].

Microbes solved the problem of H_2 activation and cleavage about 4 billion years ago with the origin of hydrogenases, enzymes that dissociate H_2 into two protons and two electrons. H_2 was the source of electrons for primary production before photosynthesis emerged [8] and hydrogenases were already present in the last universal common ancestor (LUCA) [17]. All hydrogenases known catalyse a reversible reaction such that they can either use H_2 as an electron source or dispose of leftover electrons as H_2. There are three different kinds of hydrogenases, all of them holding transition metals coordinated by varying ligands in their active sites: [NiFe], [FeFe] and [Fe] [4,18–20]. Most of the reaction intermediates in the active site of hydrogenases have been determined [20]. All three perform a heterolytic cleavage of H_2, but do so in mechanistically different ways; the same applies to the reverse reaction, the formation of H_2. The bond is polarised at an open metal site, such that a proton (H^+) is accepted by a nearby base ligand, while the hydride (H^-) remains bound to the metal, transiently altering its oxidation state. In the case of [FeFe] hydrogenases, H^- is bound end-on to one of the two Fe atoms [9,20,21]. In [NiFe] hydrogenases, H^- binds to both Ni and Fe (with help of an extra electron coming from Ni), making the hydride more stable than the one in [FeFe] hydrogenases [9,20,21]. The mechanism of [Fe] hydrogenases involves a direct hydride transfer from H_2 to an organic substrate, in contrast to the other two hydrogenases which need the assistance of FeS centres for this procedure [22].

Hydride carriers in biology

Though the reaction mechanisms of hydrogenases differ, their result is similar: Electrons from H_2 are transferred to soluble electron acceptors such as NAD^+, $F_{420,}$ or iron-sulfur clusters of oxidised ferredoxins (Fd_{ox}) for entry into metabolism [23]. While NAD and F_{420} donate and accept electron pairs as hydrides (two-electron reactions), ferredoxin donates and accepts single electrons (one-electron reactions). In this paper, we focus on NAD and the H^--transfer reaction that reduces its oxidised form, NAD^+, to its reduced form NADH [24,25]. NADH is a universal redox cofactor present in all cells, it is one of the most central molecules of metabolism, being essential in autocatalytic metabolic networks, meaning that without NADH metabolism cannot take place [26].

While hydrogenases make the electrons of H_2 accessible in modern cells [1,5,27], at the emergence of metabolism, before the existence of enzymes, electrons from H_2 that participated in CO_2 reduction and organic synthesis must have been activated by inorganic means such as mineral and metal surfaces [28–31]. Minerals could have served as prebiotic protohydrogenases ('geozymes') producing surface-bound hydrides (e.g. Fe–H) from dissociated H_2 which, in turn, are a prebiotic version of NADH. That this abiotic catalysis works for the reduction of CO_2 was shown previously [32,33]. Here our question was whether geochemical hydrides can reduce NAD^+ to NADH which could have served as a soluble electron carrier at the origin of metabolic pathways. The existence of soluble electron carriers at origins is of interest for early metabolic evolution because catalytically active sites on minerals are immobile, whereas soluble organic hydride carriers such as NADH can transport activated H to mineral surfaces that might, for

example, catalyse organic redox reactions but not H_2 activation *in situ*.

Connecting geochemical with biological hydride carriers

To address possible transition points between abiotic and biotic hydride donation and the question of why NAD^+/NADH became one of the universal hydride donors and acceptors in metabolism, we investigated geochemical conditions under which NAD^+/NADH can (a) accept (and donate) hydrides and (b) remain stable. From earlier studies, it is known that NAD^+ can be reduced heterogeneously on industrial catalysts [34,35] at neutral to slightly alkaline pH, so an approach under more rugged and natural conditions seemed possible. In a prebiotic context, this would entail a constant supply of environmental H_2 and accessible mineral surfaces. Serpentinizing systems harbour geochemical sites that reduce H_2O to H_2 via the oxidation of Fe(II)-containing minerals and thus provide these conditions [36,37]. Most but not all of these systems are alkaline because of the accumulation of metal hydroxides during serpentinization reactions [36,38]. Some microbes inhabiting such environments appear to lack hydrogenase enzymes [39], suggesting that even today, there might be alternative (abiotic?) and probably metal-based entry points for H_2 into metabolism.

To reduce NAD^+ with H_2, we investigated three different metals as potential H_2 activating agents: Fe, Co and Ni. We started with native metals to keep the reactions as simple as possible. Both Fe and Ni are employed in the active centres of hydrogenases, but are also found in serpentinizing systems in oxidised and reduced forms [40,41]. We included Co because it is a crucial transition metal in autotrophic CO_2 fixation [1] and it is assumed to have been abundant in the Archean anoxic ocean [42]. In addition, Co complexes are being tested for its properties as artificial hydrogenases [43]. The hydrides of all three metals are known to have approximately the same hydricity (tendency to transfer hydrides) [44] as NADH and are ultimately direct neighbours in the periodic system of elements, so this choice of metals would also allow us to set their experimental behaviour in relation to their electrochemical potential.

Results

NAD^+ reduction with H_2 and transition metals under alkaline conditions

We used conditions approximating those found in alkaline serpentinizing systems (starting pH 8.5, 5 bar H_2 atmosphere, 40 °C), with metals Fe, Ni and Co [40,45] in powdered form, (1 M). The resulting aqueous H_2 concentration of 3.6 mM (calculated using Henry's law, s. Equation S1) is comparable to that found in hydrothermal effluent [38,40]. Under these conditions, the reduction of 3 mM NAD^+ to NADH was facile (Fig. 1A,B). Fe, Ni and Co also play important catalytic roles in autotrophic metabolism [46–48]. That all three metals promote the reaction between NAD^+ and H_2 to NADH was observed with ^{1}H-NMR, enabling us to make a detailed structural determination of our products as various prospective hydride acceptor sites exist on the NAD^+ molecule and also degradation products such as nicotine amide are possible [49,50]. Our results show that indeed the biological form of NADH, 1,4-NADH, was the main product. We also observe some degradation products (mainly nicotinamide), but in low concentrations under the reaction conditions tested.

