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Bioenergetics of early life

Coupling of reaction networks and compartments may have sparked the first life forms

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How can chemistry turn into biology? How can living cells be built from molecules? These are fundamental questions in biology and, despite much research efforts, remain unanswered. Yet, the past two decades have seen considerable advances in our knowledge of how and which (bio)physical and (bio)chemical processes could have driven the emergence of the first living cells. These achievements have led not only to a better understanding of the molecular origins of life, but also spurred significant developments in synthetic biology, biophysics and supramolecular chemistry. Although the exact events that sparked life on Earth will quite likely remain a mystery, at least partially, exploring the chemical origins of life offers clues about our primordial past and could contribute to shaping our future.

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A systems approach to the chemistry of life

Ever since the discovery of cells by Robert Hooke in 1665, researchers across all fields have sought to understand how cells work and elucidate design principles towards the synthesis of artificial life. In 1828, Friedrich Wöhler reported the synthesis of urea from inorganic matter (cyanic acid and ammonia), demonstrating for the first time that biological molecules can be made from simple precursors. Chemists and biologists have since tried to replicate and mimic even more complex cellular functions *ex novo*, including nucleic acid and protein synthesis, cell signalling, environmental sensing and metabolic networks.

During the past two decades, chemists interested in the origins of life applied a systems approach to prebiotic chemistry—that is, the chemistry on the primordial Earth before life started—to explore how biologically relevant functions could have emerged from mixtures of simple prebiotic molecules (Kroiss *et al*, 2019). Complex systems chemists have been exploring and combining individual molecular processes, building up biomimetic networks of reactions to address key steps, such as information processing, energy production and compartmentalization, on the way to biological phenomena. In most cases, this approach helped to identify multiple stepwise pathways for synthesizing life's building blocks, including nucleotides, amino acids, lipids and metal-based cofactors. Prebiotic systems chemistry has also described minimal catalysts and the optimal conditions required to drive more complex metabolic processes.

"Metabolism" is the set of interconnected life-sustaining reactions that take place in living organisms. Cellular metabolism comprises linear and cyclic chemical pathways, often connected by shared intermediates and kinetically coupled reactions. To keep the chemistry of life out of equilibrium, metabolism couples spontaneous reactions that break down food molecules and release energy (catabolism) to non-spontaneous processes that consume energy and synthesize nucleic acids, proteins, carbohydrates and lipids (anabolism). It follows that modern metabolic pathways are built from and sustained by two interconnected and complementary features: a **biosynthetic** one, that is the ability to convert simple and complex chemicals into biological building blocks, and a **bioenergetic** feature: the ability to generate and control the energy flow that sustains such synthesis.

*"It follows that modern metabolic pathways are built from and sustained by two interconnected and complementary features: a **biosynthetic** one [. . .] and a **bioenergetic** feature. . ."*

In principle, to uncover how primitive metabolism or protometabolism emerged on Earth, one should identify synthetic processes that can be energetically coupled with each other and organized into complex reaction networks in an out-of-equilibrium setting. Whether these primitive reaction networks should predominantly resemble or differ from current metabolism, and whether the employed catalysts should be biomimetic or not, are still matters of ongoing debate. Regardless, recent efforts have led to identifying protometabolic **biosynthetic** processes, which transform simple, abiotic ingredients into life's building blocks (see Box 1).

A few pioneering experimental studies have recently recapitulated some of the fundamental aspects of modern metabolism.

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Box 1. Further reading

Recent examples aimed at mimicking extant pathways include:

i For glycolysis/pentose phosphate pathway, see Powner (*Nat Chem* 2016), Ralser (*Mol Sys Bio* 2014, *Sci Adv* 2016)

ii For Krebs cycle, see Ritson (*Sci Adv* 2021), Moran (*Nat Ecol Evol* 2017, *Nature* 2019), Ralser (*Nat Ecol Evol* 2017), Springsteen (*Nat Chem* 2020), Krishnamurthy (*Nat Comms* 2018), Sutherland (*Nat Chem* 2021)

iii For acetyl CoA pathway, see Moran (*Nat Ecol Evol* 2018), Tüysüz, Moran & Martin (*Nat Ecol Evol* 2020)

iv For proton gradients, see Sojo (*Proc Natl Acad Sci USA* 2020), Mast (*Nat Comms* 2017)

