Abstract

Background. Life depends on small subsets of chemically possible reactions. A chemical process can hence be prebiotically plausible, yet be unrelated to the origins of life. An example is the synthesis of nucleotides from hydrogen cyanide, considered prebiotically plausible, but incompatible with metabolic evolution. In contrast, only few metabolism-compatible prebiotic reactions were known until recently. Here, we question whether technical limitations may have contributed to the situation.

Methods: Enzymes evolved to accelerate and control biochemical reactions. This situation dictates that compared to modern metabolic pathways, precursors to enzymatic reactions have been slower and less efficient, yielding lower metabolite quantities. This situation demands for the application of highly sensitive analytical techniques for studying ‘proto-metabolism’. We noticed that a set of proto-metabolism studies derive conclusions from the absence of metabolism-like signals, yet do not report detection limits. We here benchmark the respective \(^1\)H-NMR implementation for the ability to detect Krebs cycle intermediates, considered examples of plausible metabolic precursors.

Results: Compared to metabolomics ‘gold-standard’ methods, \(^1\)H-NMR as implemented is \(i\) at least one hundred- to thousand-fold less sensitive, \(ii\) prone to selective metabolite loss, and \(iii\) subject to signal suppression by Fe(II) concentrations as extrapolated from Archean sediment. In sum these restrictions mount to huge sensitivity deficits, so that even highly
Concentrated Krebs cycle intermediates are rendered undetectable unless the method is altered to boost sensitivity.

**Conclusions** 

$^1$H-NMR as implemented in several origin of life studies does not achieve the sensitivity to detect cellular metabolite concentrations, let alone evolutionary precursors at even lower concentration. These studies can hence not serve as proof-of-absence for metabolism-like reactions. Origin of life theories that essentially depend on this assumption, i.e. those that consider proto-metabolism to consist of non-metabolism-like reactions derived from non-metabolic precursors like hydrogen cyanide, may have been derived from a misinterpretation of negative analytical results.

**Keywords**
Metabolism, origin of life, Krebs cycle, nuclear magnetic resonance spectroscopy, selective reaction monitoring, limit of detection
Amendments from Version 1

**Results:** Results have not changed, except we have added a new Figure 4 that provides further evidence. In sum, we show that 1H-NMR, as implemented in several origin of life studies, is several orders of magnitude less sensitive compared to LC-SRM, a gold standard metabolomics method, to detect metabolites and non-enzymatic reactions, under conditions considered important for the evolution of metabolism.

**New:** Figure 4: We add new results that (partially) explain why the sensitivity of 1H-NMR, as implemented in several origin of life studies, is unexpectedly low. We find the sample preparation method as used, is prone to selective sample loss, under the assayed conditions.

**Text:** We have re-written the text, to make the implications clearer for the broader origin of life community. In Version 1, we have used our results solely to explain why another laboratory active in chemical origin of life research was unable to detect non-enzymatic Krebs-cycle like reactions as detected by LC-SRM, by using 1H-NMR (as the latter does not reach the required sensitivity). In Version 2, we elaborate now an obvious implication for other studies into origins of life research. As the 1H-NMR method as implemented in several origin of life studies fails to detect crucial Krebs cycle intermediates due to low sensitivity, it will also have missed out on other, similarly concentrated metabolites that are required for metabolic evolution. We hence discuss that it has been an analytical mistake of several origin of life studies, to use the absence of a signal, as a claim for absence of a particular metabolite or non-enzymatic reaction (i.e. as the necessary limit-of-detection measurements were missing). We discuss that this situation may have led to unsupported conclusions. Claims concerning the absence of metabolism-like non-enzymatic reactions under prebiotically plausible conditions are not experimentally supported.

**See referee reports**

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**Introduction**

Metabolism is the biological process of nutrient uptake and biochemical utilization in order to enable cell growth and survival. In cells, this task requires a large biochemical system, the metabolic network, which interconverts the available metabolites through a series of connected enzymatic and non-enzymatic reactions\(^1,2\). From the structure of this network, one can derive principles important for its origins. For instance, in the traditional terminology, biochemical reactions have been associated with the ‘build-up’ of metabolites (anabolism), or their ‘breakdown’ (catabolism). The view of anabolism and catabolism as two separate processes triggered a decade-long debate whether early life was of heterotrophic origin (and hence started with catabolism), or autotrophic (and hence started with anabolism). However, biochemical research over the last two decades has revealed that metabolism is organised as a balanced and tightly interconnected network, in which anabolism and catabolism are inseparably intertwined. Hence it is unlikely that anabolism and catabolism evolved separately. Moreover, the study of metabolic networks has revealed that chemical reactions that operate in ‘modern’ metabolism involve only a fraction of the potential chemical space. Only a small number of chemical elements are incorporated in metabolites, enzymes can be divided into only seven mechanistic categories, and the topology of the metabolic network uses only a small fraction of all theoretically possible chemical reactions\(^3,4\). This situation dictates that only a subset of all chemical elements, and a tiny fraction of all possible chemical reactions, were essential for (the origins of) metabolism and its evolution.

In biology metabolic reactions proceed under ‘ambient’ conditions as a result of the catalytic facilitation of enzymes. Provided the existence of appropriate starting materials, the majority of those reactions could proceed in the absence of enzymes (on an overall thermodynamically favourable basis) but in general do not at relevant rates, because of unfavourable activation barriers. In the search for the origin of metabolism it seems sensible to seek a ‘non-biological’ (i.e. non-enzymatic) basis under which those reactions could have proceeded, and at least allow for the evolutionary selection of appropriate catalysts that could sustain the earliest development of life. As Darwinian evolution can only select for functional products, metabolic pathways evolve as units\(^5\). This situation dictates that metabolic pathways can not come into being one step at a time, at least if intermediate steps are necessary for the formation of the functional product. The presence of non-enzymatic reactions that interconvert a substrate into a product without a step-change in condition is a proposed solution, rendering the evolution of enzyme-catalysed pathways possible by acting as a ‘template’\(^6\).