In order to probe the nature of H_2–metal interactions in these reactions, we performed four different experiments with each metal (for visual overview s. Fig. S1). In two reactions, we first pretreated the metal powders in a dry state with H_2 gas for 16 h at 50 °C before adding a buffer/NAD^+ solution. Chemisorption and dissociation of H_2 is feasible at these temperatures [51–53]. This approximates the situation in serpentinizing systems, where H_2 is being produced continuously, such that the minerals could be constantly hydrogenated (i.e. organic redox cofactors and not hydrogenated minerals would be rate-limiting in the geochemical reaction). In addition, and in accordance with previous industrial investigations of heterogeneous NAD^+ reduction [35], a thermal (> 350 °C) pretreatment of the catalyst with H_2 increased the yield of reduced NADH due to more or less saturating hydrogenation of the catalyst. Pretreated and non-pretreated metal powders were mixed with the phosphate buffer/NAD^+ solution and reactions from 0.5 to 4 h were performed either under 5 bar of Ar or H_2 at 40 °C. In the Ar experiments, NAD^+ reduction can be attributed to the activity of preformed hydrides on the metal surface. Controls without metals were conducted in each run, showing that metals are needed for the reduction of NAD^+. All experiments were repeated at least three times. All the corresponding spectra can be found in Fig. S2.

The results summarised in Fig. 2 show how each metal responds to the different experiments over a 4-h course. The corresponding table with all individual measurements and standard deviation values can be found in the supplementary information (Table S1). Pretreating the metals with H_2 has a positive effect on

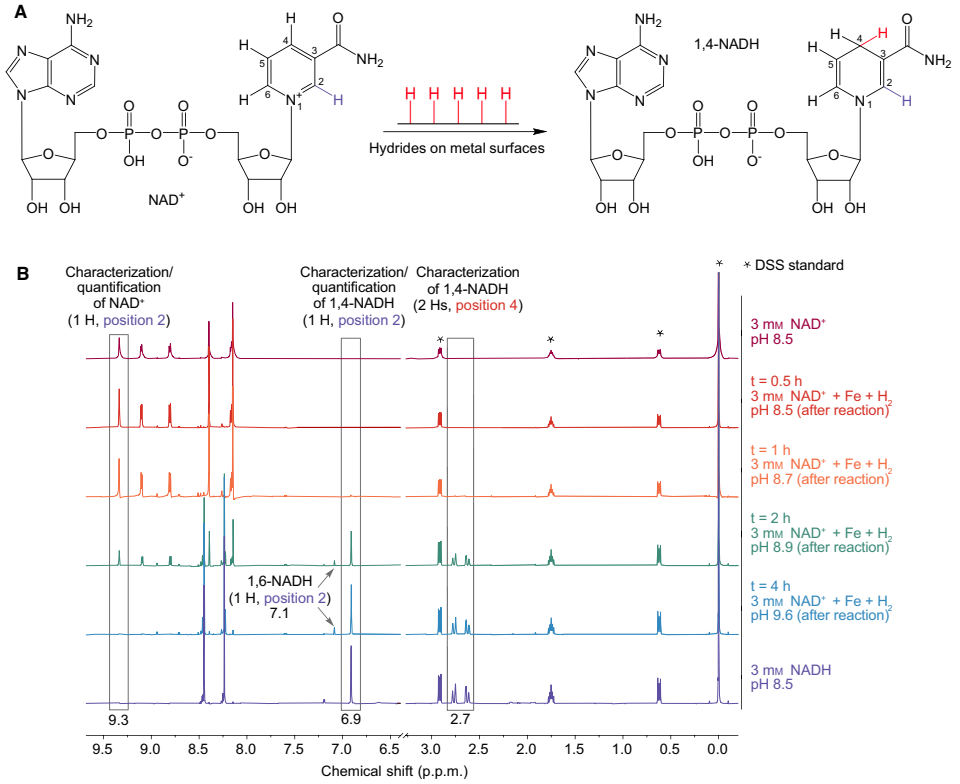

Fig. 1. Reduction of NAD^+ with H_2 and Fe powder over time. (A) NAD^+ is reduced to 1,4-NADH, the naturally occurring reduced form of nicotinamide adenine dinucleotide (NAD). This means a hydride is added to the carbon in position 4 of the aromatic cycle of NAD^+. NAD^+ can also be reduced at two further positions, the second and the sixth, thus leading to 1,2-NADH and 1,6-NADH respectively [84]. (B) Within 4 h, NAD^+ is reduced to 1,4-NADH as monitored via ^{1}H-NMR. A pH shift from 8.5 to 9.6 is observed, probably due to the oxidation of Fe powder coupled to the reduction of H_2O to hydrides/H_2 (accumulation of OH^-). The peaks of the used NMR standard sodium trimethylsilylpropanesulfonate (DSS) are marked with asterisks.

the NAD reducing activity of all three metals: Fe reaches high NADH yields quickly (Fig. 2A), as does Co (Fig. 2B), but the clearest effect can be seen with Ni, where only with H_2-pretreatment NADH yields approaching 50% can be reached. With both Fe and Co nearly 100% conversion from NAD^+ to NADH is observed, but with one important difference: while Co clearly needs H_2 gas during the reaction to reach a high yield of NADH, Fe shows almost identical results under Ar as it does under H_2. Under Ar, Co can still convert almost 50% of NAD^+ to NADH. The amount of metal used in comparison to NAD^+ is very high (1 M of metal to 3 mM NAD^+, so 333 : 1), but experiments with a ratio of 20 : 1 (62.5 mM to 3 mM NAD^+) and 10 : 1 (62.5 mM to 6 mM NAD^+) (Fig. S3) showed that all three metals still yield NADH at the lower ratios. While Fe yields about 40% NADH at 10 : 1, Ni only reaches 0.6% at the same metal to NAD^+ ratio.

