RESEARCH ARTICLE

Primordial Krebs-cycle-like non-enzymatic reactions detected by mass spectrometry and nuclear magnetic resonance [version 1; peer review: 1 approved, 1 approved with reservations]

Markus A. Keller\textsuperscript{1,2*}, Paul C. Driscoll\textsuperscript{3*}, Christoph B. Messner\textsuperscript{4}, Markus Ralser\textsuperscript{2,4}

\textsuperscript{1}Division of Human Genetics, Medical University of Innsbruck, Innsbruck, 6020, Austria
\textsuperscript{2}Department of Biochemistry, Cambridge Systems Biology Centre, Cambridge, CB2 1GA, UK
\textsuperscript{3}Metabolomics Science Technology Platform, The Francis Crick Institute, London, NW1 1AT, UK
\textsuperscript{4}The Molecular Biology of Metabolism Laboratory, The Francis Crick Institute, London, NW1 1AT, UK

\* Equal contributors

Abstract

\textbf{Background:} Metabolism is the process of nutrient uptake and conversion, and executed by the metabolic network. Its evolutionary precursors most likely originated in non-enzymatic chemistry. To be exploitable in a Darwinian process that forms a metabolic pathway, non-enzymatic reactions need to form a chemical network that produces advantage-providing metabolites in a single, life compatible condition. In a hypothesis-generating, large-scale experiment, we recently screened iron and sulfur-rich solutions, and report that upon the formation of sulfate radicals, Krebs cycle intermediates establish metabolism-like non-enzymatic reactivity. A challenge to our results claims that the results obtained by liquid chromatography-selective reaction monitoring (LC-SRM) would not be reproducible by nuclear magnetic resonance spectroscopy (\textsuperscript{1}H-NMR).

\textbf{Methods:} This study compared the application of the two techniques to the relevant samples.

\textbf{Results:} We detect hundred- to thousand-fold differences in the specific limits of detection between LC-SRM and \textsuperscript{1}H-NMR to detect Krebs cycle intermediates. Further, the use of \textsuperscript{1}H-NMR was found generally problematic to characterize early metabolic reactions, as Archean-sediment typical iron concentrations cause paramagnetic signal suppression. Consequently, we selected non-enzymatic Krebs cycle reactions that fall within the determined technical limits. We confirm that these proceed unequivocally as evidenced by both LC-SRM and \textsuperscript{1}H-NMR.

\textbf{Conclusions:} These results strengthen our previous conclusions about the existence of unifying reaction conditions that enables a series of co-occurring metabolism-like non-enzymatic Krebs cycle reactions. We further discuss why constraints applying to metabolism disentangle concentration from importance of any reaction intermediates, and why evolutionary precursors to metabolic pathways must have had much lower metabolite concentrations compared to modern metabolic networks. Research into the chemical origins of life will hence miss out on the chemistry relevant for metabolism if its focus is restricted solely to highly...
abundant and unreactive metabolites, including when it ignores life-compatibility of the reaction conditions as an essential constraint in enzyme evolution.

**Keywords**
H-NMR, LC-SRM, Krebs cycle, reproducibility

This article is included in the The Francis Crick Institute gateway.

**Corresponding author:** Markus Ralser (markus.ralser@crick.ac.uk)

**Author roles:** Keller MA: Conceptualization, Investigation, Methodology, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Driscoll PC: Conceptualization, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; Messner CB: Investigation, Writing – Review & Editing; Ralser M: Conceptualization, Funding Acquisition, Project Administration, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

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

**Grant information:** This work was supported by the Wellcome Trust [093735] to MR; the Francis Crick Institute, which receives its core funding from the Wellcome Trust, UK Medical Research Council and Cancer Research UK [FC001134]; and the European Research Council [260809], starting grant to MR.

**Copyright:** © 2017 Keller MA et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**How to cite this article:** Keller MA, Driscoll PC, Messner CB and Ralser M. Primordial Krebs-cycle-like non-enzymatic reactions detected by mass spectrometry and nuclear magnetic resonance [version 1; peer review: 1 approved, 1 approved with reservations] Wellcome Open Research 2017, 2:52 (https://doi.org/10.12688/wellcomeopenres.12103.1)

**First published:** 21 Jul 2017, 2:52 (https://doi.org/10.12688/wellcomeopenres.12103.1)
**Introduction**

Metabolism is the biological process of nutrient uptake and biochemical conversion in order to enable cell growth and survival. In cells, this task obliges a large biochemical system, the metabolic network, which interconverts the available metabolites through a series of connected enzymatic and non-enzymatic reactions. The origins of this metabolic network in evolution are barely understood. However, the chemical and biological properties of the living cell’s metabolic networks provide plentiful information about the fundamental and universal constraints that apply to achieve a functional metabolism. First, the metabolic network of every living cell contains many important metabolites of low concentration that are reactive and quickly turned over. These reactions assemble in a tightly interconnected, biochemical network of a few hundred reactions co-occurring in, broadly speaking, a single chemical condition. As the intermediates that successfully participate in this metabolic network are in a constant flux of being formed and consumed, they typically do not accumulate to high concentrations. This property prevents the formation of ‘carbon sinks’ that reduce metabolic efficiency and create toxicity, as most accumulated metabolites inhibit enzymes due to a confined structural diversity that prevails within the metabolome. 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. As a result, the importance of a metabolite in metabolism does not correlate with its concentration. While some relatively unreactive metabolites, such as citrate, can achieve millimolar concentrations, many of the life-essential metabolites, such as NADP+, adenosine, cytidine, and guanosine, are present at thousand- to hundred-thousand fold lower concentrations, even in the fully nourished, rapidly growing cell.

Most of the important biochemical reactions in modern cells are catalyzed by enzymes. However, these macromolecules possess highly sophisticated structures that could not have existed at the time of metabolism’s origins. These structures being the consequence of Darwinian selection, it was debated for a long time whether the topological organisation of metabolism itself originated through genetic selection principles (‘genetics first’), or emerged on the basis of a non-enzymatic chemistry (‘metabolism first’). Recently, systematic metabolomics-type experiments that address metabolic networks or prebiotic reaction conditions have provided evidence for the latter scenario. First, 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. Second, there have been demonstrations of two comprehensive and metabolism-like non-enzymatic chemical networks — a non-enzymatic glycolysis and a non-enzymatic pentose phosphate pathway. Both operate on the basis of a single inorganic catalyst that was readily available across the early Earth, namely soluble iron cations, Fe(II). Fe(II) and other metal ions are ideal candidates to enable multi-step reaction sequences that can serve as evolutionary precursors to enable the selection of enzymes. Indeed, metabolic pathways cannot originate by reaction, but only evolve as functional units. This is since Darwinian evolution can operate only by selection on the functional (end-) product. In the absence of a chemical ‘template’ that forms the selectable product, there emerges a chicken-or-egg problem for the evolutionary origin of enzymes, as chemical intermediates, required to form the product, could not themselves be selected before the product itself is formed. This property dictates that to form a selectable network, the reactions need to co-occur in the same chemical condition, which must permit the persistence of life so that enzymes can be selected. Further, the result of a function-based selection process is reflected in the modern metabolic network, which possess a modular structure.