The challenge has led researchers to seek ‘prebiotically plausible’ chemical reaction conditions that might provide at least part of the basis for the metabolic origins of life. This activity has generated a plethora of claims for such prebiotic plausibility, with starting reactants and reactions that could be described as covering a chemical ‘space’ that is very significantly greater than that which encompasses biological metabolism as conserved across the kingdoms of life.

It should be taken as given that modern biological metabolism represents the product of the action of evolution, over all time, upon this necessarily limited reaction space. It therefore seems sensible to conclude that many if not the majority of chemical reactions that could have taken place on the prebiotic planet bear no direct relationship with the origins of metabolism. Put another way, whilst a chemical reaction might be deemed to be prebiotically plausible, it should not be automatically considered as important to the origin of life. Rather, one is obliged to consider whether such a reaction could provide a basis upon which the principles of natural selection could operate.

To identify these reactions, origin of life research does not need to operate ‘in the blind’. While it is true that we know too little about the primordial planet, the chemical and biological properties of the living cell’s metabolic networks provide ample information about the fundamental and universal constraints that apply to the function of every metabolic system. These principles can be used to identify chemical reactions that could have been evolutionarily important. Here we would like to highlight the two key constraints that are important in context of this manuscript. We highlight the significance of low metabolite concentrations, and the fact that in order to enabling both evolutionary and metabolic processes, only those reactions that can occur without a step-change in conditions can play a role.
First, the metabolic network of every living cell contains many important metabolites at low concentration that are reactive and quickly turned over. These reactions assemble in a tightly interconnected biochemical network of a few hundred reactions that co-occur in, broadly speaking, a single (or very few) set of reaction conditions\textsuperscript{11-13}. The intermediates that successfully participate in this metabolic network are in a constant flux of being formed and consumed, so that typically they do not accumulate to high concentrations (or expressed in the terminology of organic chemistry, they are not present in high yield). The specific contribution of these reactions to metabolism prevents the formation of ‘carbon sinks’ that reduce metabolic efficiency and create toxicity, as most accumulating metabolites would inhibit enzymes due to a confined structural diversity that prevails within the metabolome\textsuperscript{1}. Second, as a consequence of this flux through a network of enzymes with different rate constants and specificity, metabolite concentrations span several orders of magnitude\textsuperscript{14-16}. As a result, the importance of a metabolite in metabolism does not correlate with its instantaneous concentration. While some relatively unreactive metabolites, such as citrate, can achieve millimolar concentrations, many of the life-essential metabolites, such as NAD\textsuperscript{+}, adenosine, cytidine and guanosine, are present at thousand- to hundred-thousand fold lower concentrations, even in the fully nourished, rapidly growing cell\textsuperscript{11}. This fact creates huge demands on the analytical technologies used to study metabolism. Specifically, if a global characterisation of metabolic processes is attempted, and if the reaction spectrum of metabolism is to be comprehensively captured, a highly sensitive analytics is essential. Even more so, a sensitive analytical technology is required for studying the evolutionary precursors to enzymatic reactions, as many precursors that served in the evolution of enzymes must have even been of even lower concentration.

From these principles, one can derive considerations important for the understanding of the early evolution of metabolism. First and of foremost importance is the situation that in order to enable enzyme evolution, chemical reactions should be compatible with one another and occur in the same set of conditions. These constraints represent a significant difference to those that often govern the practice of chemical synthesis procedures typically employed in organic chemistry. In these, one allows one reaction to come to completion, and then (leaving aside whether the intermediate product is isolated or not) the next reaction is induced by a step-change in conditions. In contrast, the reaction sequences important for metabolism need to overcome the formation of intermediates that can not react further and therefore could accumulate to high concentration. In other words, it is unlikely that metabolism evolved from a set of chemical processes in which each intermediate is stable, and enzymes can not evolve if after each reaction step conditions need to change. While in organic synthesis such reactions are typically favoured as they provide high yields, they are problematic for metabolism and its evolution. Second, the reaction condition that successfully promotes one part of a metabolic network can not be so ‘extreme’ as to lead to the loss of functionality of other essential parts of that network. Some chemical processes deemed prebiotically plausible, like the synthesis of activated pyrimidine nucleotide that requires precursors such as hydrogen cyanamide\textsuperscript{21}, violate each individual one of these essential constraints. It has hence been proven difficult to imagine any scenario that could bring such processes in context with any scenario under which such processes could serve as a realistic precursor for the origin of metabolic reactions\textsuperscript{8}. Indeed, as Sutherland notes, biology ‘would have had to overwrite almost the entire reaction network’ in order to make such chemistry compatible with metabolism\textsuperscript{18}. However, Chemical networks largely lack evolvability\textsuperscript{19}, and in addition enzymatic pathways can not evolve in conditions that are so extreme that life itself can not persist. The most obvious answer is that chemical networks which do not resemble each other in a single node, and can not have evolved from one another, look not only unrelated, but are in fact unrelated. In other words, it is difficult to conceive that a chemical network which, like the hyrogen-cyanide network, bears no resemblance to the metabolic network, is its evolutionary precursor.