We also observed a significant pH increase (s. Fig. S4) during the Fe and Co reactions and the development of coloured colloids (Fe: dark green, Co: pink; s. Fig. S5), especially when there was no H_2 gas added to the reaction or when not pretreated with H_2, while the pH remained stable and the solution colloid free (though coloured yellow) during the Ni reactions. The

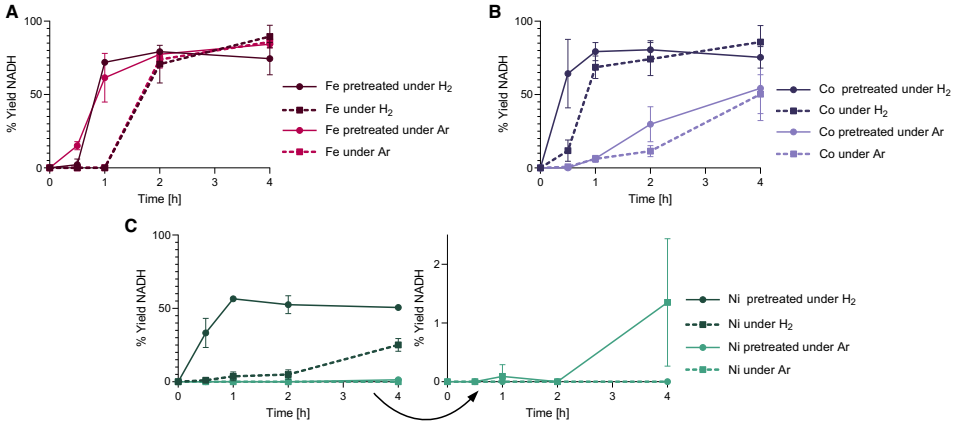

Fig. 2. NADH synthesis with Fe, Co and Ni under four different settings. In the experiments, the three metal powders were either pretreated with H_2 gas overnight at 50 °C before the reactions under 5 bar H_2 or Ar between 0.5 and 4 h (solid lines and circles), or the reactions took place without the pretreatment (dashed lines and squares). Data points shown are mean ± SD. (A) Fe does not need an external H_2 source under the given conditions, NADH synthesis is equally efficient in both cases. Pretreated Fe reduces NAD^+ faster but does not lead to a higher yield overall. Repetitions from 0.5 h to 4 h: n(pretreated, H_2) = 3, 3, 4, 3; n(H_2) = 3, 4, 4, 6; n(pretreated, Ar) = 2, 3, 2, 3; n(Ar) = 1, 4, 3, 3. (B) Co can reduce up to roughly 50% of NAD^+ without an external H_2 source, but the presence of H_2 gas improved the yield and accelerated the conversion immensely. Pretreatment with H_2 also decreased the reaction time of the conversion visibly as long as there is an H_2 source during the reaction. Repetitions from 0.5 h to 4 h: n(pretreated, H_2) = 4, 4, 4, 4; n(H_2) = 4, 4, 4, 5; n(pretreated, Ar) = 2, 5, 4, 6; n(Ar) = 4, 4, 4, 4. (C) Ni powder cannot reduce NAD^+ under the absence of H_2 as an electron source. Pretreated with H_2, Ni shows a 50% yield of NADH. Pretreated Ni can convert a very small amount of NAD^+ to NADH under Ar, suggesting that hydrides are covering the surface of the metals after the pretreatment. Repetitions from 0.5 h to 4 h: n(pretreated, H_2) = 4, 3, 3, 4; n(H_2) = 4, 4, 4, 6; n(pretreated, Ar) = 2, 5, 4, 6; n(Ar) = 0, 4, 4, 4.

pH shift observed was also the reason we chose to work with a 1 M phosphate buffer. With buffers of lower concentrations, the pH of the Fe and Co samples shifted far into the alkaline range (> pH 10 and > pH 9 respectively), conditions under which NAD^+ is unstable and decomposes [54] (s. Figs. S6,S7A). NADH, however, is far more stable under alkaline conditions, as we were able to determine in additional experiments, the results of which are summarised in Fig. S7B. The phosphate buffer can, however, lead to a degradation of NADH [55], which can explain minor losses we observed in NAD^+ conversion (Table S1).

These observations led us to the conclusion that while Ni clearly catalysed the dissociation of H_2 gas and is thus making electrons from H_2 accessible to NAD^+, both Fe and Co themselves can serve as the electron source instead of H_2 gas and are being oxidised (directly connected to the developing colouration), especially during the experiments without external H_2 source (under Ar). We undertook additional experiments to further investigate the role of Fe and Co as reductants.

Probing the role of metals with ^2H: H_2 activation, H_2 synthesis, both or something else?

The observed pH shifts and colouration were a hint for following redox reactions taking place (the hydroxides generated are partly soluble [56] and lead to an pH increase):

$$Fe + 2\ H_2O \rightarrow Fe(OH)_2\ (\text{green rust}) + H_2 \quad (1)$$

$$Co + 2\ H_2O \rightarrow Co(OH)_2\ (\text{pink}) + H_2 \quad (2)$$

Fe and Co powder are able to produce nascent H_2: freshly synthesised H_2 from two hydrogen atoms. The intermediates of this reaction are surface-bound hydrides [57] which could, in theory, be directly transferred to NAD^+. It is also known that Fe readily produces H_2 from water under mild alkaline conditions [58]. To clarify the present mechanisms, Fourier-transform infrared spectroscopy (FTIR) was performed on all three metals (Fe, Co and Ni) before and after the reaction under Ar (expecting the highest

oxidation rate in these experiments in contrast to the ones under H_2 gas and hence more reducing conditions). The respective FTIR spectra (s. Fig. S8) show the differences between the metals very clearly, in line with our other results. Ni does not show any visible changes, Co does, but not clearly enough to specify the nature of the change. The spectrum of Fe after the reaction shows a new Fe-O bond (in the region of 500–600 cm^-) as well as stretching and bending bands of -OH groups located around 3200 and 950 cm^- respectively, which indicates transformation of metallic iron into iron hydroxides such as $Fe(OH)_2$, confirmed by the dark green colouration of the Fe samples ('green rust').