Is the origin of life based on the same chemical reactions as those that mediated the origin of the metabolic network? One theory suggests that life was of heterotrophic origin. A heterotrophic origin of life requires metabolites to accumulate first so that they can be ‘consumed’. Experiments of organic chemistry have explored hypothetical prebiotic processes that could lead to such non-enzymatic formation and accumulation of biomolecules. Hypothetically, a photoredox/cyanide chemistry could form nucleotides, small sugars and amino acids, a reductive Krebs cycle could have provided citrate, and the formose reaction could form larger sugars. However, in order for the metabolites to be obtained at high concentrations, these organic processes operate within substantially different constraints than those that apply to metabolic pathways. Most importantly, in order to accumulate to a high concentration, many of the proposed reaction systems depend on changes in reaction conditions before the final product is formed.

Second, at least the processes suggested to enable an accumulation of sugars or nucleotides involving photoredox chemistry, or the reductive reactions resembling the Krebs cycle, involve reactants, such as cyanide and titan, and reaction conditions, such as intense UV light, that are metabolism atypical and would render the survival of early cells highly questionable. Indeed, even the most resistant modern cells do not survive exposure to strong UV light or in a concentrated HCN solution - and one should keep in mind that early cells could not have had the sophisticated mechanisms that allow some highly modern microbes to persist in relatively extreme environments. However, enzymes cannot evolve in conditions where life itself cannot persist. Consistently, most of the reaction topologies observed under such extreme conditions are not relevant for the origin of metabolism despite providing high yields, by being not reminiscent of reactions that play a role in the metabolic backbone. Hence, if life was predominantly of heterotrophic origin, the formation of first biomolecules was most likely driven by very different chemical processes to those that underlie the early forms of the metabolic network.

Alternatively, metabolism, or key parts of it, could evolve without the prior accumulation of metabolites. Metabolic pathways could also descend from metabolic cycles that favour the parallel occurrence of anabolism and catabolism and do not require the formation of high concentrations of intermediates. In this case, the chemistry enabling the origin of life and the chemistry leading
to the metabolic network could be similar. However, whilst theories incorporating an autotrophic origin of the metabolic network might overcome the need of chemical processes leading to primordial metabolite accumulation, they do not revoke the question of how early enzymes evolved. Until the recent discovery of non-enzymatic glycolysis and pentose phosphate pathways, chemical networks, based on a simple metabolism-typical non-enzymatic catalyst that show a topological similarity with metabolism, were not known. For instance, the plausibility of the required single, inorganic catalyst in the context of a metabolic pathway, such as the Krebs cycle, was deemed an ‘appeal to magic’.

How could catabolism and anabolism emerge in parallel and in the same environment?

An example here is provided by the essential pathways of central carbon metabolism. Next to a non-enzymatic glycolysis, we recently reported non-enzymatic reactivity that resembles gluconeogenesis. At room or higher temperature, the glycolytic catabolites glyceraldehyde 3-phosphate and dihydroxycetone phosphate non-enzymatically react into pyruvate, just as in glycolysis. This picture changes upon freezing the solution or upon repeated desiccation-rehydration. Under these circumstances, as in gluconeogenesis, a non-enzymatic aldol reaction proceeds from the same metabolites to form fructose 1,6-bisphosphate instead. Gluconeogenesis is an anabolic pathway that structurally overlaps, but metabolically antagonises glycolysis and is essential for glucose metabolism. Furthermore, while non-enzymatic reactions resembling glycolysis and the pentose phosphate pathway are catalysed by Fe(II), the gluconeogenic reaction is accelerated by amino acids. Therefore, freeze-thaw, desiccation-rehydration, or metal ion/amino acid availability cycles all might be able to induce a metabolism-like cycling between a non-enzymatic anabolism and catabolism. Together, glycolysis and gluconeogenesis could have evolved from this juncture by forming a metabolic system without need for the pre-accumulation of a particular glycolytic substrate.

The conditions enabling these non-enzymatic reactions were all identified by hypothesis-generating (unbiased) methods similar to those employed in functional metabolomics. Such methods provide the necessary throughput to systematically screen a large spectrum of reaction conditions. Further, as one assumes that metabolite concentrations were lower in early forms of metabolism before the evolution of sophisticated enzymes could increase metabolic efficiency, these methods provide the sensitivity and dynamic range to characterize metabolite concentrations at realistic (i.e., metabolism-typical) concentration levels. Hence, in contrast to the logic of an organic chemistry experiment to elaborate a hypothetical prebiotic accumulation process with a pre-set outcome (i.e. to obtain a high concentration of a specific metabolite hypothesized to be important for the origins of life), these systematic experiments strictly follow a network rationale to define conditions in which a maximum number of metabolism-like chemical reactions could co-occur. A logical consequence of a reaction-condition defining approach is that in any given condition, not all of the reactions are equally fast or efficient, but indeed, a range of different reaction rates and specificities are identified to co-occur. In other words, for a set of conditions, some of the co-occurring reactions are fast, some are slow, some efficient, some not, just as in typical chemical or metabolic networks. Furthermore, as in every reaction network, intermediates accumulate only when the metabolite consuming reaction is slower than the metabolite forming reaction. In a perfectly random, linear network this situation applies to 50% of the intermediates. However, in biological networks that possess a non-random structure, this measure could be much lower, down to the extreme case where no single intermediate accumulates (i.e., when the rate limiting reaction is the first in a linear network). Consequently, in the analysis of each reaction network, the sensitivity of the analytical method determines to what degree the whole spectrum of co-occurring reactions can be comprehensively characterized. In other words, the less sensitive the analytical methodology used to characterize a reaction network, the less likely it is to fully capture the set of reactions.