Most of the important biochemical reactions in modern cells are catalyzed by enzymes. As enzyme structures are a consequence of Darwinian selection, it has long been debated whether the topological organisation of metabolism itself originated through the formation of these enzymes (‘genetics first’), or emerged on the basis of a non-enzymatic chemistry that existed irrespective of the existence of enzymes (‘metabolism first’)\textsuperscript{20-22}. Recently, systematic metabolomics-type experiments have provided evidence for the latter scenario. First, for many enzyme-promoted metabolic reactions there exist non-enzymatic reactions that can occur in parallel. The latter are typically intrinsically slower or less specific than the corresponding enzymatic reaction, yet still occur in cells and constrain the metabolic network\textsuperscript{6,14-26}. Indeed, recently, there have been experimental demonstrations of metabolism-like non-enzymatic chemical networks that operate on the basis of environmentally available molecules. In particular, the discovery of a non-enzymatic glycolysis and a non-enzymatic pentose phosphate pathway implies that central metabolism reflects non-enzymatic reactions that occur in the presence of soluble iron(II) cations, Fe(II), readily available across the early Earth\textsuperscript{27,28}. Further, recently described metabolism-like non-enzymatic reactions resemble large parts of oxidative\textsuperscript{29} and reductive Krebs cycle\textsuperscript{30}, chemical reactions that form S-adenosylmethionine\textsuperscript{31}, key gluconeogenic reactions\textsuperscript{32}, and a reductive acetyl-CoA pathway\textsuperscript{33}. Each of these possesses contains reactions that, to a different extent, can proceed without multiple changes in reaction conditions. Fe(II) and other metal ions are ideal candidates to enable multi-step reaction sequences that can serve as evolutionary precursors leading to the development and evolutionary selection of ‘organic’ catalysts, namely enzymes.

If non-enzymatic reactions are broadly required in the evolution of metabolic pathways, one can question why until recently they barely been identified in origin of life studies. Indeed, such reactions were unknown for a long time. Not long ago, Leslie Orgel stated about the probability that a metabolic pathway might have a non-enzymatic precursor, ‘To postulate one fortuitously catalyzed reaction, perhaps catalyzed by a metal ion, might be reasonable, but to postulate a suite of
them is to appeal to magic. As several metabolism-like non-enzymatic reaction networks were discovered recently, one can consider this prediction of this giant in the origin of life field, disproven. Indeed, a non-enzymatic glycolysis and pentose phosphate pathway are driven by Fe(II)\(^{32,33}\). Also, many of the other recently discovered metabolism-like networks, which include key reactions resembling gluconeogenesis, the oxidative and reductive Krebs cycle, a non-enzymatic acetyl-CoA pathway, as well as an unexpected reaction that non-enzymatically provides S-adenosylmethionine\(^{29,30-33,37}\), involve or are affected, by metal ions.

In this manuscript we aim to provide an explanation why metabolism-like non-enzymatic reactions have been missed in many origin of life studies, and in this way pave the way to solve a long-standing, and in part emotionally held debate. We noticed that most of the recent discoveries of metabolism-like non-enzymatic reactions emerged from the application of sensitive analytical technologies, such as liquid chromatography selective reaction monitoring (LC-SRM) that can reach zeptomolar limit of detection (LOD) values. Moreover, many of the reactions discovered recently involve metal ions, in particular native or ferrous iron, that are known to broaden \(^1\)H-NMR signals\(^{27-30}\). Could it be that in previous studies metabolism-like reactions were missed due to technical reasons, either due to an insufficient absolute sensitivity, or being susceptible to signal suppression by the prebiotic drivers of metabolism-like reactions, like metal ions or free-radical donors? We therefore benchmarked an analytical protocol as implemented in studies that formed the basis to claim the absence of metabolism-like reactivity under prebiotic conditions\(^{33}\). We revealed that the \(^1\)H-NMR technology as implemented in studies that formed the basis to claim the absence of metabolism-like reactivity under prebiotic conditions\(^{33}\). We therefore benchmarked an analytical protocol as implemented in studies that formed the basis to claim the absence of metabolism-like reactivity under prebiotic conditions\(^{33}\). We therefore benchmarked an analytical protocol as implemented in studies that formed the basis to claim the absence of metabolism-like reactivity under prebiotic conditions\(^{33}\). We therefore benchmarked an analytical protocol as implemented in studies that formed the basis to claim the absence of metabolism-like reactivity under prebiotic conditions\(^{33}\). We therefore benchmarked an analytical protocol as implemented in studies that formed the basis to claim the absence of metabolism-like reactivity under prebiotic conditions\(^{33}\). We therefore benchmarked an analytical protocol as implemented in studies that formed the basis to claim the absence of metabolism-like reactivity under prebiotic conditions\(^{33}\). We therefore benchmarked an analytical protocol as implemented in studies that formed the basis to claim the absence of metabolism-like reactivity under prebiotic conditions\(^{33}\).

Results

Analytical techniques dramatically vary in sensitivity, and absence to signals can only be interpreted with knowledge of the limit of detection.

The analytical chemistry textbook teaches that the absence of a signal is only scientifically relevant in context of a limit of detection measurement. Despite this principle being broadly known, it was concluded from the absence of metabolism-like signals in some origin of life studies\(^{17,33,37}\), that early forms of metabolism did not resemble the chemistry that operates in modern metabolism\(^{13}\). In order to obtain insight into whether conclusions are robust also in the absence of LOD values, we benchmarked the analytical protocol in comparison to a ‘gold-standard’ LC-SRM technology used for sensitive, absolute quantification in metabolomics and proteomics\(^{20,69}\). To conduct this benchmarking exercise we focus on Krebs cycle intermediates: First, because the Krebs cycle is a frequently studied metabolic pathway in the context of the origins of life. Second, because non-enzymatic reactions that resemble the Krebs cycle have been described recently, and require metal ions, ferrous sulfide, or the generation of free radicals, reasonable catalysts to proceed enzymatic metabolism\(^{29,30,33}\).

We started with a comparison of the analytical sensitivity in the absence of a complex sample matrix. Under these conditions, \(^1\)H-NMR, that can be susceptible to signal suppression under particular conditions (vide infra) is expected to perform as well as it can. Nonetheless, using the typical instrument and measurement settings for both technologies, LC-SRM was up to one thousand times more sensitive in the detection of TCA intermediates (Table 1, Figure 2). Therefore in the context of origin of life studies, LC-SRM would be able to detect a much broader range of reactions spectrum.
Table 1. Limits of detection (LOD) for TCA intermediates as obtained with $^1$H-NMR and LC-SRM techniques in water.