For example, the fact that metabolic pathways often share substrates and intermediates inspired Springsteen and Krishnamurthy to design interconnected non-biological cycles, analogues of the tricarboxylic acid cycle, based on simple molecules that were likely present on early Earth (Springsteen *et al*, 2018). In parallel, Moran and coworkers reported an anabolic biochemical pathway—the reverse tricarboxylic acid cycle—in which two or more consecutive reactions occur under common conditions (Muchowska *et al*, 2017). The need for the molecular constraints operating on one chemical reaction to apply to all other interconnected processes is, indeed, one of the crucial aspects of modern metabolic networks. Yet, little is known about the bioenergetic aspect of protometabolism, and its ability to drive and regulate biosynthetic pathways.

The role of gradients in the bioenergetics of life

One of the universal features of all life forms is the conversion of chemical energy into biological energy through the synthesis of adenosine triphosphate (ATP), which is then used to fuel biosynthetic processes. Modern cells extract electrons from nutrients and convert the energy released during this process into a transmembrane concentration gradient that drives the synthesis of ATP. To do this, cells rely on metabolic pathways that control the spatial distribution of the involved molecules, the kinetic barriers of individual biochemical processes, and the energetic constraints of the reaction network as a whole. Examples of such cellular energy-handling/storage systems are mitochondria and chloroplasts; prokaryotes also comprise complex biological machinery with similar metabolic functionality.

In mitochondria, a series of metal-based proteins transfer electrons extracted from biological fuels via reduced nicotinamide adenine dinucleotide (NADH) to oxygen, and concomitantly translocate protons across the inner mitochondrial membrane to generate a proton gradient. Conversely, chloroplasts capture light energy and use it to release oxygen from water molecules. The electrons thus released convert nicotinamide adenine dinucleotide phosphate ($NADP^+$) to its reduced form; this reaction is also coupled with the transfer of protons across the thylakoid membrane. In either case, the resulting proton gradient is then used by the universal ATP synthase to replenish the cellular ATP pool (Fig 1).

Concentration gradients are among the simplest forms of disequilibrium states created and harnessed by cells. To generate concentration gradients, modern metabolism involves extremely complex biological machinery that relies upon the combination of three key components: enzymes to provide selectivity over multiple substrates and to accelerate biochemical processes; high-energy molecules, such as ATP, to provide the energy needed to drive thermodynamically unfavourable biochemical reactions; and compartments to provide enclosed reaction sites and to separate biochemical processes.

> *"Concentration gradients are among the simplest forms of disequilibrium states created and harnessed by cells."*

While efforts in elucidating the individual origin of these key ingredients have significantly reduced the gap between abiotic and biotic systems, primitive catalysts, energy-rich molecules and compartmentalization have rarely been combined to better understand the origins of bioenergetics in early cells. The reason being the number of experimental challenges, including selectivity, orthogonality and organization (see Box 2), when one aims at recapitulating complete metabolic networks within prebiotic constraints.

On the way towards a primitive minimal metabolism

The complexity of the modern biochemical machinery, together with the lack of any historical record of the major prebiotic transitions, makes it also extremely difficult to untangle the sequence of events that led to the emergence of metabolism. In an attempt to describe a clearer trajectory between complex chemistry and biology, Ruiz-Mirazo and colleagues recently proposed a model for minimal metabolism (Lauber *et al*, 2021). This hypothesis offers a two-level concept, based on the indissoluble links between (bio)chemical processes within our cells, and the molecular constraints that result from and, at the same time, control those processes. According to their model, a minimal metabolism would stand at the interface between out-of-equilibrium complex chemistry and living systems.