Just as glycolysis and the pentose phosphate pathway, also the Krebs cycle could date back to a non-enzymatic chemical network

Recently, we have addressed the plausibility of a chemical reaction condition that could enable a critical amount of Krebs cycle-like reactions to co-occur, a situation that could enable the evolutionary selection of Krebs cycle enzymes. Non-enzymatic reactions that replicate the reductive version of the Krebs cycle, or that form several of its intermediates, have been previously described in the literature. It is possible that such reactions provided metabolic precursors in the event that life was of heterotrophic origin. However, the conditions described lead to the accumulation rather than metabolism-like reactivity of the Krebs-cycle-like metabolites. They further depend on non-metabolism typical catalysts like semiconductor particles in combination with UV light, or a change of condition, several situations that all forestall the Darwinian selection of enzymatic pathways for the reasons outlined above. In order to test for the plausibility of a metabolism-sensitive catalytic milieu, we therefore designed a hypothesis-generating screen, in the sense that the analysis was not biased by previous hypotheses for how a primordial Krebs cycle should look (i.e., in terms of directionality or completeness) or which function it might have had. We instead focussed on identifying conditions in which a majority of Krebs cycle intermediates show metabolism-like reactivity. Knowing that the Krebs cycle requires iron-sulfate cluster catalysis, we combinatorially screened a broad panel of iron- and sulfur-containing chemicals and assessed the resultant chemistry using liquid chromatography-selective reaction monitoring (LC-SRM). Out of a large number of systematically tested conditions, the more than 6,000 sample study essentially yielded a single hit. Namely, we observed that Krebs cycle-like non-enzymatic reactions are enabled in the presence of free radicals that form upon the activation of peroxodisulfate, a reactive sulfur-containing chemical that can be activated by metal species, in our case in the form of iron(ii) sulfide, FeS. A critical subset of the reactions were also enabled by reactive oxygen species derived from hydrogen peroxide. However, the reactions that proceeded in the presence of hydrogen peroxide were much slower and less specific as those observed in the presence of FeS and peroxodisulfate.

Recently, a chemist active in the origin of life field has requested the retraction of our work, claiming the non-reproducibility of our results by nuclear magnetic resonance spectroscopy (¹H-NMR).
After critically reviewing our procedures (Results), we joined forces with a highly experienced biomolecular NMR spectroscopist (PCD) to address potential differences between ¹H-NMR with LC-SRM technologies in comprehensively characterizing reaction milieus that could have enabled Krebs-cycle like reactions to occur. This cooperation led to both a ¹H-NMR confirmation of Krebs-cycle like reactivity as enabled by peroxydisulfate, and also, in the identification of the most likely source of the discrepancy: a lack of sensitivity of the ¹H-NMR technology to comprehensively characterize metabolism-like non-enzymatic reactions.

We herein report substantially differing levels of sensitivity between LC-SRM and ¹H-NMR. In determining the limits of detection (LOD) values, we find that that ¹H-NMR is at least one hundred- to one thousand-times less sensitive than LC-SRM in detecting Krebs cycle intermediates in water. Further, these experiments highlight a general difficulty in the application of ¹H-NMR to characterize prebiotically relevant metabolic reactions. Iron concentrations in the range that, according to current geoscientific knowledge, were dominating the Archean oceans and other aquatic environments, have the potential to effectively suppress ¹H-NMR signals of metal-binding metabolites due to paramagnetic line broadening. Metabolites that react on the basis of a metal catalyst, among the most likely precursors of enzymatic reactions, are likely missed by ¹H-NMR, even when metabolites are at relatively highly concentration. For instance, we show that in the presence of iron, some of the metabolite concentrations used in our previous study as starting conditions, are not detected.

Having determined the quantitative limits for the application of ¹H-NMR to detect Krebs cycle-like non-enzymatic reactions in the prebiotically plausible conditions, we then selected a subset of the Krebs cycle-like reactions that are amenable to monitoring by ¹H-NMR as they a) realistically fall within its detection limits in water, and b) do not require the presence of iron species, so that the risk of paramagnetic signal suppression is avoided. Further, as intermediates in a metabolic network are both formed and consumed, we recorded time series. The results of the ¹H-NMR analysis unequivocally confirmed all of these reactions proceeded. Further, the spectra unambiguously confirmed the identity of all the product metabolites to be TCA intermediates. Therefore the NMR measurements confirm the outcome of our previous study (as conducted by LC-SRM), and show that TCA intermediates are specifically forming in the presence of peroxydisulfate. Further, the results underline that it is important to determine LODs when one is attempting to compare the outcomes of different analytical technologies. The hundred- to thousand-fold difference in the LODs dictates that ¹H-NMR will be less able to comprehensively capture the spectrum of non-enzymatic TCA intermediate reactions under any given set of reaction conditions. It remains a good analytical practice to report the specific LODs, when scientific conclusions are to be drawn from the absence of a (¹H-NMR) signal.

In this context, we also review key constraints that apply to the origins of metabolic pathways. The first is an unfounded perception that only highly abundant and prebiotically stable metabolites should be considered important for the origin of metabolism. This is not the case, as the metabolic network is a dynamic reaction system in which essential network properties disentangle concentration and importance. Further, enzymes evolve only if a reaction can be improved with a simple structure, meaning many of them will be derived from slow, unspecific, or low-yield chemical reactions. Indeed while the concentrations of metabolites span several orders of magnitude in any organism, some of the most important metabolites are in the low concentration range, and there is no overall correlation between the importance of a metabolite and its concentration in the metabolic network topology. Vice versa, often the non-essential, unreactive, secondary metabolites are the most concentrated metabolites in the cell, as only these can accumulate without compromising the metabolic system. The second problem we address is a perception that it would be acceptable to include catalysts atypical of metabolism and conditions incompatible with life such as exposure to intense UV light or high cyanide concentrations, in research into the early forms of metabolism or ‘proto-metabolism’. However, enzymes can only evolve under conditions in which life itself can evolve, while non-enzymatic reaction networks cannot be switched between inorganic catalysts without losing their topology. We therefore conclude this article with a plea that chemists and biologists in the field should cooperate and share their knowledge about the constraints that apply to metabolism in order to solve this most fascinating problem of biology.

**Methods**

We recorded no new LC-SRM data. In order to fairly compare the techniques in regards of the non-enzymatic Krebs-cycle like reactions, all LC-SRM analysis represented herein is based on re-analysis of our previous study data, which contained all controls and dilution series required for a comparison with ¹H-NMR.