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>LOD - $^1$H-NMR (pmol)</th>
<th>LOD - LC-SRM (pmol)</th>
<th>Fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate</td>
<td>190</td>
<td>0.202</td>
<td>940.6</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>205</td>
<td>0.202</td>
<td>1014.9</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>100</td>
<td>0.686</td>
<td>145.8</td>
</tr>
<tr>
<td>Succinate</td>
<td>17</td>
<td>0.105</td>
<td>157.1</td>
</tr>
<tr>
<td>Fumarate</td>
<td>47</td>
<td>0.401</td>
<td>116.0</td>
</tr>
<tr>
<td>Malate</td>
<td>240</td>
<td>0.778</td>
<td>308.5</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>47</td>
<td>0.061</td>
<td>762.3</td>
</tr>
</tbody>
</table>

Second, the most critical parameter in this comparison is however not the performance of the technologies in pure water, which is not a plausible scenario for the origin of metabolism. Rather, what is important is the absolute sensitivity of the analytical measurement for the reaction products in the relevant reaction matrices. For instance, it is well known that transition metal ions, including soluble Fe(II), can appear to suppress $^1$H-NMR signals due to paramagnetic effects that lead to the broadening of $^1$H NMR resonance lines$^{41,42}$. For instance, as we have shown previously, even highly concentrated ribose-5-phosphate and 6-phosphogluconate, metabolic precursors to ribonucleotides, are undetectable (to the extent that the $^1$H-NMR signals are broadened to the noise level) in the presence of FeCl$_2$, with a metabolite/Fe(II) molar ratio as high as 0.3$^{28}$. As iron is highly abundant...
in Archean sediment\textsuperscript{44–45}, and was hence present in large quantities in prebiotically plausible milieus, it becomes imperative to carefully assess the capability of \textsuperscript{1}H-NMR to detect, and faithfully quantify, proto-metabolic reactions under such conditions. In particular it is important to be aware of the risk of a false-negative assessment based on the apparent absence of NMR signals without carefully reporting LOD values. We therefore tested to what extent this situation may also be a problem for the detection of TCA intermediates. We observed clear and molecular species-dependent broadening of \textsuperscript{1}H-NMR resonances in the presence of iron species, when compared to iron-free controls (Figure 3). The signal broadening was extensive; metabolites were rendered undetectable even at concentrations in the high micromolar range, which is much more than plausibly required for the evolution of enzyme function.

Whilst the early atmosphere was ‘reducing’ in nature, any metabolic network that arose must have included both oxidative and reductive chemical transformations. Indeed, the early atmosphere was not devoid of oxidative reactions as induced by UV light or geothermal activity, for instance. Moreover, the Krebs cycle and glycolysis are ancient and essential oxidative pathways that existed long before the oxygenation of the atmosphere, and also today are found in aerobic as well as anaerobic microorganisms. Oxidative Krebs cycle-like non-enzymatic reactions are observed in the presence of either sulfate or hydroxyl radicals\textsuperscript{29}. In another recent study, hydrogen peroxide was found to be the enabling agent in a hypothetical prebiotic metabolic process. Oxidizing reactions hence have to be considered factors in early metabolic evolution\textsuperscript{35}. Therefore, we and other have included oxidizing substances like hydrogen peroxide or peroxydisulfate in our screens for protometabolic reactions. In this context, we noticed that the sample preparation procedure used in the origin of life studies, involves lyophilisation of the reaction mixture\textsuperscript{37,38}. We speculated that the presence of the specific sample matrix could affect samples during the \textsuperscript{1}H-NMR due to the formation of high local concentration of both the analyte and the free radical donor, a situation which might deplete, or might cause reactivity, of the analyte. Indeed, we found that the lyophilisation procedure is prone to metabolite loss. Even otherwise stable Krebs cycle metabolites, like pyruvate or acetate, were rendered undetectable if lyophilized in the presence of peroxydisulfate in the conditions under which that enable non-enzymatic Krebs cycle-like reactions (Figure 4).

\textsuperscript{1}H-NMR confirms that non-enzymatic Krebs cycle reactions that fall within its limits of detection proceed as evidenced by LC-SRM

The strong differences in the LC-SRM and NMR LOD values revealed that the NMR measurement paradigm will be compromised to an ill-defined extent in being able to comprehensively reveal the full range of reaction products that might emerge due to non-enzymatic reactions, for instance considering Krebs-cycle like non-enzymatic reactions that occur in the presence of a free radical source\textsuperscript{29}. In other words, in a complex reaction environment, LC-SRM will be able to detect more metabolites and reactions than \textsuperscript{1}H-NMR on account of its much greater sensitivity. However, having determined the LOD differences between the two measurement methods and demonstrated the potential interference of soluble iron species allowed us to select a subset of non-enzymatic Krebs cycle-like reactions that were realistically detectable by \textsuperscript{1}H-NMR, specifically upon altering the sample preparation procedure to avoid lyophilisation of the metabolites in the presence of the sulfate radical donor peroxydisulfate. The reactions selected were those starting from isocitrate leading to \(\alpha\)-ketoglutarate, isocitrate leading to succinate, and \(\alpha\)-ketoglutarate leading to succinate; all of these reactions were analysed in a time series over several hours. The NMR data showed that substrates are consumed in a time-dependent manner and \(\alpha\)-ketoglutarate and succinate were formed (Figure 5 and Figure 6). In addition, both NMR experiments indicated the formation of acetate. NMR spiking experiments with chemically pure standards confirmed the identity of the products formed (Figures 5B and C, Figure 6B).
Figure 3. FeS-induced signal suppression of $^1$H-NMR signals obtained for TCA intermediates. TCA metabolites at 100 µM concentration, which is within range of their typical cellular concentration, were combined with FeS, and measured at $^1$H 700 MHz field strength. This field strength is higher as the one applied in several origin of life studies, and the analysis presented here might hence be of higher sensitivity as the one achieved in the previous studies. Even at this high metabolite concentration, and in the mixture of low chemical complexity, the presence of FeS led to the suppression of malate and citrate NMR resonances. Of note, malate and citrate are Krebs-cycle intermediates, a pathway that depends in cells on Fe-S clusters.