> *"During the transition from a prebiotic to a biotic Earth, the complexity of the corresponding chemical reactions would increase, together with means to control these."*

During the transition from a prebiotic to a biotic Earth, the complexity of the corresponding chemical reactions would increase, together with means to control these. Along the evolutionary trajectory, chemical diversity would eventually decrease, as only a subset of all possible reactions is currently exploited by modern cells. In contrast, functional diversity would increase through the generation and combination of a wide variety of transformation processes and interdependent components. Among different possible combinations and implementations, the authors suggest an example of this minimal metabolism that, if experimentally achieved, would integrate aspects of biosynthesis and bioenergetics. Such a hypothetical protometabolic model would involve chemical networks capable of producing compartments and catalysts, while coupling endergonic-exergonic processes to achieve spatial, kinetic and energetic control.

Box 2. Challenges to overcome to build biomimetic metabolic pathways, combining biosynthetic and bioenergetic aspects

i Primitive catalysts, such as metals and metal ions, are rarely as efficient and substrate-selective as modern enzymes. Moreover, the limitations of primitive catalyst may result in pathways operating in fundamentally different ways, potentially altering the outcomes, thermodynamics and kinetics of the linear or cyclic metabolic processes to be replicated.

ii Modern metabolism relies upon multifaceted biochemical systems, made of different enzymes and capable of performing multiple functions simultaneously. Such catalytic complexity and orthogonality—the ability of each reaction to proceed without interfering with other reactions—are not easily achievable in a one-pot scenario.

iii Biomimetic high-energy molecules, notable thioesters and polyphosphates, synthesized by prebiotic means, must be able to drive endergonic reactions and pair them with exergonic processes. Enzymes facilitate this process by keeping all substrates and energy-rich molecules, such as ATP, in proximity, and organize and couple multiple reactions at once at their active site. Primitive catalysts such as metals or metal ions cannot replicate this organizing function of enzymes.

iv Notwithstanding the high selectivity of enzymes, modern biochemical reactions occur in different environments within a cell to keep substrates or products separated and are often coupled with one another across compartments' boundaries, such as membranes. Such features have not been achieved yet with primitive compartments.

Prebiotic systems chemistry is already developing ideas on how to experimentally approach this protometabolism problem as a whole—including both the biosynthetic and bioenergetic sides of it—in line with the model proposed by Ruiz-Mirazo. Trying to understand the origin of metabolism from a geochemical standpoint has already inspired numerous studies on protometabolic processes and naturally occurring concentration gradients that are potentially capable of driving endergonic reactions. The adsorption of substrates and products on mineral surfaces has also highlighted the importance of minerals as primitive organizational scaffolds. How enzyme-regulated metabolism could have emerged from such mineral-driven chemistry is, however, largely unexplained. Still, a geochemically based protometabolism is necessary to build a scenario that combines some of the relevant synthetic and energetic aspects of modern cells. In an effort to bridge the geochemical and biochemical domains, Mansy and coworkers have recently proposed a plausible path towards modern metabolism, exploiting a combination of soluble biomimetic and metal-based catalysts and lipid membranes (Bonfio *et al*, 2018).

A recent publication showed that membrane-bound peptide-coordinated iron–sulphur clusters—among the most ancient and ubiquitous biological cofactors—catalyse the transfer of electrons from NADH to various biologically relevant acceptors, including ubiquinone, molecular oxygen and hydrogen peroxide (Bonfio *et al*, 2018). These prebiotically-assembled electron transport chains mimic their modern counterparts without the complex multiple cofactor-containing enzymes present in contemporary bacteria and organelles. Electron shuttling to electron acceptors, such as molecular oxygen, within relatively impermeable lipid membranes led to an internal pH increase, thus generating a proton gradient. This shows that the generation of biologically relevant redox processes and concentration gradients does not require sophisticated enzymes and complex protein pumps but can be realized with a few simple chemicals (Fig 2). A follow-up work (Basak *et al*, 2021) demonstrated that simple metabolites, pyruvate and oxaloacetate, can drive the reduction of NAD^+ under prebiotic conditions. Electrons were shuttled from the substrate to final acceptors, while α-ketoacids underwent oxidative decarboxylation. The two studies offer a range of experimental opportunities to those interested in building protometabolic networks combining biosynthetic and bioenergetic ingredients.