For NMR spectroscopy, TCA intermediate interconversion experiments were conducted similarly, with some adaptation necessary to account for the much larger volumes necessary. Briefly, 100gM isocitrate or α-ketoglutarate were incubated in a water bath at 70°C in the presence of 200 μM ammonium peroxydisulfate for 0, 45, 105, 180, 285, and 320 minutes (total volume for each time point 1 ml). Incubations were conducted in 2 ml microcentrifuge tubes, and stopped by cooling the reaction mixture.

For ¹H-NMR analysis, samples were prepared at a volume of 500 μl in 5 mm NMR tubes, containing 5% D₂O for field-frequency lock. One-dimensional ¹H-NMR experiments were conducted at either 600 (Avance III) or 700 MHz with (Avance IIIHD) spectrometers (Bruker Daltonics) equipped with 5mm TCI or QCI cryoprobes at 25°C, as indicated. A standard excitation sculpting (zgesgp) pulse sequence was employed with typical acquisition parameters: sweepwidth 20 ppm; relaxation delay 2 s; acquisition time 3 s (84584 complex data points); 4 dummy scans; 256 transients; total measurement time 22 mins. Free induction decays were apodized with 2 Hz line broadening, and zerofilled to 128K complex points prior to Fourier transformation. For LOD measurements conducted on the Avance IIIHD, the number of transients was increased up to 1440; total measurement time 2 h 2 m.
Results
Self-assessment of the previous study design\textsuperscript{31}, leading to the identification of Krebs-cycle like non-enzymatic reactions

The hypothesis-generating screen used to define chemical conditions under which Krebs-cycle intermediates interconvert non-enzymatically is based on a series of \textit{per se} very simple experiments. One combines a TCA intermediate (pure chemical standard) with a large panel of iron and/or sulfate species, one by one, and incubates the reaction mixture for the given period of time. The samples are then analysed by LC-SRM. The study gains complexity however by the high number of such experiments necessary. We generated 5 times 88 different combinations of TCA intermediates with iron- and sulfate-containing chemicals, and measured each sample at two different time-points. Each condition that gave a positive signal was then replicated in a triplicate, and sampled in a time series in order to be able to determine their respective maximum reaction rates with mathematical models best describing their modes of product formation (Methods section, in \textsuperscript{31}). Furthermore, as we corrected for batch effects, each measurement series was controlled by multiple blank injections and standard dilution series. On this basis, we constantly monitored the instrument performance, detection limits and dynamic range. Eventually, our study involved the measurement of around 6,100 samples by LC-MS/MS. Therefore the throughput of the LC-SRM method, measuring each sample within minutes, was highly important. With a slower technology, like \textsuperscript{1}H-NMR, the processing of such a number of samples would require years of measurement time.

We first considered possible systematic errors that could have occurred during the large study. The chemical suppliers (most standards were purchased from Sigma-Aldrich) might have delivered the wrong molecules. However, we did work with all, not just a single TCA intermediate, and the study was conducted over a long period of time, so that we re-ordered several chemicals multiple times. Further, in all cases the MS/MS spectra and retention times matched expectations. Hence, a failure of our screen for this reason would mean all delivered standards were incorrect, and instead all be isomers of TCA cycle metabolites; an improbable situation. Also, some reactions were found not to require the presence of iron species, and others do not require peroxydisulfate, so even a problem with the purity of these chemicals would not explain our results. Next, LC-SRM cannot distinguish isomers that have the same mass, fragmentation pattern and chromatographic retention time. However, we detected all, not just some, TCA intermediates in at least one reaction, and employed several different chromatographic methods. Therefore it is highly unlikely that we systematically confused all TCA intermediates with hypothetical isomers possessing the same mass and fragmentation patterns, and which eluted at the same retention time in all LC gradients in all samples. Even if we had in some cases overestimated the rate of formation of a product metabolite due to the interference of isomers indistinguishable by LC-SRM, this would not affect our conclusion about the existence of a single condition in which the spectrum of TCA cycle-like non-enzymatic reactions can occur.

Next, we considered the potential for systematic human error. However, three scientists (MK, SH, and DK), with the help of two internship students, independently prepared samples and conducted the measurements. The five individuals obtained consistent results. Indeed, something might easily have gone wrong during a single LC-SRM measurement. However, we only considered metabolite-forming reactions to be significant when they were detected in at least three replicate experiments. Furthermore the formation of TCA cycle intermediates was observed during the initial screen, once more in a triplicated time-series verification experiment, and finally again in an additional triplicated time-series experiment to investigate the impact of radical scavenging agents. Further, in 6,100 measurements that were run over the period of a year, with all necessary controls being included, human error seems highly unlikely.

Analytical technologies vary by detection limit

Given that the challenge to our previous work centred on the reproducibility of our data generated by LC-SRM, we next considered whether something might have gone wrong in the attempt to reproduce the results using \textsuperscript{1}H-NMR. Important in this context is to determine whether LC-SRM and \textsuperscript{1}H-NMR would be equally suitable to capture the broad reaction spectrum of TCA cycle intermediates in the presence of FeS and peroxydisulfate. The most critical parameter for this is the sensitivity of the analytical measurement that could strongly depend on the reaction matrix. As a consequence, the absence of signal in an analytical experiment is broadly considered relevant only if the respective LOD has been determined. We approached the scientist challenging our study to provide the LOD values he obtained by \textsuperscript{1}H-NMR; but we were unable to access it. An assumption that \textsuperscript{1}H-NMR (or any analytical technology) detects and quantifies all biologically relevant metabolites irrespective of the matrix is scientifically naive. Indeed, most reaction conditions addressed in our previous article contain high concentrations of iron and sulfur species. It is well known that transition metal ions, including Fe(II), can suppress \textsuperscript{1}H-NMR signals due to paramagnetic effects that lead to the broadening of resonance lines\textsuperscript{35,41}. For instance, as we have shown previously, even highly concentrated ribose-5-phosphate and 6-phosphogluconate are undetectable to the extent that the \textsuperscript{1}H-NMR signals broaden to the noise level in the presence of FeCl\textsubscript{2}, with a metabolite/iron molar ratio as low as 0.3\textsuperscript{69}. In several instances in our Krebs-cycle screen, iron was however present at hundred- to thousand-fold higher concentration than the TCA cycle products formed. Under such iron-rich conditions, it becomes imperative to carefully assess the capability of \textsuperscript{1}H-NMR to detect, and quantify, the relevant metabolites, in particular when conclusions are derived from the apparent absence of signals. We therefore tested whether this situation may also be a problem for TCA intermediates. Indeed, we observed clear, and molecular species-dependent broadening of \textsuperscript{1}H-NMR resonances in the
Figure 1. FeS induced signal suppression of $^1$H-NMR signals obtained for TCA intermediates. TCA metabolites at 100 µM concentration, which were the starting conditions of our previous work$^{31}$, were combined with FeS, and measured at 700 MHz field strength. Even at this high metabolite concentration, and in the mixture of low chemical complexity, the Archean sediment-typical iron level can suppress the signals of malate and citrate. In the presence of iron, $^1$H-NMR would hence be problematic not only to comprehensively characterize the formation of TCA metabolites as in our previous study, but indeed to fully picture the (metabolism-typical) starting concentrations chosen.