Figure 4. The sample handling procedure used in origin of life studies is prone to selective loss, specifically under conditions in which metabolism-like non-enzymatic reactions are observed. A pyruvate standard was dissolved in an ammonium peroxydisulfate (APS)-containing solution, or processed as in Ritson & Sutherland, 2012, or without the lyophilisation step. Pyruvate reacts further to acetate in the presence of APS, an interconversion that is part of the metabolic network. Both pyruvate and acetate signals are depleted by the sample preparation procedure employed in Ritson & Sutherland, 2012, while they are unequivocally detected in the absence of the lyophilisation step. This example illustrates why it is considered an essential practice in analytical chemistry not to derive conclusions from the absence of $^1$H-NMR signals without validating the sample preparation procedure or detection limits.
Figure 5. NMR based evaluation of the Krebs cycle-like non-enzymatic interconversion of isocitrate to succinate and of isocitrate to α-ketoglutarate in the presence of peroxodisulfate. (A) 100 µM isocitrate was mixed with 200 µM APS and incubated at 70°C in a closed microcentrifuge tube for 0, 45, 105, 180, 285, and 320 minutes. Each mixture was then subjected to 1H-NMR analysis. Time-dependent formation of succinate, α-ketoglutarate and acetate was observed. Chemical shifts were observed to drift during the course of each reaction due to a small change in pH. (B) Spiking experiments with ~20 µM succinate, ~10 µM acetate and (C) ~20 µM α-ketoglutarate confirmed the identity of the Krebs cycle intermediates formed.
Figure 6. NMR based evaluation of the non-enzymatic Krebs cycle like interconversion of α-ketoglutarate to succinate in the presence of peroxodisulfate. (A) Experiments were conducted with α-ketoglutarate as substrate using the same procedure adopted for Figure 5. A time dependent accumulation of succinate and acetate was observed and (B) their chemical identity was verified in subsequent spiking experiments.

Discussion

In this study, we discuss constraints important for the early evolution of metabolism that emerge from the functional principles of the metabolic network, and benchmark an 1H-NMR protocol implemented in several origin of life studies, for the suitability to detect non-enzymatic precursor reactions to metabolism. Non-enzymatic Krebs cycle reactions serve as a good example. The cycle is extensively discussed in the context of the origin of life. Furthermore, next to glycolysis, the pentose phosphate pathway, and the formation of S-adenosylmethionine, non-enzymatic reactions that cover both the Krebs cycle as well as the reverse Krebs cycle have become known recently.

In comparison to LC-SRM experiments, broadly considered the gold-standard in quantitative metabolomics, the 1H-NMR protocol tested falls short in terms of sensitivity and dynamic range in providing a comprehensive quantification. The two techniques exhibit substantially different LODs for TCA cycle intermediates, by a factor of at least one hundred- to one thousand-fold, even in samples of low complexity and when any matrix-dependent signal suppression can be ruled out -
hence under the conditions in which $^1$H-NMR is expected to perform well. However, the more important benchmark is however the one in the presence of either metal ions or a free radical source, as the origin of metabolism is associated with such non-enzymatic catalysts. The most important molecule in this context is certainly ferrous iron. Not only is ferrous iron the most concentrated metal ion in Archean sediment and considered to be present in Archean oceans up to millimolar concentrations, but many of the so-far described metabolism-like non-enzymatic reactions are iron dependent$^{26-29,35}$. Under either the presence of Fe(II), or peroxysulfate that is a source of sulfate radicals, the $^1$H-NMR experiments as conducted in the origin of life studies, were signal-suppressed. The consequence is an enormous absolute difference in sensitivity, adding at least two more orders of magnitude compared to the sensitivity differences already detected in water. Specifically under the conditions considered important for the origin of metabolism, the $^1$H-NMR protocol used in several origin of life studies will hence miss out on at least a major fraction of ongoing chemical reactions.

Certainly, our experiment, or better the absolute values obtained, cannot serve as a universal comparison between $^1$H-NMR and LC-SRM, and concern specifically the protocols used in the origin of metabolism studies both for LC-SRM$^{36}$ and $^1$H-NMR$^{37}$. Indeed, LC-SRM experiments can be operated with different chromatographic conditions and MS settings. Also on the NMR side, the sensitivity is not only determined by the magnetic field strength employed, but equally depends on measurement time (i.e. the extent of signal averaging), repetition rate (relative to nuclear relaxation times), sample temperature, volume, pH, the quality of the NMR sample-tube and other instrument-specific parameters. In order to provide a fair comparison that leaves little room for argument, we decided to compare the exact values from which non-enzymatic Krebs cycle-like reactions were first discovered$^{38}$ (Figure 1, Figure 3), measured on our well maintained, but merely -- in MS terms -- averagely sensitive triple quadrupole mass spectrometer (Agilent 6460, acquired in 2011) for comparison with the NMR data obtained using an above-average magnetic field-strength spectrometer ($^1$H frequency 700 MHz). We used 5 mm diameter NMR sample tubes for achieving sensitivity, and summed measurements over a typical period of 2 hrs per sample to obtain a reasonable signal-to-noise estimate. The effective $^1$H NMR LOD was estimated from the S/N of a well resolved resonance in the spectrum of the metabolite obtained at 2 μM concentration, assuming a bona fide linear response of the NMR receiver technology as claimed for modern NMR spectrometers. Hence, this is an ‘effective’ LOD, relevant only to these typical NMR measurement conditions. In principle it might be possible to enhance the absolute LOD for a given metabolite by optimization of the acquisition parameters, e.g. pulse flip angle, relaxation delay, and number of transients collected. However, variation of the pulse and delay parameters from those that we employed cannot be expected to change the effective LOD by even a factor of ~3. Moreover, although one can improve the sensitivity by recording and summing more transients, the S/N ratio can only grow at a maximum of the square root of the number of transients. Namely, to improve the LOD this way by a factor of 10 would require, unreasonably, 200 hours measurement time per sample. However, we would like to emphasize that more sensitivity is equally be possible at the LC-SRM site. If one were to employ a triple-quadrupole mass spectrometer of the latest generation, or use a nanoflow-chromatography setup, the reported differences in LOD sensitivity would further increase by two orders of magnitude, or more. Hence, even upon extensive optimisation $^1$H-NMR, would not reach the sensitivity of LC-SRM.