To further probe the mechanisms of NAD^+ reduction, we replaced H_2O in the solvent with heavy water (^2H_2O, D_2O) to determine the source of the hydrides added to NAD^+. There were two reasonable options: either protons from H_2O and metal electrons (formed to surface-bound hydrides) or surface-bound hydrides from H_2 gas. Previous work on reducing NAD^+ and ^2H sources [59,60] provided us with mechanistic details and reference ^1H-NMR spectra for NAD^2H species, the presence of which can be monitored via the duplet of duplet at 2.7 p.p.m. (position 4 as shown in Fig. 1). In our case, we would expect the formation of NAD^2H instead of NADH if the hydride comes from $^2H^+$ in ^2H_2O plus electrons from metal, while NADH should be formed if the H_2 gas is the prevalent hydride source. Three 2-hour NAD^+ reduction experiments with Fe, Co and Ni were performed in ^2H_2O (otherwise using the same conditions as described above): metals under Argon, H_2-pretreated metals under Argon and metals under H_2. The results are shown in Fig. 3 with a focus on the 2.7 p.p.m. peaks described above (s. Fig. S2 to see the full range of the shown spectra). When no (under Argon Fig. 3A) or only little H_2 (pretreated and reaction under Argon, Fig. S9 and

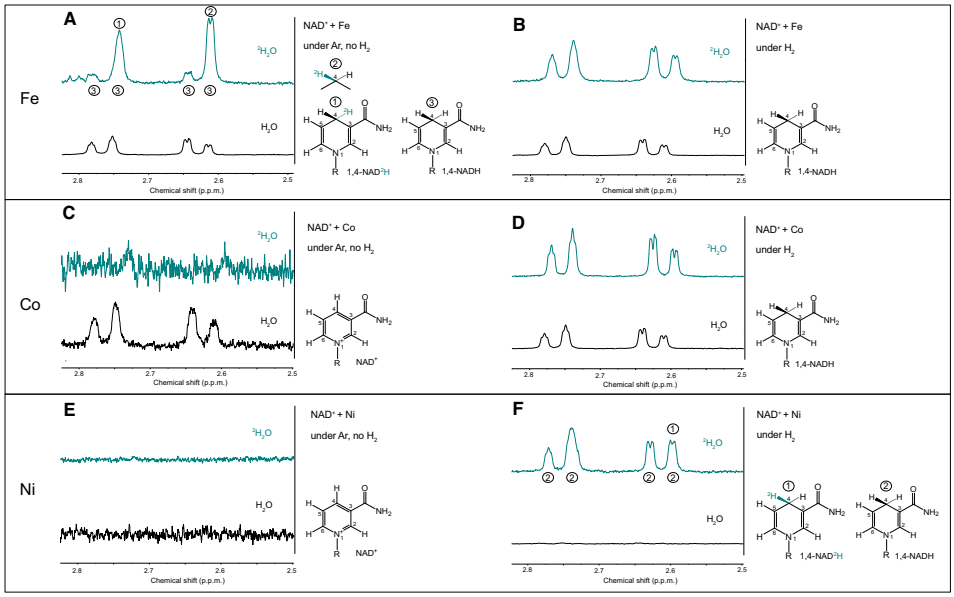

Fig. 3. Two-hour experiments in ^2H_2O to determine the source of the H^- reducing NAD^+. (A) When there is no external H_2 source, Fe delivers the electrons to form $^2H^-$ from ^2H_2O which is transferred to NAD^+. The Deuterium (^2H) at position 4 in the nicotinamide ring of NADH changes the proton coupling visibly. (B) The mechanism for NADH formation apparently changes when H_2 is added to the reaction. NADH becomes the main product, suggesting that H_2 is catalytically activated by Fe. (C) Co does not produce NAD^2H (or NADH) over the detection limit under Ar gas in ^2H_2O. (D) When H_2 is added to the reaction, Co promotes NADH formation. (E) Ni does not produce NAD^2H (or NADH) over the detection limit under Ar gas in ^2H_2O (or ^1H_2O). (F) In contrast to the ^1H_2O experiments, Ni highly promotes NADH formation in ^2H_2O under H_2 after 2 h. Note that (C) and (E) are more vertically zoomed in than the rest of the panels as the product concentration was significantly lower.

Table S1) is in the experimental system, NAD^2H is the prevalent product of the reactions with Fe. This indicates that Fe reduces ^2H_2O to $^2H^-$ which then is transferred to NAD^+. When the reaction is performed under $^1H_2$ atmosphere (Fig. 3B), however, the product does not contain any measurable amount of ^2H, suggesting that $^1H_2$ is the hydride source in this case. We confirmed these observations additionally by integrating the duplet of duplet at 2.7 p.p.m. in both the Ar and the H_2 experiments (the peak integrals correspond to the number of associated ^1H; ^2H is not detected). The integral values were compared to those of the singlet at 6.9 (1 ^1H-proton), ultimately showing that in ^2H_2O and under Ar only one ^1H-proton is detected at position 4 of NADH (Table S2). In combination with the other findings above, the reaction path involving Fe-dependent reduction of water (or ^2H_2O) to metal-bound hydride (or $^2H^-$) appears reasonable. Fe seems to employ both described mechanisms, depending on the conditions. The positive effect of Fe pretreatment with H_2 is not explained by the present results. However, it is worth to mention that the overall yield of NADH with Fe under Ar is substantially lower and variable in ^2H_2O (0–50%, Table S1) than it is in ^1H_2O (60–70%), while pretreatment increases the yield of NAD^2H in the ^2H_2O experiments (~ 80%, Table S1). We still registered a pH shift and slight green colouration suggesting the formation of $Fe(OH)_2$ (Figs. S4, S5). We will return to this observation shortly.