Some metabolites were rendered undetectable at the starting concentrations as used in our previous work$^{31}$. Sensitivity of LC-SRM and $^1$H-NMR in detecting TCA intermediates in the absence of iron

A subset of our non-enzymatic Krebs cycle reactions are also observable in the absence of iron, and could hence be studied by $^1$H-NMR without concern for paramagnetic signal suppression. We thus continued by comparing the limits of detection of $^1$H-NMR and LC-SRM for the intermediates of the Krebs cycle-like chemical network. Certainly, this is never a totally perfect comparison, because LC-SRM experiments can be operated with different MS settings and chromatography. Also on the NMR side, the sensitivity is not only determined by the magnetic field strength, but also measurement time (extent of signal averaging), sample temperature, volume, pH, the quality of the NMR sample-tube and other parameters. In order to provide a fair comparison that leaves little room for speculation, we decided, however, to compare the exact values from which the conclusions have been derived$^{31}$ (Figure 2), measured on our only averagely sensitive, but well maintained, Triple Quadrupole mass spectrometer (Agilent 6460, six years old), and compare it with an above-average magnetic field-strength spectrometer (700 MHz). We used 5 mm diameter tubes for good sensitivity, and summed measurements over a typical period of 2 hrs per sample to obtain a reasonable signal-to-noise estimate. Here, the LOD was estimated from the S/N of a well resolved resonance in the spectrum of the metabolite obtained at 2 µM concentration, and assuming a bona fide linear response of the NMR receiver technology as claimed for modern NMR spectrometers. This argument is however only applicable to purified metabolites; the sensitivity is likely be much lower in complex matrices, and might hence overestimate the effective sensitivity of the $^1$H-NMR. The LOD calculations were based on 1 µL injections for LC-SRM and 500 µl sample volume for $^1$H-NMR. Hence, whilst further increase in sensitivity is possible on both the $^1$H-NMR and the LC-SRM side, our LC-SRM settings are much further from the maximally obtainable sensitivity of the technique than our $^1$H-NMR settings. Furthermore, in these calculations we ignore the fact that while the LC-SRM analysis is conducted with small injection volumes, much larger sample volumes are required for any form of $^1$H-NMR. Our comparison hence underestimates the effective LOD differences in favor of $^1$H-NMR. Yet, despite these compromises, which all favour $^1$H-NMR over LC-SRM, and despite performing the comparison in the absence of iron, the results clearly show that LC-SRM remains hundred to thousand-fold more sensitive than $^1$H-NMR for all TCA intermediates (Table 1, Figure 3).

$^1$H-NMR confirms non-enzymatic Krebs cycle reactions that fall within its limits of detection

The strong differences in the LOD values revealed that the NMR technology will characterize any non-enzymatic reaction spectrum in a prebiotic Fe/S environment substantially less comprehensively than LC-SRM. In other words, in a complex reaction environment, LC-SRM will detect more metabolites and reactions than $^1$H-NMR by simply being at least a hundred- to a thousand-fold more sensitive. However, having determined the LOD differences between the two technologies and revealing the potential interference of iron allowed us to select a subset of reactions from our previous study that are realistically detectable by $^1$H-NMR, and that do not depend on iron in order that paramagnetic interfer-
Table 1. Limits of detection (LOD) for TCA intermediates as obtained with \(^1\)H-NMR and LC-SRM techniques in water.

| Intermediates  | LOD - \(^1\)H-NMR (pmol) | LOD - LC-SRM (pmol) | Ratio  |
|----------------|---------------------------|---------------------|--------|
| Citrate        | 190                       | 0.202               | 940.6  |
| Isocitrate     | 205                       | 0.202               | 1014.9 |
| \(\alpha\)-ketoglutarate | 100                  | 0.686               | 145.8  |
| Succinate      | 17                        | 0.105               | 157.1  |
| Fumarate       | 47                        | 0.401               | 116.0  |
| Malate         | 240                       | 0.778               | 308.5  |
| Pyruvate       | 47                        | 0.061               | 762.3  |

Figure 2. Determination of limit of detection (LOD) and limit of quantification (LOQ) for the LC-SRM measurement of TCA intermediates. A dilution series of citrate, \(\alpha\)-ketoglutarate, succinate, fumarate, malate, and pyruvate was measured down to a lowest amount of 3.2e-08 µmol. Blanks were measured in parallel to quantify noise levels. Integrated blank area values (n=4) were used to determine the LOD and LOQ (mean+3×SD, mean+10 × SD respectively). A third order polynomial fit was used to describe the behavior close to the LOD and was used to quantified LODs and LOQs.

Figure 3. Limit of detection (LOD) comparison between \(^1\)H-NMR and LC-SRM. LOD values for \(^1\)H-NMR and LC-SRM on typical Krebs-cycle intermediates, as determined in water. Please note that the graph is depicted by using a logarithmic scale.
ence is ruled out. The reactions selected were those starting from isocitrate forming α-ketoglutarate, isocitrate forming succinate, and α-ketoglutarate forming succinate, all analysed in a time series over several hours. The substrates are consumed in a time dependent manner and succinate and α-ketoglutarate are formed as previously reported (Figure 4 and Figure 5). In addition, both experiments showed formation of acetate, a metabolite that has not been monitored in our previous study, but could account for

Figure 4. NMR based evaluation of the non-enzymatic interconversion of isocitrate to succinate and of isocitrate to α-ketoglutarate in the presence of peroxydisulfate. (A) 100 µM isocitrate was mixed with 200 µM peroxydisulfate and incubated at 70°C in a closed microcentrifuge tube for 0, 45, 105, 180, 285, and 320 minutes. Mixtures were then subjected to ¹H-NMR analysis and time dependent formation of succinate and α-ketoglutarate was observed. Also a peak indicating acetate was observed. Chemical shifts were observed to drift during the course of the reactions due to a small change in pH. (B) Spiking experiments with with ~20 µM succinate, ~10 µM acetate and (C) ~20 µM α-ketoglutarate confirmed the approved the identity of the TCA-cycle intermediates formed.
Figure 5. NMR based evaluation of the non-enzymatic interconversion of \( \alpha \)-ketoglutarate to succinate in the presence of peroxydisulfate. (A) Experiments were conducted with \( \alpha \)-ketoglutarate as substrate using the same procedures as adopted for Figure 4. A time dependent accumulation of succinate and acetate was observed and (B) their chemical identity was verified in subsequent spiking experiments.