Irrespective of this difference, we have shown that $^1$H-NMR has a key restriction for comprehensive characterization of prebiotic metabolic reactions. Iron, which is not only one of the metabolically most important inorganic catalyst, and implicated in most so-far discovered proto-metabolic reactions, was also - by far - the most highly abundant transition metal in the Archean sediment, broadens the $^1$NMR signals of metal binding metabolites, which includes many metalic intermediates. Our data published herein, as well as previously$^{34}$, show that in the presence of iron(II) ions, the $^1$H-NMR technique could fail to detect those metabolites that interact with iron. This aspect provides a problem for research on early metabolic reactions, keeping in mind that in the largely oxygen-free, reductive atmosphere of the Archean sediment, iron solutes were mostly kept in the reduced, Fe(II) form. Fe(II) is water soluble, and recent estimates predict that Archean seawater contained iron(II) up to millimolar concentration$^{41-46}$. Any analysis of similar solutions by $^1$H-NMR would need to take the presence of such high levels of paramagnetic ions into account, particularly when the target molecules would have a propensity to coordinate the transition metal, as many TCA intermediates would do. For instance, one could increase the absolute analytical NMR sensitivity in the presence of iron, by using $^{13}$C-NMR$^{47,48}$ which could be specifically important in origin of life research. Also here however, the modifications will not allow to reach the sensitivity of LC-SRM.

Finally, we reveal that a typically employed sample preparation procedure is prone to selective metabolite loss (Figure 4). We hence urge that researchers in future origin of life studies pay attention to the adherence to analytical standards, and to include positive and negative controls, and most importantly, to determine and consider LOD values when attempting to interpret any absence of signals.

Is it the case that only highly abundant and unreactive metabolites are important for metabolic evolution?

Last but not least, we take the opportunity to discuss why low abundant metabolites are important in the evolution of metabolism, and hence, why sensitive analytical technology is required to uncover the chemistry underlying proto-metabolism. In the literature it is often implied that only metabolites that are stable on geological time frames are important for the origins of life, as only with those one can achieve a metabolite rich ‘soup’ for heterotrophic origin of metabolism theories. If this were true, how could the metabolic network, which in essence operates on the basis of reactive intermediates, and of low metabolite concentrations, evolve at all? No sensible
scientific scenario for the origin of life starts with the pre-availability of hundreds of highly sophisticated enzymes. Indeed, the need for reactive intermediates and a biology that operates with low metabolite concentrations is a direct consequence of basic network properties essential for metabolism, and second a requirement for the evolution of enzymes and the regulation of metabolism. Intermediates in chemical and biological networks need not only to be formed, but also converted further. The formation of stable intermediates would simply lead to their accumulation and block metabolic flux. Another basic network property dictates that all intermediates for which the rate of consumption rate is faster than the rate of production will never accumulate to a high concentration. Depending on its organisation, a reaction network in which none or just very few of the intermediates reach substantial concentration can readily be envisaged. To our knowledge, there is no scientific evidence that metabolite concentrations higher than those prevailing in modern cells were required at any stage of metabolic evolution. Instead, there are multiple lines of evidence that imply that the availability of intermediate metabolites increased with the evolution of more specific and efficient enzymes.

The most obvious indicator that metabolism emerged from initially lower metabolite concentrations is the existence of the enzymes themselves: an enzyme would not provide selective advantage if it did not improve metabolite production compared to the non-enzymatic reaction. Another important aspect of this issue is that modern metabolism is regulated through modulation of enzyme activity. Such mechanisms could only have evolved in situations where the non-enzymatic rate of metabolite formation is slower than by the enzyme-catalysed route, as a change in enzyme activity or abundance has only a regulatory effect on metabolism when that enzyme activity is limiting. In conclusion while several essential metabolites are present only at low concentration in the modern metabolic network, all the biological evidence points to a situation wherein the levels of metabolite formation needed to be lower to enable the evolution of modern enzymes. One can therefore argue that, at least for most metabolites, the concentrations of metabolites in the modern metabolic network are the only available indicators of the maximal metabolite concentration required during the evolution of metabolism. Therefore, we would like to comment on whether one can give recommendations on how sensitive an analytical approach should be for the effective conduction of origin of metabolism studies. Despite the fact that so far no one has a deep understanding about the origins of life, the metabolism community has obtained a fairly good understanding of metabolite concentrations prevailing in living cells. As there is no evidence that metabolite concentrations needed to be higher for the origins of life, an ideal analytical method for origin of life studies should at least reach the sensitivity to detect cellular metabolite concentrations, and ideally, be much more sensitive. The \(^1\)H-NMR analysis implemented in several origin of life studies, at least according to the benchmark exercise conducted herein, does not achieve this minimum level of sensitivity.

