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Research Article

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Do Soluble Phosphates Direct the Formose Reaction towards Pentose Sugars?

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Abstract

The formose reaction has been a leading hypothesis for the prebiotic synthesis of sugars such as ribose for many decades but tends to produce complex mixtures of sugars and often tars. Channeling the formose reaction towards the synthesis of biologically useful sugars such as ribose has been a *holy grail* of origins-of-life research. Here, we tested the hypothesis that a simple, prebiotically plausible phosphorylating agent, acetyl phosphate, could direct the formose reaction towards ribose through phosphorylation of intermediates in a manner resembling gluconeogenesis and the pentose phosphate pathway. We did indeed find that addition of acetyl phosphate to a developing formose reaction stabilized pentoses, including ribose, such that after 5 h of reaction about 10-fold more ribose remained compared with control runs. But mechanistic analyses using liquid chromatography–mass spectrometry showed that, far from being directed towards ribose by phosphorylation, the formose reaction was halted by the precipitation of Ca^{2+} ions as phosphate minerals such as apatite and hydroxyapatite. Adding orthophosphate had the same effect. Phosphorylated sugars were only detected below the limit of quantification when adding acetyl phosphate. Nonetheless, our findings are not strictly negative. The sensitivity of the formose reaction to geochemically reasonable conditions, combined with the apparent stability of ribose under these conditions, serves as a valuable constraint on possible pathways of sugar synthesis at the origin of life. Key Words: Origin of life—Sugars—Formose reaction—Phosphorylation—Protometabolism—Astrobiology. Astrobiology 22, xxx–xxx.

1. Introduction

The question of how and why life emerged on early Earth around 4 billion years ago is experiencing a resurgence of scientific interest. A range of hypotheses are jostling to explain life's emergence, from extraterrestrial delivery (Chyba *et al.,* 1990; Svettsov, 2002; Osinski *et al.,* 2020) of organics to cyanide-rich subaerial pools (Powner *et al.,* 2009; Becker *et al.,* 2018; Damer and Deamer, 2020). Submarine alkaline hydrothermal systems have been proposed as a possible cradle of life on Earth for their unique physicochemical properties (Russell *et al.,* 1994; Martin and Russell, 2003; Martin *et al.,* 2014) which promote the synthesis and accumulation of organics from CO_2 and H_2 (Muchowska *et al.,* 2017; Preiner *et al.,* 2020). These vent systems offer tantalizing similarities between their geochemical organization and extant biological pathways and cellular structures (Martin and Russell, 2007; Sojo, *et al.,* 2016). The route by which any of these environments gave rise to the universally conserved core metabolism of cells has become a burning question.

In this context, sugars and their origins have been highly debated. They have multiple, universally conserved and essential functions in cells, suggesting that they played an important role in the emergence of life. From this perspective, the most prominent sugar derivatives are nucleotides, which are the basis for the coding molecules of life, RNA, and DNA. In addition, sugars serve as starting materials for the synthesis of a range of biomolecules and key cofactors including aromatic amino acids and pyridoxal-5-phosphate, respectively. However, there is still no consensus on the