Cobalt does not deliver a picture as clear as that obtained for Fe. H_2 was necessary during the Co catalysed reaction to detect NADH formation in the ^2H_2O experiments. Co reduces NAD^+ to NADH under Ar, without H_2 and with ^1H_2O as proton source (Fig. 3C, lower trace), although with a far lower yield than Fe (Fig. 2B or Table S1). In the ^2H_2O experiments however, H_2 was necessary for NADH formation. We could not observe any NAD^2H formation after 2 h (Fig. 3D). Performing the same experiment for 4 h yielded detectable amounts of NADH, but not enough to investigate the peak at 2.7 p.p.m. (Table S1) and thus the status of deuteration.

In the case of Ni, the experiments under Ar confirm the conclusion from the previous experiments (no NADH formation). Nevertheless, a surprising effect of ^2H_2O was observed: while only very little NADH formation was observed after 2 h in H_2O under H_2, almost all NAD^+ is converted to NADH in ^2H_2O (Fig. 3F) and there seems to be a very small amount of NAD^2H formed. We have no thorough explanation for this puzzling observation, but it is possible that H_2O absorbing on the Ni surface occupies sites needed for H_2 dissociation—while ^2H_2O does not as easily [61–63]. The formation of NAD^2H is probably the result of an exchange between H^+ and $^2H^+$ as the peak distribution is by far not as clear as in the case of Fe (Fig. 3A).

Overall, we were able to derive some valuable conclusions from the experiments performed under ^2H_2O, but there are also some observations (e.g. yield loss with Fe and Co) that we cannot properly explain so far and are most likely the result of isotopic effects.

Being aware that metal ions are going into solution during our experiments, we also tried to exclude these dissolved species as being the true catalysts in our reactions. Therefore, we performed a 'hot filtration test' for all metals, that is, we separated the solid from the liquid phase after a 1 h reaction under H_2 (so we could test whether hydride-bearing, soluble complexes are being formed), added a new NAD^+/buffer solution onto the solid phase and resumed the reaction under 5 bar for both parts for another 1 h and compared the NADH yields (Fig. S10). The separated liquid phase did not show any additional NADH formation for any of the metals, while the separated solid phase was still able to promote a very high $NAD^+ \rightarrow$ NADH transformation yield (95% for Fe, 81% for Co, 35% for Ni after 1h of reaction time). This indicates that the solid metals are the (far) more crucial species for NAD^+ reduction in our experiments.

In addition, we also observed that the reaction with fresh NAD^+ and 'used' metal powders (powders that had already been used in a previous H_2 reaction) yielded more NADH in 1 h than pristine metal powder would in that time (Fig. S11). The same experimental order, but with the first step under Ar instead of H_2 lead to similar results. This could mean one of several things. On one hand, it is possible that the oxidised forms of the metals (Equations 1 and 2) are better catalysts for the hydrogenation reaction than the native metal forms. We performed additional experiments (16 h pretreatment with H_2, 4 h reaction time under H_2) with magnetite (Fe_3O_4), cobalt(II,III)oxide (Co_3O_4) and nickel (II)oxide (NiO) using the same amount of metal atoms per mol to test their ability to promote NAD^+ reduction. In this case, however, no oxide yielded any NADH (Fig. S12). On the other hand, it is also possible that the 1 h reaction served as a pretreatment step (e.g. hydride formation on metal surfaces from either H_2 gas or oxidation of the metals), resulting in the observed higher yield of NADH after 1 h of reaction.

Discussion

We tested Fe, Co and Ni for their ability to reduce NAD^+ with H_2 gas to establish a connection between

a biotic H^- donor (NADH) and an abiotic donor (metal hydride). The reduction was performed by all three metals, which however showed differences in their reactivity and response to H_2-pretreatment. The detailed effects of H_2-pretreatment on each of the metals under the present conditions will be investigated further. In this publication, we were able to collect some insights concerning the possible mechanisms in the performed experiments.

Mechanisms: where do the hydrides come from?

Of all three metals, Ni produced the lowest (but still substantial) NADH yields with around 50% after H_2 pretreatment (Fig. 2C), but it employs the most straightforward hydrogenation mechanism, additionally backed up by the results of the experiments with heavy water. Known to enable the dissociation of H_2 [15,64–67], Ni was able to reduce NAD^+ under H_2 gas and showed the strongest positive response to pretreatment with H_2 gas out of the three metals. This suggests that Ni-bound hydrides are transferred to NAD^+ to produce mainly 1,4-NADH (s. Fig. 4A). The redox potentials calculated for the experimental conditions (40 °C, 5 bar H_2, pH 8.5) back up the observations (Equation S2) [68,69]: the potential of $Ni(OH)_2 + 2\ e^- + 2\ H^+ \leftrightarrow Ni^0 + 2\ H_2O$ ($E = -390$ mV) is not reducing enough to enable $2\ H_2O + 2\ e^- \leftrightarrow H_2 + 2\ OH^-$ ($E = -510$ mV).

Iron also dissociates H_2 to metal-bound hydrides, but can also produce fresh H_2 from water via metal-bound hydrides under mild hydrothermal conditions [57,58,70]. This is consistent with the midpoint potential calculated for the experimental conditions: $Fe(OH)_2 + 2\ e^- + 2\ H^+ \leftrightarrow Fe^0 + 2\ H_2O$ ($E = -550$ mV) [68], which is more negative than the redox potential for H_2 formation from H_2O ($E = -510$ mV). In our experiments, 100% NAD^+ reduction was reached under all conditions after 4 h with Fe (Fig. 2A). The Fe reactions also benefit from pretreatment with H_2 gas, reaching high NADH yields in shorter times. The mechanistic effect of pretreatment, however, could not be determined. Through further experiments in heavy water (^2H_2O) we were able to confirm that the reaction depends on the abundance of $^1H_2$ gas: if the reactions take place under a 5 bar $^1H_2$ atmosphere, surface dissociation of $^1H_2$ seems to be the main pathway, while under Ar atmosphere freshly produced hydrides are apparently transferred from the metal surface (Fig. 4B). Fe is still visibly oxidised (formation of green rust $Fe(OH)_2$; Fig. S5) under the reducing H_2 atmosphere, but much less so than under Ar. We cannot exclude at this point that these oxidised forms of iron catalytically enhance the reaction with H_2 gas, but experiments we conducted with metal oxides as a proxy suggest they cannot promote NAD^+ reduction by themselves (Fig. S12).