The bioenergetics of minimal metabolism

It is just a matter of time before these or similar *in vitro* systems will be integrated into a single reaction network that efficiently couples endergonic and exergonic prebiotic reactions: the Bonfio *et al* study offers a pathway for NADH consumption, while Basak *et al* provides a way to regenerate it. As such, a reaction network driven by the NAD^+/NADH redox cycle would couple the generation of a transmembrane concentration gradient with other protometabolic processes, such as the production and consumption of α-ketoacids. If the system was continuously supplied with fuel and products/waste removed, such an extended protometabolic network would occur in an out-of-equilibrium similar to *in vivo* conditions.

> *"... one of the major complications in building complex reaction networks is related to the orthogonality of the processes involved and the availability of substrates/metabolites."*

This approach could, in principle, lead to the first example of protometabolic networks that achieve energetic control to connect linear or cyclic reaction pathways through energetically coupled reactions or common intermediates. However, to make multiple reactions work efficiently together requires overcoming kinetic restrictions as the requirements of one pathway become relevant to others; if one consumes a shared metabolite faster, it would deplete other pathways of their substrate. That is, the kinetic constraints of all interconnected protometabolic reactions must be tested first individually and then simultaneously, for compatibility and orthogonality, that is the ability of two processes to occur independently without interfering with each other. The challenge is to find catalysts—metals, peptidomimetics or ribozymes—for accelerating individual reactions that benefit the out-of-equilibrium conditions and avoid non-productive deadlocks.

Indeed, one of the major complications in building complex reaction networks is related to the orthogonality of the processes involved and the availability of substrates/metabolites. Living systems therefore evolved enzymes with specific substrate-binding pockets, while exerting a strict control on the spatial distribution of the different reactions. In eukaryotic cells, for example glycolysis takes place in the cytosol, the tricarboxylic acid cycle in the matrix of the mitochondrion, and ATP production across the mitochondrial inner membrane. Physical separation of metabolic pathways avoids cross-reactivity between the substrates in different chemical processes, tackling both the thermodynamic and the kinetic aspects of each reaction.

Compartments and concentration gradients

In addition, the spatial organization of metabolic pathways allows to make the products of one pathway readily available for neighbouring processes, thus minimizing the need for transporting them across the cell. Prebiotic chemists have explored a variety of physicochemical methods to keep molecules and reactions separated within the same environment, ranging from thermophoresis to compartmentalization. Among biomimetic compartments, membrane-less and membrane-bound assemblies were used to host various biochemical processes, including nucleic acid polymerization and ribozyme catalysis. Exploring protometabolic processes within compartments would thus not only help to build complex networks with nonorthogonal reactions but also to analyse how concentration gradients could be generated and exploited, and how molecular transport and exchange could be regulated at the compartment interface.

Two key aspects need to be considered for generating concentration gradients by prebiotic means. First, primitive membranes, mostly comprised of fatty acids, were likely more permeable to ions and small molecules their modern phospholipid-based descendants. Passive diffusion would therefore have made it more difficult to maintain a concentration gradient. Second, modern transmembrane gradients mostly result from translocation of protons and ions across lipid membranes. Prebiotic versions of membrane transporters must have thus started playing a pivotal role not only in generating transmembrane gradients through ion transport, but also in associating chemical processes, occurring on one side of the membrane, to a chemical response on the other side. The prebiotic synthesis of lipids capable of self-assembling into those more impermeable membranes, as well as primitive transmembrane transporters, would thus be required to generate, maintain and take advantage of concentration gradients in a biomimetic fashion.

This calls for further exploration of how such gradients could be exploited in a prebiotic context. At first, one could think of exploiting a local pH increase or decrease to drive *in situ* acid- or base-driven reactions. Several prebiotic processes, such as RNA strand displacement and peptide synthesis, are catalysed by acids or bases and take advantage of fluctuations in environmental pH. Similarly, the accumulation or removal of charged species, including mono- and divalent cations, on one side of the membrane could affect metal-based chemical reactions, intermolecular interactions and self-assembly processes.