In summary, we found that an \(^1\)H-NMR experiment as conducted in some key origin of life studies is subject to signal suppression in the analysis of key metabolites under conditions that can be considered important for the origin of metabolism, in particular by the presence of iron. As a consequence, the studies lack sensitivity and dynamic range to provide a comprehensive characterisation of prebiotically plausible chemical reaction networks. Other technologies available today, like LC-SRM, achieve sensitivity values that are orders of magnitude higher, specifically in the analysis of prebiotic conditions that are rich in iron, for instance. Our findings have specific implications for the origin of life research that utilizes low-sensitivity analytcs to screen for non-enzymatic reactions that could be potential precursors of metabolism. By being unable to detect metabolism-like non-enzymatic reaction in several origin of life studies, it has been concluded that early chemical reactions must have been fundamentally different to those that operate in modern metabolism. Our analysis clearly shows that the analytics that was used in the underlying studies does not justify such conclusions. Specifically in the presence of iron, perhaps the most important catalyst in forming precursors to metabolic pathways, the protocol misses out on orders of magnitude of relevant metabolite concentrations, and hence on large parts of the available reaction space. As a consequence, the conclusion that metabolism-like non-enzymatic reactions could not occur under prebiotically conditions feels premature, and may have emerged as consequence of the analytical procedures used. We hence strongly recommend to derive conclusions from the absence of signals only if appropriate limit of detection values and analyte controls are available.

Data availability
\(^1\)H-NMR raw data generated in this study has been deposited in the Mendeley Data repository at http://dx.doi.org/10.17632/kr7tfsryjz.1. All data relating to LC-SRM, as previously detailed, can be found here and has been deposited in the Mendeley Data repository at http://dx.doi.org/10.17632/vgpmnzd55.1.

Competing interests
No competing interests were disclosed.

Grant information
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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
The NMR spectra were recorded at the MRC Biomedical NMR Centre at the Francis Crick Institute.
Supplementary material

Supplementary File 1: LC-MS/MS method for TCA metabolite quantification. The full methodology is available here and is open access on PMIC.

Click here to access the data.

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38. Butcher Y, Tassan K, et al.: Multiple reaction monitoring for...


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Amy A. Caudy
Donnelly Centre and Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada

This manuscript is a response to criticism of the author’s previous screen to identify conditions in which reactions of the TCA cycle proceed spontaneously. The authors have used 1H NMR to analyze reactions carried out under conditions which they had previously shown using LC-MS to spontaneously accumulate certain TCA cycle intermediates. They demonstrate that beyond the decrease in sensitivity expected of NMR as compared to LC-MS, that the presence of iron leads to peak broadening that makes it extremely difficult to use NMR to detect metabolites that had been readily observed by LC-MS.

A few points would significantly improve the manuscript:

1. In the introduction, the authors write, “for many metabolic enzymatic reactions there exist non-enzymatic reactions that can occur in parallel. These reactions are typically intrinsically slower or less specific than the enzymatic reaction, yet are still part of the metabolic network.” This, and some of the surrounding text, would benefit readers by more clearly explaining that enzymatic catalysts can only alter the speed of an existing favorable reaction. All reactions that we observe as enzyme-catalyzed have some inherent rate, although that one may be vanishingly slow under typical biological conditions. For the benefit of the trainees and outsiders who may read this, please revise the introduction to make it more clear that enzymes are simply increasing the rate of the already possible. As the authors rightly point out, some reactions proceed at appreciable non-enzymatically catalyzed rates in cells, but, as is not adequately clean the current text, all of these reactons mus proceed at some rate.

2. As another point for trainees, the authors should make somewhat more clear that the sensitivity of NMR is determined by data collection time. If there was infinite time, some of these analytes would be observed (although those affected by band broadening might not be).

3. The second and third paragraphs of the results section could be significantly edited down; they come across as defensive and for the most part do not strengthen the points in the paper.

4. In the section “Sensitivity of LC-SRM and 1H-NMR in detecting TCA intermediates in the absence of iron” the discussion of injection volumes should be made more specific so that readers can
better appreciate the issues at hand. (Some readers may not realize that 2-10 ul injections are employed in many LC-MS studies, in comparison to the hundreds of microliters required in a typical NMR sample tube.)

5. The authors should also address the potential for 13C analysis, which has been used in the field to address the problem of paramagnetic centers. (see, for example, references 7-10 in 10.1021/ja054902h, as well as that paper itself)

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Metabolomics and enzyme analysis

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
catalysts can only alter the speed of an existing favorable reaction. All reactions that we observe as enzyme-catalyzed have some inherent rate, although that one may be vanishingly slow under typical biological conditions. For the benefit of the trainees and outsiders who may read this, please revise the introduction to make it more clear that enzymes are simply increasing the rate of the already possible. As the authors rightly point out, some reactions proceed at appreciable non-enzymatically catalyzed rates in cells, but, as is not adequately clear in the current text, all of these reactions must proceed at some rate."

Response: We could not more agree with this statement. We have therefore re-written this paragraph accordingly, now explaining this important aspects concerning non-enzymatic reactivity and metabolic networks in more detail.

Reviewer: "As another point for trainees, the authors should make somewhat more clear that the sensitivity of NMR is determined by data collection time. If there was infinite time, some of these analytes would be observed (although those affected by band broadening might not be)."

Response: We have expanded this part significantly (it now covers the main part of the discussion); we provide a detailed description how the sensitivity of 1H-NMR could be boosted to be able to detect more non-enzymatic reactions. We hope this section might help stimulate future origin of life studies to increase the sensitivity of their 1H-NMR protocols. We would like to emphasize that the difference in LODs between NMR and LC-MS measurement paradigms is large and that it would be practically impossible to raise the sensitivity of the NMR experiments to a level that even begins to approach that of LC-SRM. (See also comment in response to Reviewer #2 below.) A specific comment to this effect has been incorporated into the revised manuscript.

Reviewer: "The second and third paragraphs of the results section could be significantly edited down; they come across as defensive and for the most part do not strengthen the points in the paper."