Following our preliminary report of metal-dependent NAD^+ reduction with H_2 [71], it was recently reported [72] that iron sulfides can reduce small amounts of NAD^+, but without an external

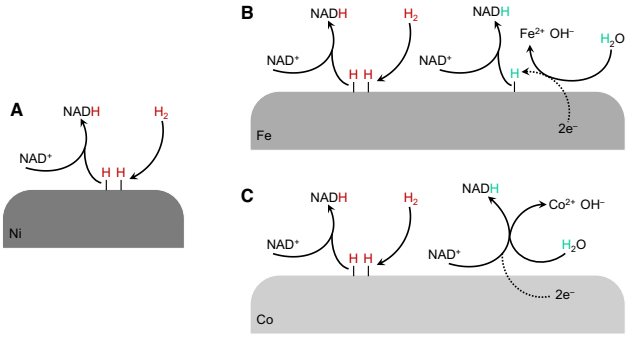

Fig. 4. Proposed mechanisms for NAD^+ reduction depending on the used metal. (A) H_2 dissociates on the Ni surface. The metal-bound hydrides can then directly reduce NAD^+ to NADH. (B) Fe employs two different mechanisms, depending on the availability of H_2 gas in the atmosphere. Without H_2, Fe itself delivers the electrons for hydride formation on its surface, the necessary proton comes from H_2O. With H_2 around, Fe is able to assist H_2 dissociation like Ni. (C) Co also is able to activate H_2 to transfer the hydride to NAD^+. But without H_2 gas in the atmosphere, Co seems to employ a different mechanism than iron. Judging from the electrochemical potentials, it is able to reduce NAD^+ directly without hydride formation. Note that the absorption of water molecules and/or hydroxides on the metal surfaces are not considered in these depictions.

electron source such as H_2, so the combination of both oxidised iron compounds such as iron sulfides, iron hydroxides or iron oxides and H_2 gas as electron donor has to be investigated further. In the conditions of serpentinizing hydrothermal vents, H_2 is abundant and ubiquitous [38,73], hence it is not clear what kinds of prebiotic environments such reactions in the absence [72] of H_2 would be modelling. In the presence of H_2 under the conditions reported here, NAD^+ reduction is complete, rapid, and facile. Our experiments were performed under an excess of metal (1 M of metal to 3 mM NAD^+, so 333 : 1), but lowering the amount of metal in relation to NAD^+ (20 : 1 and 10 : 1, Fig. S3) did not automatically decrease the NADH yield by the same magnitude—at least for Fe and Co. This either means that the amount of metal does not directly influence the yield, underlining the catalytic character of the metals or that the NAD^+ concentration is the limiting factor in our 333 : 1 experiments. Nickel is not spent during the reaction and depends on the abundance of H_2 to reduce NAD^+ which strongly suggests that Ni is acting as a catalyst —an inorganic precursor of an enzyme, a geozyme— for the biochemical reaction. This made the drop of NADH yield between the 333 : 1 (50%) and 20 : 1 (1%) experiments surprising and suggests that the area of the hydrogenated Ni surface has to be quite large to efficiently reduce NAD^+. As we also observed that Ni yields substantially more NADH under H_2 gas when the reactions take place in ^2H_2O instead of H_2O (Fig. 3F), we posit that water molecules quickly cover Ni's surface and thus block the catalytic sites needed for H_2 dissociation. This also could explain why Ni is performing better when pretreated with H_2 before adding the aqueous NAD^+/buffer: here, metal-bound hydrides can form before the binding sites are blocked by water molecules. Once NADH is being formed, these sites can be taken by water molecules again, which would explain why experiments with Ni do not exceed a 50% yield of NADH. Under geochemical conditions with lower water activity (also found in serpentinizing systems [74,75]), this process might be less of an issue than in the aqueous solutions investigated in our experiments.

Judging from our time–course experiments, we assumed that Co is also able to both dissociate H_2 and produce H_2 *in situ*, as pretreated Co gave the highest yield of 1,4-NADH in the shortest amount of time (Fig. 2B). Co is visibly far more dependent upon the presence of H_2 gas to attain higher NADH yields than Fe. But there is a third mechanism to be considered here, looking at the following midpoint potentials (calculated for the given experimental conditions) [68,69,76]: although $Co(OH)_2 + 2\ e^- + 2\ H^+ \leftrightarrow Co^0 + 2\ H_2O$ (E = –407 mV) cannot directly enable $2\ H_2O + 2\ e^- \leftrightarrow H_2 + 2\ OH^-$ (E = –510 mV), it would be able to reduce $NAD^+ + 2\ e^- + H^+ \leftrightarrow$ NADH (E = –390 mV) directly. This might explain why we were not able to demonstrate both mechanisms for Co with the heavy water experiments as we could with Fe. Without H_2, Co did not show any reduction during the 2 h ^2H_2O experiments. The data thus indicate that Co is able to dissociate H_2 [77] and transfer electrons to NAD^+ (either in hydride form or directly), but the results concerning the possibility of a nascent H_2 pathway for Co are not conclusive (Figs. 3C,4C). In case Co is indeed directly transferring electrons to NAD^+, there might be a kinetic inhibition in the ^2H_2O experiments due to slower proton transfer [78].