In addition, one could also try to couple concentration gradients to the synthesis of high-energy molecules. It has been recently shown how prebiotic agents, such as methyl isocyanide, enable the formation of activated phosphate species, required for nucleic acid polymerization, under acidic conditions within permeable liposomes (Bonfio *et al*, 2020). That is, if compartments were composed of more impermeable lipids and prebiotic ion transporters, a metabolically generated proton gradient could drive the synthesis of highly reactive species needed for (proto)cellular biosynthetic processes.

In modern eukaryotic cells, the proton gradient generated across the inner mitochondrial membrane fuels the synthesis of ATP via ATP synthase. This is another challenge: the chemical synthesis of ATP—or its prebiotic analogues, such as thioesters—is not trivial. However, the greatest challenge in recapitulating the gradient-driven production of ATP is the ATP synthase itself: it is a large protein assembly that combines catalytic and ATP-binding units with pH-sensitive modules. Insight into how this complex, yet elegant machinery evolved remains elusive. A minimal version of such a biomolecular motor would need to exploit a concentration gradient to synthesize high-energy molecules. That is, a primitive ATP synthase would need to "sense" variations in pH on both sides of the membrane and, in response to it, drive the synthesis of ATP or its prebiotic analogues.

Primitive metabolism in synthetic biology

The quest to mimic complex biological machinery is not just exclusive to prebiotic chemists, but also tackled within the synthetic biology and nanotechnology communities. For example, Judy Hirst and her group recently showed that key

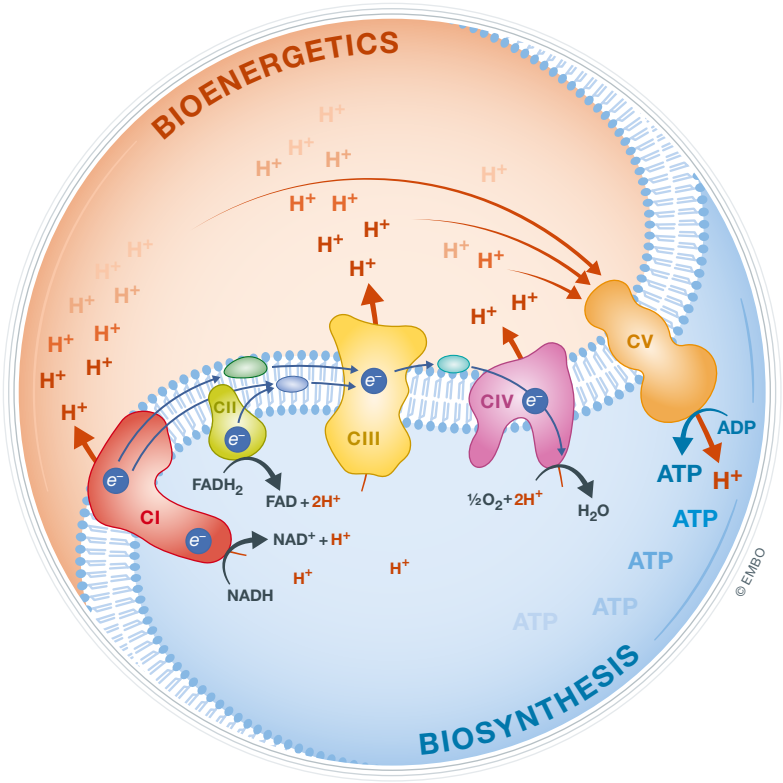

Figure 1. An illustration of how gradients over membranes could have evolved to couple biosynthetic and bioenergetic pathways.

functionalities of mitochondrial respiration could be recapitulated through a partly biological and partly synthetic electron transport chain (Biner *et al*, 2020). Such a biomimetic system was integrated into upstream metabolic pathways, by coupling metabolite oxidation to the reduction of NAD^+, and downstream pathways, by using ATP to drive endergonic reactions. In parallel, supramolecular chemists and nanotechnologists try to build biomimetic machinery mostly through the assembly of artificial and non-natural ingredients. An integrated approach, which builds upon prebiotic chemistry, nanotechnology and bottom-up synthetic biology, could provide comprehensive design principles to recapitulate the emergence of modern metabolism and, ultimately, the abiotic synthesis of life.