A time dependent accumulation of succinate and acetate was observed and (B) their chemical identity was verified in subsequent spiking experiments.

Discussion

This study was conducted as a response to claims in the origin of life research community questioning the use of mass-spectrometry of peroxydisulfate, in essence, leads to the formation of other metabolites important in the metabolic pathway, but barely to the formation of non-metabolic intermediates. This further strengthens our previous speculation that the TCA cycle, like glycolysis and the pentose phosphate pathway\(^{18,19,29}\), obtained their structure by being made of most likely reaction products in a given chemical environment.

Some of the non-TCA cycle reactivity (expressed as carbon loss) observed previously in the iron-free reaction conditions\(^3\). Spiking experiments with chemically pure standards confirmed the identity of the products formed (Figures 4B and C, Figure 5B). Of note, the \(^1\)H-NMR experiments confirmed the high specificity in which the non-enzymatic reactions specifically form TCA cycle metabolites. Although many other metabolite forming reactions would be thermodynamically feasible, the \(^1\)H-NMR signals appearing in essence corresponded to TCA cycle metabolites. These results hence confirm the second key conclusion of our previous article that the reactivity of Krebs cycle intermediates in the presence...
to reveal non-enzymatic Krebs cycle reactions\textsuperscript{1}. In replicating TCA cycle-like reactions by \textsuperscript{1}H-NMR, we unequivocally confirm the non-enzymatic formation and identity of TCA cycle intermediates within a single reaction condition, thereby validating the key result of our previous work.

We also identify the most likely source of discrepancy when comparing \textsuperscript{1}H-NMR and LC-SRM experiments in detecting non-enzymatic metabolic reactions. The two techniques possess 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 is largely ruled out. This difference has the potential to limit the ability of \textsuperscript{1}H-NMR to achieve a comparable depth in the characterisation of early metabolic chemical reaction networks when compared to LC-SRM. Indeed, in the future this difference will amplify; if one were to employ a more powerful Triple-Quadrupole mass spectrometer of the latest generation, the reported differences in LOD sensitivity might easily increase to 10,000-fold. Irrespective of this difference, we show that \textsuperscript{1}H-NMR has a further restriction for comprehensively characterizing prebiotic metabolic reactions. Iron is not only the metabolically most important inorganic catalyst, but also - by far - the most highly abundant transition metal in the Archean sediment, suppresses the \textsuperscript{1}H-NMR signal of metal binding metabolites. Our data show that in the presence of iron, the \textsuperscript{1}H-NMR technique might even miss out on detecting metabolites, to the extent that not even complement of metabolites in the starting conditions used in our previous work are faithfully captured\textsuperscript{1}. This 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 is mostly kept in its reduced, Fe(II) form. Fe(II) is water soluble, and recent estimates assume that Archean seawater contained up to millimolar iron concentrations\textsuperscript{13,16}. This iron would need to be removed before the analysis by \textsuperscript{1}H-NMR, which is analytically speaking a difficult procedure, keeping in mind that the metabolites affected by signal suppression are specifically the iron binding metabolites.

Are only highly abundant and unreactive metabolites important for metabolic evolution?

Last but not least, we take here the opportunity to address important constraints applying to the evolution of metabolic networks, or ‘proto-metabolism’, which explain why sensitive analytical technology is required to uncover its underlying chemistry. In heterotrophic theories for the origin of life it is often considered that only metabolites stable in geological timeframes are important. But if this would be true, how could the metabolic network, which in essence operates on the basis of reactive intermediates, 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 first 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 inert metabolites would just lead to their accumulation, could block metabolic flux, and eventually deprive the cell of its nutrients. Further, intermediates for which the consumption rate is faster than the production rate will also not accumulate to high concentrations. In a randomly organised linear network this situation applies to 50% of the metabolites. Indeed, in the extreme case that the rate-limiting reaction is the first one in a linear network, or in a multiplex network that enables multiple conversion reactions per metabolite, none of the downstream metabolites would be formed faster as they react further. Depending on its organisation, a reaction network in which none or just very few of the intermediates reach substantial concentration can form. In any case, to our knowledge, there is no scientific evidence that higher metabolite concentrations than those prevailing in modern cells were required at any stage of metabolic evolution. Instead, there are multiple lines of evidence which imply that the availability of intermediates increased with the evolution of better enzymes. The most obvious indicator for the emergence of metabolism from initially lower metabolite concentrations is the existence of the enzymes themselves: an enzyme has no selective advantage if it produces less metabolite than would be available through a non-enzymatic reaction. Further, it is important to keep in mind that the early precursors of metabolic enzymes can not have possessed the same sophisticated structures as modern enzymes; hence there must be a selective advantage that can be provided by a simple structure.

Another, evidence to the same argument is that some non-enzymatic reactions are sufficient for the needs of metabolism such that they continue be non-enzymatic in the modern metabolic network. Similarly, for many enzymatic reactions, a parallel, slower, non-enzymatic reaction remains part of metabolism, and many enzymes evolve from low-abundant promiscuous enzymatic reactions\textsuperscript{4,14,42}. Finally, another argument is that metabolism is regulated through modulation of enzyme activity. Such mechanisms can only evolve in situations where the non-enzymatic 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 if the enzyme activity is limiting\textsuperscript{43,44}. Finally, a much more indirect and speculative, but highly illustrative indicator of the importance of low-abundant metabolites comes from chemical origin of life research itself. Starting with the experiments of Miller and Urey\textsuperscript{45}, chemists have worked for decades to build up life-essential metabolites. But only with the recent introduction of highly sensitive mass spectrometers, and in essence accepting the relevance of low abundance intermediates, have chemical networks been identified that are reminiscent of metabolic networks\textsuperscript{13,14,20,46}.