In a geochemical context, it should be mentioned that in serpentinizing systems we can expect both abiotic hydride donor mechanisms to take place if the electron acceptor will permit: H_2 is constantly produced from H_2O on mineral surfaces (bound hydride intermediate) and dissolved H_2 gas (as a source for freshly chemisorbed hydride) is present in the hydrothermal effluent.

By separating the liquid and the solid phase mid-experiment we were trying to exclude homogeneous catalytic mechanisms (catalysis by metal ions in solution), which are known for hydrogenation reactions [9]. Our results indicate that reactions of the divalent metals do not play a major role in the present reactions. For Ni, this is in line with the role of Ni^0 in the reaction mechanism of CODH proposed by Ragsdale (2009) [46], although in that reaction, electrons stem from ferredoxin rather than directly from H_2. We explicitly are not excluding colloidal hydroxides as potential catalysts for Fe and Co as we were not able to reliably separate them from the solid phase without changing the rest of the solid phase. However, experiments with Fe_3O_4, Co_3O_4 and NiO under H_2 atmosphere as a proxy for oxidised phases show that they do not promote NADH synthesis at all (Fig. S12).

NAD stability under alkaline conditions and the possibility for reversible reactions

In order to have been useful at life's emergence, NAD had to serve as both hydride donor and hydride acceptor. That means the oxidation/reduction reaction has to be able to proceed in both directions under the given environmental conditions. Very small pH fluctuations could bear upon this issue. From the present data (Fig. S7) we observe that NADH is more stable under (very) alkaline conditions than NAD^+ which will degrade within a few hours under alkaline

conditions [54]. Also in our reduction experiments (at pH 8.5, usually increasing during the reaction), we observe a loss of NAD^+ in reactions in which it is not quickly converted to substantial amounts of NADH (Table S1). Under slightly acidic conditions, however (pH < 7), NADH will be oxidised quite quickly. Thus, in an environment in which pH can slightly vacillate around neutral pH, both reduction and oxidation of NAD are possible. Mineral catalysts could probably direct the reversibility at even smaller pH ranges. In serpentinizing systems, local pH microenvironments have an influence on serpentinization rates [79], which might mean serpentinization rates could also have an influence on pH. Depending on the exact mineral composition of a serpentinizing system, pH depends on the buffering characteristics of mineral compounds, such that pH is not a static parameter. Under specific rock compositions, there are even a few acidic serpentizing systems on modern Earth [38], although the chemical reactions of serpentinization are bound to ultimately make a hydrothermal system alkaline.

Abiotic and biotic hydride donors

Our study shows that metal surfaces can serve as abiotic hydrogenases (or 'geozymes') that can transfer the electrons from H_2 directly to an organic cofactor. This direct transfer of two electrons from H_2 to an organic cofactor, without intervention of one-electron transfer through FeS centres, has only been recently observed for a hydrogenase, namely the F_{420} reducing [Fe] hydrogenase of methanogens and some anaerobic bacteria [22]. Metal hydrides could not only have served as abiotic hydride donors for biological molecules in early evolution, but also bring into focus a possible transition point from abiotic to biotic hydride donors in a prebiotic context. Prior to the origin of hydrogenases, early metabolic systems [6,26,80] were possibly still dependent upon metal hydrides from their geochemical surroundings but at some point an organic molecule that was able to integrate the hydride and transfer it to acceptors such as CO_2 under the given environmental conditions and made the protocell independent of metal hydrides. Though this is a strong hypothesis in need of testing, we note that there are cells living in H_2-rich environments today that appear to lack hydrogenases [39]. Our findings suggest that early redox cofactors might have interacted with H_2 rich environments in a far more complex manner than previously suspected. Our findings also show that in the case of Ni, an essential element of acetogens and methanogens [46], there exists a direct, rapid, and facile reaction between NAD^+ and metal hydrides. Overall, H_2 gas is able to convert an organic molecule (NAD^+) to an organic hydride carrier (NADH) in the absence of enzymes [81], in the presence of native metals [41] under the conditions of H_2 rich hydrothermal vents, which naturally deposit native Ni (and Fe) in the form of the mineral awaruite (Ni_3Fe) [40,82,83].

Materials and methods

Samples with pretreatment

4 mmol of iron powder (Fe^0; 99.9+% metals basis, particle size < 10 μm, Alfa Aesar, Thermo Fisher Scientific, Lancashire, UK), cobalt powder (Co^0; metal basis, particle size 1.6 μm, Alfa Aesar) and nickel powder (Ni^0; metal basis, particle size 3–7 μm, Alfa Aesar) were placed in reaction glass vials, closed with a PTFE-membrane bearing crimp cap, equipped with a syringe needle for gas exchange, and lastly exposed to 5 bars of H_2 overnight (16 h, 400 r.p.m. and 50 °C) in a stainless-steel high-pressure reactor (BR-300, Berghof Products + Instruments GmbH, Eningen, Germany). Afterwards, for NAD^+ reduction, a solution of 12 μmol of NAD^+ (free acid, Merck Millipore, Darmstadt, Germany) in 4 mL of 1 M phosphate buffer (pH 8.5; potassium phosphate monobasic and sodium phosphate dibasic, Honeywell Fluka, Fisher Scientific, Schwerte, Germany; in HPLC-grade water) was prepared and added via a disposable syringe through the needle in the membrane, leading to an overall concentration of 1 M metal. As an experimental control, one additional sample was prepared for every reaction without any metal powder. The samples were reintroduced in the reactor, which was closed tightly and pressurised again with either 5 bar of Ar or 5 bar of H_2, depending on the experiment. For the controls with metal oxides (Fe_3O_4, 50–100 nm, 97% trace metals; Co_3O_4, < 10 μm; NiO, > 99.995% trace metals; Sigma-Aldrich, Taufkirchen, Germany) we used 1 M worth of metal atoms according to each oxide (0.333 M for Co_3O_4 and Fe_3O_4 and 1 M for NiO).