In an effort to combine the biosynthetic and bioenergetic aspects of modern metabolism, advancements in replicating redox processes and generating transmembrane proton gradients are already leading to a better understanding of how living cells are fuelled and sustained. Yet, the potential of such protometabolic studies is possibly even wider. One can wonder if abiotically-synthesized metabolic building blocks could be employed for reaction networks, other than those they have been biologically designed for—or if entirely artificial modules can replace natural ones within commonly explored cellular pathways.

Along these lines, a recent study led by Tobias Erb and coworkers (Miller *et al*, 2020) describes a microfluidic-based platform for the automated assembly of cell-sized bioreactors, which mimic the function of chloroplasts by enabling the light-driven conversion of carbon dioxide (CO_2) into more complex compounds. A thylakoid membrane-based energy module was isolated, tested for its ability to produce NADPH and ATP, and coupled with natural or artificial CO_2-fixing metabolic cycles. By interfacing biological and synthetic modules in these cell-sized compartments, such

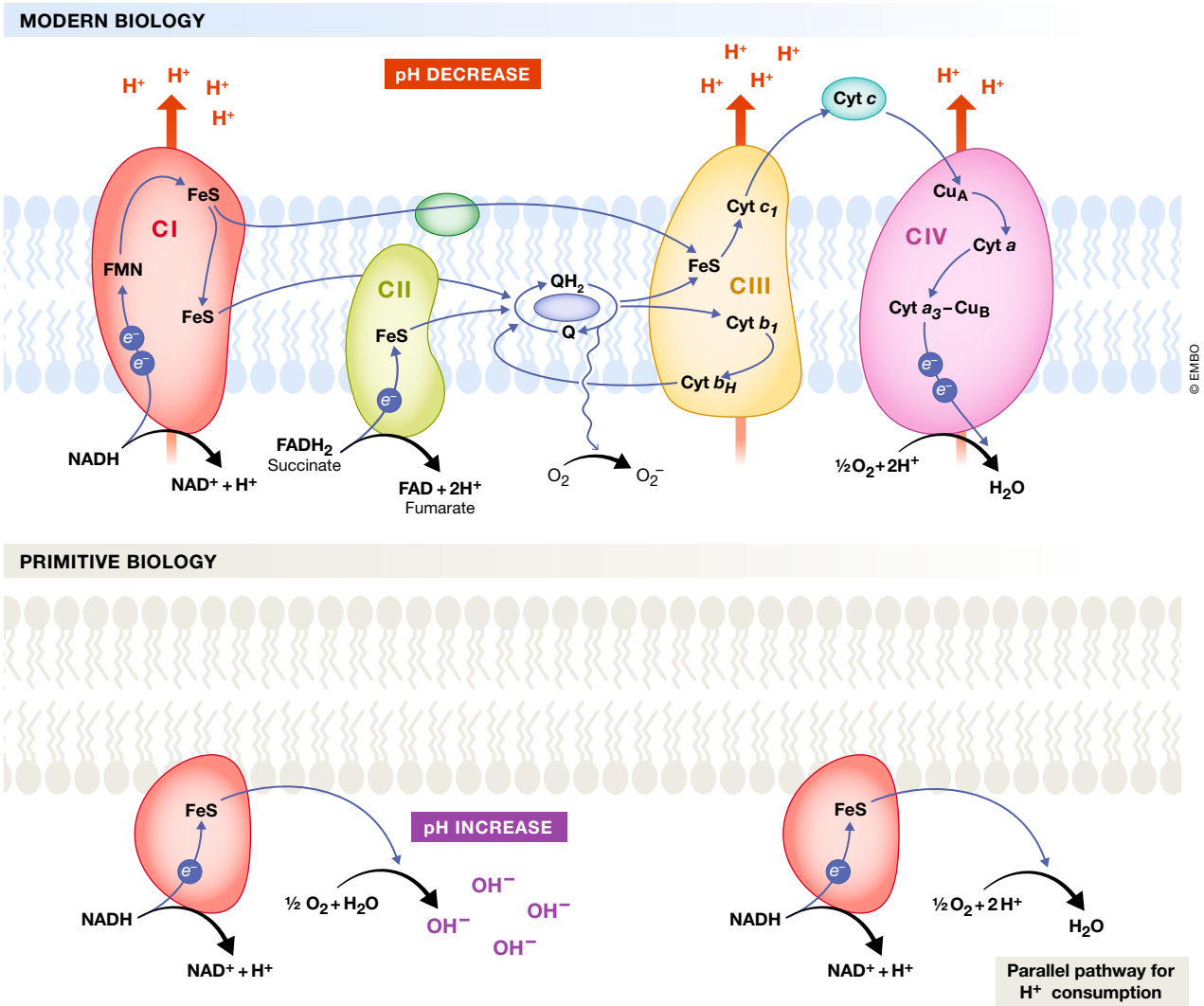

Figure 2. A plausible evolutionary precursor to modern electron transport chains: primitive membrane-bound catalysts could have mediated electron transfer and generated proton gradients across compartments.

photosynthetic entities were shown to be capable of mimicking, and potentially outcompeting, natural photosynthesis.

> *"Prebiotic chemists have explored a variety of physicochemical methods to keep molecules and reactions separated within the same environment, ranging from thermophoresis to compartmentalization."*

Similarly, whole-genome synthesis paves the way towards the assembly of synthetic cells. Pioneering efforts by Daniel Gibson, Craig Venter and coworkers led to the design and construction of JCVI–syn1.0 in 2010, the first man-made bacterial cell with a completely synthetic genome. Specifically, they synthetically assembled the genome of *Mycoplasma mycoides* in yeast and then transplanted it into a *Mycoplasma capricolum* cell, replacing the original genome. The *Mycoplasma mycoides* synthetic genome was further minimized to create JCVI–syn3.0, which is smaller than that of any autonomously replicating living cell (Hutchison *et al*, 2016). While most of the incorporated genes are involved in information processing, membrane function and metabolism, about a third of the synthetic genome codes unknown