In summary, while several essential metabolites are present at low concentration in the modern metabolic network, all the biological evidence points to a situation wherein metabolite formation before the evolution of modern enzymes were even lower. This means that the concentrations of metabolites in the modern metabolic network are the best available indicators of the maximal metabolite concentrations required for metabolic evolution. On the other hand, focussing on providing a proof for (sometimes decades-old) aspects of theories for the origin of life, research into its chemical origins has greatly emphasised the accumulation of metabolites to high concentrations. But a reac-
tion mixture, even if full of stable metabolites, is no metabolic network, and a (purely) heterotrophic origin of life remains a debatable concept for exactly this reason\textsuperscript{42}. Hence it is possible that research into the chemical origins of life has, specifically for its preoccupation with allowing metabolites to accumulate and to achieve yield, so far specifically missed out on the reactions important for the evolution of enzymes and hence metabolism.

**Data availability**

\textsuperscript{1}H-NMR raw data generated in this study has been deposited in the Mendeley Data repository at [http://dx.doi.org/10.17632/tk7tsryjz.1\textsuperscript{37}]

All data relating to LC-SRM, as previously detailed, can be found here\textsuperscript{38} and has been deposited in the Mendeley Data repository at [http://dx.doi.org/10.17632/vgpmnzdz55.1\textsuperscript{39}].

---

**Competing interests**

No competing interests were disclosed.

**Grant information**

This work was supported by the Wellcome Trust [093735] to MR; the Francis Crick Institute, which receives its core funding from the Wellcome Trust, UK Medical Research Council and Cancer Research UK [FC001134]; and the European Research Council [260809], starting grant to MR.

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\textsuperscript{41} and will become open access on PMC on September 13\textsuperscript{th} 2017.

Click here to access the data.

---

**References**

1. Joeng H, Tomber B, Albert R, et al.: The large-scale organization of metabolic networks. Nature 2000; 407(6804): 651-654. [PubMed Abstract] [Publisher Full Text]

2. Francke C, Siezen RJ, Teusink B: Reconstructing the metabolic network of a bacterium from its genome. Trans Microbiol. 2005; 13(11): 550–558. [PubMed Abstract] [Publisher Full Text]

3. Thiele I, Swainston N, Fleming RM, et al.: A community-driven global reconstruction of human metabolism. Nat Biotechnol. 2013; 31(5): 419–425. [PubMed Abstract] [Publisher Full Text]

4. Price ND, Reed JL, Palsson BO: Genome-scale models of microbial cells: evaluating the consequences of constraints. Nat Rev Microbiol. 2004; 2(11): 886–897. [PubMed Abstract] [Publisher Full Text]

5. Stelling J, Klamt S, Bettenbrock K, et al.: Metabolic network structure determines key aspects of functionality and regulation. Nature 2002; 420(6912): 190–193. [PubMed Abstract] [Publisher Full Text]

6. Alam MT, Olin-Sandoval V, Stinocone A, et al.: The self-inhibitory nature of metabolic networks and its alleviation through compartmentalization. Nat Commun 2017; 8: 16018. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

7. Bennett BD, Kimball EH, Gao M, et al.: Absolute metabolite concentrations and implied enzyme active site occupancy in Escherichia coli. Nat Chem Biol. 2009; 5(8): 593–599. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

8. Ideker T, Thorsson V, Ranish JA, et al.: Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science. 2001; 292(5518): 929–934. [PubMed Abstract] [Publisher Full Text]

9. Blank LM, Kuepfer L, Sauer U: Large-scale \( ^{13} \text{C} \)-flux analysis reveals mechanistic principles of metabolic network robustness to null mutations in yeast. Genome Biol. 2005; 6(6): R49. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

10. Lusis PL: An open question on the origin of life: the first forms of metabolism. Chem Biodivers. 2012; 9(11): 2635–2647. [PubMed Abstract] [Publisher Full Text]

11. Lusis PL: Prebiotic metabolic networks? Mol Syst Biol. 2014; 10: 729. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

12. Orgel LE: The implausibility of metabolic cycles on the prebiotic Earth. PLoS Biol. 2008; 6(1): e18. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

13. Antet FA: The place of metabolism in the origin of life. Curr Opin Chem Biol. 2004; 8(6): 654–659. [PubMed Abstract] [Publisher Full Text]

14. Keller MA, Piedrafita G, Raiser M: The widespread role of non-enzymatic reactions in cellular metabolism. Curr Opin Biotechnol. 2015; 34: 153–161. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

15. Piedrafita G, Keller MA, Raiser M: The Impact of Non-Enzymatic Reactions and Enzyme Promiscuity on Cellular Metabolism during (Oxidative) Stress Conditions. Biomolecules. 2015; 5(3): 2101–2122. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

16. Laszlo L, Van Schaftingen E, Hanson AD: Metabolite damage and its repair or pre-emption. Nat Chem Biol. 2013; 9(5): 72–80. [PubMed Abstract] [Publisher Full Text]

17. D’Ari R, Casadodes J: Underground metabolism. Bioessays. 1998; 20(2): 181–186. [PubMed Abstract] [Publisher Full Text]

18. Keller MA, Turchyn AV, Raiser M: Non-enzymatic glycolysis and pentose phosphate pathway-like reactions in a plausible Archean ocean. Mol Syst Biol. 2014; 10: 725. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

19. Keller MA, Zylstra A, Castro C, et al.: Conditional iron and pH-dependent activity of a non-enzymatic glycolysis and pentose phosphate pathway. Sci Adv. 2016; 2(1): e1501326. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

20. Horowitz NH: On the Evolution of Biochemical Syntheses. Proc Natl Acad Sci U S A. 1946; 31(6): 153–157. [PubMed Abstract] [Publisher Full Text] [Free Full Text]

21. Raiser M: The RNA world and the origin of metabolic enzymes. Biochem Soc Trans. 2014; 42(4): 985–988. [PubMed Abstract] [Publisher Full Text] [Free Full Text]
22. Schönheit P, Buckel W, Martin WF: On the origin of heterotrophy. Trends Microbiol. 2016; 24(1): 12–25. PubMed Abstract | Publisher Full Text

23. Powne MW, Gerland B, Sutherland JD: Synthesis of activated pyrimidine ribonucleotides in prebiotically plausible conditions. Nature. 2009; 459(7244): 239–242. PubMed Abstract | Publisher Full Text

24. Patel BH, Percivalle C, Ritson DJ, et al.: Common origins of RNA, protein and lipid precursors in a cyanosulfidic protometabolism. Nat Chem. 2015; 7(4): 301–307. PubMed Abstract | Publisher Full Text | Free Full Text