Response: After careful considerations, we agree with the Reviewer, indeed this comment does not apply to the results section. Indeed, the first version of this manuscript was structured to advocate some our earlier work, as we have been exposed to a challenge. An organic chemistry lab active in the origin of life field, did derive conclusions from the absence of 1H-NMR signals but without determining limit of detection values. Without noticing what we think is a severe analytical mistake (one can not claim a signal obtained using a high-sensitivity technology is wrong, on the basis if a ~thousand-times lower sensitive method does not give the same result); this laboratory has however broadly distributed letters within the Research community, funders and hiring committees relevant for career progression of our lab members, claiming that non-enzymatic Krebs cycle reactions would not exist, and accused us of publishing scientifically inappropriate data. In the course of this study we realized that without the necessary controls, the absence of certain NMR signals has been overinterpreted not only in this distributed letter, but in several other contexts as well. We think the over-interpretation of the absence of signals, led to misleading conclusions in the origin of life field. This situation renders our results much more relevant for the broader research community working on the chemical origins of life, as we had initially anticipated. We thank the Reviewer for triggering this line of thought - we have now re-written the entire manuscript to put this central aspekt into context.
Reviewer: "In the section “Sensitivity of LC-SRM and 1H-NMR in detecting TCA intermediates in the absence of iron” the discussion of injection volumes should be made more specific so that readers can better appreciate the issues at hand. (Some readers may not realize that 2-10 ul injections are employed in many LC-MS studies, in comparison to the hundreds of microliters required in a typical NMR sample tube.)"

Response: We have improved this part, and thank the reviewer for pointing this out.

Reviewer: "The authors should also address the potential for 13C analysis, which has been used in the field to address the problem of paramagnetic centers. (see, for example, references 7-10 in 10.1021/ja054902h, as well as that paper itself)"

Response: This is an excellent suggestion. Many origin of life studies that depend on 1H-NMR could indeed switch to 13C-NMR to overcome signal suppression issues. We agree with the reviewer that alternative non-standard approaches to NMR detection of metabolite NMR resonances might give a greater chance to detect signals from metabolites that directly coordinate paramagnetic metal ions. We note that such experiments as those highlighted by the referee employ both specialised hardware (for optimal RF detection of X-nuclei) and 13C-isotope enrichment of the analyte (often a stable protein-metal ion complex, not weak equilibrium interaction with a solvated metal ion, as is likely the case in our samples). We understand that these approaches rely on the lower magnetogyric ratio of the X-nucleus and the impact on the effective nuclear relaxation time constants T1 and T2. However while such experiments might aid the detection of paramagnetic metal-ion coordinated species, this phenomenon does unfortunately not help to resolve the differing claims for the detection of metabolites by different laboratories using differing measurement paradigms 1H NMR and LC-SRM. In a more general sense, it seems impossible however that a 13C-NMR approach would improve metabolite NMR LODs, particularly in the absence of isotopic enrichment, over that obtainable by 1H NMR and those LODs will still be orders of magnitude higher than for LC-SRM.

Competing Interests: No competing interests were disclosed.
This is a very interesting paper, describing experiments that could shed light on the formation of prebiotic chains of chemical reactions resembling today's cellular metabolic networks such as glycolysis, the pentose phosphate pathway and the TCA cycle.

As the authors note quite openly, this paper is a response to claims of non-reproducibility of LC-SRM data by the proton-NMR. In our opinion, the authors convincingly address those claims and demonstrate, despite various problems with $^1$H NMR due to the presence of Fe(II), that NMR data support the data interpretation derived from the previously acquired LC-SRM data. As the authors state, they “unequivocally confirm the non-enzymatic formation and identity of TCA cycle intermediates”.

While we think that this very interesting article could be indexed as it is, we would like to make one suggestion. As most of the argumentation about the usability of $^1$H NMR refers to limits of detection, it would be useful if the authors discussed their particular choice of the repetition time (broadly speaking the relaxation delay plus acquisition time). While 5 seconds are not extremely short, depending on the longitudinal relaxation times of the metabolites, this choice could lead to a signal attenuation in the NMR spectrum and consequently lead to an underestimation of the sensitivity of NMR. However, we are well aware that the presence of Fe(II) could shorten $T_1$ times substantially, in which case a repetition time of 5s could be enough to restore equilibrium magnetisation.

We don’t think that this suggestion is in any way critical for the conclusions drawn by the authors and therefore recommend the indexing of this article without changes.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests**: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

**Author Response 27 Apr 2018**

Markus Ralser, Cambridge Systems Biology Centre, Cambridge, UK
We thank the reviewer for his suggestions, these have been incorporated in the manuscript. The reviewer is correct to highlight that a strict LOD determination for any given metabolite, indeed any given NMR resonance for any given metabolite, will be affected by the NMR scan repetition rate relative to the rate of longitudinal nuclear relaxation (whose time constant is denoted T1). In setting up ‘standard’ NMR experimental conditions one has to choose a combination of excitation pulse angle and relaxation recovery time to obtain a ‘sensible’ signal-to-noise ratio for the majority of peaks in the spectrum (acknowledging that different chemical moieties could have different T1 values). We believe that the reviewers are happy with the choice of parameters in the measurements presented in this study. In our experience the formal LODs for any given resonance might vary if different parameters were chosen, but in our hands this would equate to a factor of less than 3 (when assessed on a ‘signal per scan’ basis; note any increase in the relaxation delay acts against the effective ‘sensitivity per unit measurement time’). This leaves a discrepancy between NMR and LC-MS LODs that can not be bridged, even when attempting to exhaustively ‘chasing down’ the formal LOD value for every resonance in every metabolite. We also note that all of this leaves aside the fact that the NMR LODs can, in theory, be improved arbitrarily by recording a higher number of scans (because in principle the S/N only improves with the square root of the number of scans). However, trying to approach the sensitivity of LC-MS measurements could be practically impossible due to measurement times that exceed the stability of the analytes that should be measured. We believe that we have been clear that the NMR LODs reported in the manuscript are ‘effective LODs obtained under typical NMR measurement conditions’. That the presence of Fe(II) ions could potentially aid sensitivity with the shortening of effective T1 times is recognised but the expectation is that this is of no overall assistance in the case when paramagnetic broadening of 1H resonances (i.e. an influence on transverse relaxation rates; time constant T2) is the dominant effect.

**Competing Interests:** No competing interests were disclosed.