biological activities. Overall, these findings suggest the presence of yet-to-be-discovered functionalities that are essential for life, and for building artificial cells *ex novo*. The next challenge in synthetic biology would then be to design and synthesize a complete eukaryotic genome, as in the case of the Synthetic Yeast Genome project (Sc2.0), with potential future applications in industrial microbiology.

Other strategies build upon expansion of the genetic code to drive the synthesis of non-canonical biopolymers. For example, Jason Chin and his group proposed innovative approaches to decode quadruplet codons through engineered bacterial ribosomes to incorporate unnatural amino acids into proteins and new polymers (Robertson *et al*, 2021). Besides being key to understanding the fundamentals of biology and possibly pioneering the development of completely orthogonal biology, the assembly of synthetic and reprogrammed genomes will provide platforms for developing novel biologically based products, including medicines, materials and renewable fuels.

However, all these strategies typically yield synthetic cells that still closely resemble and relate to their biological ancestors, without comprehensively understanding how life can be synthesized *ex novo* from abiotic ingredients. In other words, approaches to "minimize" or rewrite the cellular genome and protein content are not aimed at building living systems from scratch.

> *"One can wonder if abiotically synthesized metabolic building blocks could be employed for reaction networks, other than those they have been biologically designed for…"*

To do that requires a holistic, multidisciplinary strategy to understand and potentially recapitulate the reaction networks that led to the emergence of life. A complete understanding of how biological components can be abiotically generated and combined within interconnected reaction pathways has the additional potential of developing a parallel, and possibly orthogonal biology, as well as to unlock alternative evolutionary routes, overcoming the boundaries of modern biology. Prebiotic chemistry and synthetic biology can thus not only provide the design principles for the synthesis of artificial living systems. A joint effort can also give clues as to how life, possibly from different ingredients, could be found, identified or generated here or elsewhere in the Universe.

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Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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