Samples without pretreatment

4 mmol of each metal powder were placed in 5 mL glass vials (beaded rim) with a polytetrafluoroethylene (PTFE)-coated stirring bar. The described NAD^+ solution in phosphate buffer was pipetted on top, and sealed with a crimp cap with a PTFE-coated membrane. One more sample was prepared without metal powder to work as a control. To allow gas exchange between the interior and the exterior of the glass vial, a syringe needle was placed through the crimp cap membrane before the vials were placed in the high-pressure reactor.

Reaction

After pressurising the reactor with either 5 bar of Ar gas (99.998%, Air Liquide, Paris, France) or 5 bar of H_2 gas (99.999%, Air Liquide), the reactions were started and regulated by a temperature controller (BTC-3000, Berghof

Products + Instruments GmbH). Reactions were performed from 0.5 h to 4 h at 40 °C. After the reaction, the reactor was depressurised and the samples (metal powders and solution) were transferred to 2 mL Eppendorf tubes and centrifuged for 15 min at 16 000 ***g***. (Biofuge fresco, Heraeus, Hanau, Germany). The supernatants (NAD^+/NADH) and pellets (metal powders) were subjected to different analyses which are described below.

Separation of metal and supernatant to check for the active catalytic species

To study the liquid and solid phases separately, we separated them after a 1-h reaction with NAD^+ (5 bar H_2, 40 °C) through hot filtration. The liquid phase was pipetted to a new glass vial with a new PTFE-coated stirring bar and sealed. The solid phase also went into a new glass vial but with a fresh NAD^+ stock solution. Then, all-glass vials were re-introduced to the reactor to perform another 1-h reaction.

^2H_2O controls

Three control experiments were made for Fe and Co: one for pretreatment under Ar, one for reactions with H_2, and another under Ar. Our controls contained ^2H_2O instead of the corresponding volume of HPLC-grade water with all other parameters and analytic procedures maintained.

Measurement of pH

The pH after the reaction was measured for all samples containing inorganic catalysts using a Lab 875 Multiparameter Benchtop Meter (SI Analytics, Xylem, Mainz, Germany) and a pH electrode (SI Analytics).

Quantitative ^{1}H-NMR analysis

To detect and quantify the formation of NADH and side products such as nicotinamide and the decrease of NAD^+ we used an existing protocol for quantitative proton nuclear magnetic resonance (^{1}H-NMR) [32,33]. The internal standard was a 7-mM solution of sodium 3-(trimethylsilyl)-1-propanesulfonate (97%, Sigma-Aldrich) in deuterium oxide (CH_3 peak at 0 p.p.m.; ^2H_2O or D_2O, D_2O 99.9 atom % D, Sigma-Aldrich), mixed 1:6 with the supernatant of our samples. qNMR spectra were obtained on a Bruker (Billerica, MA, USA) Avance III 600 using a ZGESGP pulse program. Thirty-two scans were made for each sample with a relaxation delay of 40 s (600 MHz) and a spectral width of 12 315 p.p.m. (600 MHz). Analysis and integration were performed using MESTRENOVA (v.10.0.2) software.

UV-Vis Spectroscopy analysis

UV-Vis Spectroscopy was performed in some experiments as a complementary analysis to ^{1}H-NMR with an Agilent Technologies (Santa Clara, CA, USA) Cary 300 UV-Vis Compact Peltier spectrometer. Two UV-Quartz cuvettes were used for each measurement, one containing the supernatant of a sample and the other 1 M phosphate buffer (pH 8.5) as a reference value. Analysis was made using the Cary UV Workstation.

Fourier Transform Infrared (FTIR) Measurements

The metals from a 4 h reaction under Ar in ^1H_2O were collected from their glass vials and individually washed with Milli-Q water through suction filtration. Then, the metal samples were dried using a vacuum desiccator overnight and homogenised with a mortar and pestle for analysis. FTIR spectra of the metal powders before and after the reaction were obtained on Perkin Elmer-Spectrum Two (Perkin Elmer, Waltham, USA) utilising an Attenuated Total Reflectance (ATR) geometry with a $LaTiO_3$ detector. For each measurement, the dry powder was directly measured on the surface of the ATR crystal at room temperature without any pretreatment. Each spectrum was collected with the resolution of 4 cm^{-1} with 32 scans in the range of 400–4000 cm^{-1}.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MP and DPHP planned experiments; DPHP, TB, and JL performed experiments; MP and DPHP analysed data; MP and DPHP wrote the paper, WFM and KK edited the manuscript; WFM, KK, HT, AV provided supervision of the lab work.

Peer review

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. Original data files (^{1}H-NMR and FTIR spectra) are available on request from the corresponding author, MP.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Table S1. Single values of performed experiments.
Table S2. Relative quantity of ^1H atoms in the fourth position of the nicotinamide ring of NADH.
Fig. S1. Overview of experimental setup for timecourse NAD^+ reduction experiments.
Fig. S2. Overview of ^1H-NMR spectra of all conducted experiments.
Fig. S3. Experiments with pretreated metals after 4 h at 40 °C under H_2 with a far lower concentration of metal powder.
Fig. S4. pH shift during experiments.

Fig. S5. Change of sample colouration during reaction.
Fig. S6. The effect of a higher concentrated buffer on pH stability and consequent NAD stability monitored via ^{1}H-NMR.
Fig. S7. Stability of NAD^+ and NADH under different pH.
Fig. S8. FTIR measurements of Ni, Co and Fe before and after reaction.
Fig. S9. ^{1}H-NMR spectra of ^2H_2O experiment with H_2-pretreated Fe under Ar.
Fig. S10. Schematic of hot filtration experiments.
Fig. S11. NADH synthesis before (Reaction 1) and after (Reaction 2) hot filtration.
Fig. S12. ^{1}H-NMR spectra of NAD^+ with iron, cobalt and nickel oxides after reaction.
Equation S1. Calculation of H_2 concentration – Henry's law.
Equation S2. Calculation of electrochemical potential of H_2 at a given partial pressure and pH – Nernst equation.

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