25. Ritson D, Sutherland JD: Prebiotic synthesis of simple sugars by photoredox systems chemistry. Nat Chem. 2012; 4(11): 895–899. PubMed Abstract | Publisher Full Text | Free Full Text

26. Zhang XV, Martin ST: Driving parts of Krebs cycle in reverse through mineral photochemistry. J Am Chem Soc. 2009; 131(4): 1205–1208. PubMed Abstract | Publisher Full Text | Free Full Text

27. Breslow R: On the mechanism of the formose reaction. Tetrahedron Lett. 1959; 1(21): 22–26. Publisher Full Text

28. Orgel LE: Self-organizing biochemical cycles. Proc Natl Acad Sci U S A. 2000; 97(23): 12503–12507. PubMed Abstract | Publisher Full Text | Free Full Text

29. Mesner CB, Driscoll PC, Piedrafita G, et al.: Non-enzymatic gluconeogenisis-like formation of fructose 1,6-bisphosphate in ice. Proc Natl Acad Sci U S A. 2017; 114(28): 7403–7407. PubMed Abstract | Publisher Full Text | Free Full Text

30. Ravasz E, Somera AL, Mongru DA, et al.: Hierarchical organization of modularity in metabolic networks. Science. 2002; 297(5586): 1551–1555. PubMed Abstract | Publisher Full Text

31. Keller MA, Kampjut D, Harrison SA, et al.: Sulfate radicals enable a non-enzymatic Krebs cycle precursor. Nat Ecol Evol. 2017; 1: 0083. PubMed Abstract | Publisher Full Text | Free Full Text

32. Cooper G, Reed C, Nguyen D, et al.: Detection and formation scenario of citric acid, pyruvic acid, and other possible metabolism precursors in carbonaceous meteorites. Proc Natl Acad Sci U S A. 2011; 108(34): 14015–14020. PubMed Abstract | Publisher Full Text | Free Full Text

33. Liang C, Brusil CJ, Marley MC, et al.: Persulfate oxidation for in situ remediation of TCE. I. Activated by ferrous ion with and without a persulfate-thiosulfate redox couple. Chemosphere. 2004; 55(9): 1213–1223. PubMed Abstract | Publisher Full Text

34. Tee AL, Ahmad M, Watts RJ: Persulfate activation by naturally occurring trace minerals. J Hazard Mater. 2011; 196: 153–159. PubMed Abstract | Publisher Full Text

35. Canfield DE, Habicht KS, Thamdrup B: The Archean Sulfur Cycle and the Early History of Atmospheric Oxygen. Science. 2000; 288(5466): 658–661. PubMed Abstract | Publisher Full Text

36. Rouxel OJ, Bekker A, Edwards KJ: Iron isotope constraints on the Archean and Paleoproterozoic ocean redox state. Science. 2005; 307(5712): 1088–1091. PubMed Abstract | Publisher Full Text

37. Camprubi E, Jordan SF, Vaslikadou R, et al.: Iron catalysis at the origin of life. IUBMB Life. 2017; 69(6): 373–381. PubMed Abstract | Publisher Full Text

38. Andreini C, Bertini I, Cavallaro G, et al.: Metal ions in biological catalysis: from enzyme databases to general principles. J Biol Inorg Chem. 2008; 13(8): 1205–1218. PubMed Abstract | Publisher Full Text

39. Hwang TL, Shaka AJ: Water Suppression That Works. Excitation Sculpting Using Arbitrary Wave-Forms and Pulsed-Field Gradients. J Magn Reson A. 1995; 112(2): 275–279. Publisher Full Text

40. Ciure I, Iwahara J: Theory, practice, and applications of paramagnetic relaxation enhancement for the characterization of transient low-population states of biological macromolecules and their complexes. Chem Rev. 2009; 109(9): 4108–4139. PubMed Abstract | Publisher Full Text | Free Full Text

41. Bertini I, Luchinat C: NMR of paramagnetic molecules in biological systems. Benjamin-Cummings Publishing Company, 1986. Reference Source

42. Khersonsky O, Tawfik DS: Enzyme promiscuity: a mechanistic and evolutionary perspective. Annu Rev Biochem. 2010; 79: 471–505. PubMed Abstract | Publisher Full Text

43. Hackett SR, Zanotelli VR, Xu W, et al.: Systems-level analysis of mechanisms regulating yeast metabolic flux. Science. 2016; 354(6311): pii: aaf7886. PubMed Abstract | Publisher Full Text | Free Full Text

44. Park JO, Rubin SA, Xu YF, et al.: Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. Nat Chem Biol. 2016; 12(7): 462–489. PubMed Abstract | Publisher Full Text | Free Full Text

45. Miller SL: A Production of Amino Acids under Possible Primitive Earth conditions. Science. 1953; 117(3046): 528–529. PubMed Abstract | Publisher Full Text | Free Full Text

46. Lurino P, Tawfik DS: Spontaneous Emergence of S-Adenosylmethionine and the Evolution of Methylation. Angew Chem Int Ed Engl. 2017; 56(1): 343–345. PubMed Abstract | Publisher Full Text

47. Ralser M, Driscoll PC: “Non-enzymatic Krebs cycle-like reactions detected by 1H-NMR”. Mendele Data, v1. 2017. Data Source

48. Keller M, Ralser M: “Sulfate radicals enable a non-enzymatic Krebs cycle precursor”. Mendele Data, v1. 2017. Data Source
Open Peer Review

Current Peer Review Status:

Version 1

Reviewer Report 31 August 2017

https://doi.org/10.21956/wellcomeopenres.13097.r24383

© 2017 Caudy A. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 confirm that I have read this submission and 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.

Author Response 27 Apr 2018
Markus Ralser, Cambridge Systems Biology Centre, Cambridge, UK

Reviewer: “This manuscript is a response to criticism of the authors’ previous screen to identify conditions in which reactions of the TCA cycle proceed spontaneously. The authors have now used 1H NMR to analyze reactions carried out under conditions which they had previously shown using LC-MS to lead to the spontaneous accumulation of certain TCA cycle intermediates. They demonstrate that beyond the decrease in sensitivity expected of NMR as compared to LC-MS, 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:

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 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.

Reviewer Report 04 August 2017

https://doi.org/10.21956/wellcomeopenres.13097.r24380

© 2017 Ludwig C et al. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Christian Ludwig
Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK
Thomas Brendan Smith
Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK
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 confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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.