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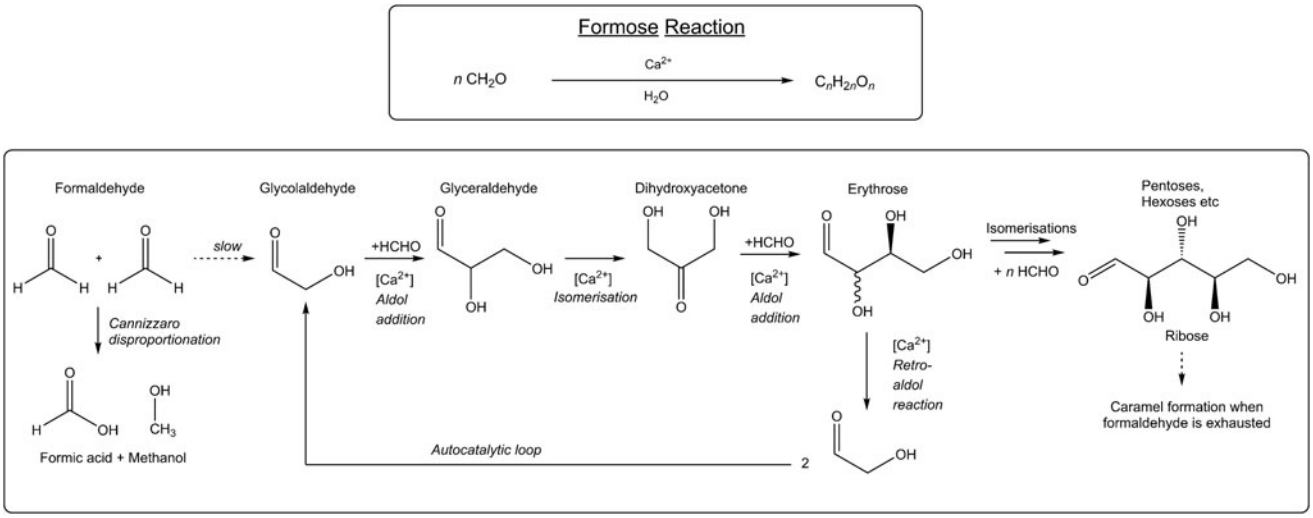

FIG. 1. Schematic representation of the formose reaction starting with the condensation of two formaldehyde molecules up to pentoses/hexoses. The reaction continues further and creates longer chain and branched sugars. Many side reactions take place alongside those depicted here but have been omitted for clarity.

prebiotic origins of sugars (Cleaves, 2008). The most popular theory, the formose reaction discovered by Butlerow (1861), shows that in alkaline conditions with a divalent metal ion catalyst, formaldehyde undergoes aldol condensation and isomerization reactions to form an autocatalytic network that generates a wide range of sugars, including biologically important species such as ribose (Fig. 1).

The formose reaction is feasible under a plethora of prebiotic scenarios, including hydrothermal (both subaerial [Deamer and Weber, 2010] and submarine [Omran, 2020]) systems, the interstellar medium (Meinert *et al.,* 2016), and small exogenous rocky bodies (*i.e.,* asteroids and comets [Pallmann *et al.,* 2018; Furukawa *et al.,* 2019; Haas *et al.,* 2020]). However, the reaction appears to be most efficient in aqueous, alkaline solutions at moderate temperatures, lending credence to an alkaline hydrothermal vent scenario. Evidence for prebiotic formaldehyde is emerging with Hudson *et al.* (2020) recently demonstrating that formic acid can be synthesized under mild pressure (<1.5 bar) in simulated Hadean hydrothermal vent conditions. Further reduction to formaldehyde may be possible at higher pressures due to higher quantities of dissolved H_2 (Wiebe *et al.,* 1932). Indeed, Herschy *et al.* (2014) did detect small amounts of formaldehyde under alkaline hydrothermal conditions but did not prove that it derived from bicarbonate.

A primary criticism of the formose reaction as a prebiotic source of sugars is that, even under ideal conditions, complex mixtures of isomeric and epimeric sugar species are produced. Biologically relevant species such as ribose are typically in the minority (Gollihar *et al.,* 2014). The formose reaction also suffers from very poor efficiency due to competition with the Cannizzaro disproportionation (Kopetzki and Antonietti, 2011) and other side reactions (Iqbal and Novalin, 2012). Worse, sugars will progress to caramelization reactions once the formaldehyde is exhausted, leading to the biochemically unproductive formation of tars (Reid and Orgel, 1967). In the presence of amine species, sugars also undergo the Maillard reaction and Amadori rearrangements.

Orgel (2004) acknowledged that if the formose reaction could be directed towards the synthesis of ribose, this would be an ideal prebiotic route to nucleotides. Mellersh and Smith (2010) suggested that a putative mechanism could be phosphorylation. In a microporous alkaline hydrothermal system, phosphorylation could promote the selective retention of sugars and funnel the formose reaction towards synthesis of ribose-phosphate. Pleasingly, because most sugars are phosphorylated in the gluconeogenic and pentose phosphate pathways, they proposed that this mechanism of directing the formose reaction towards selective synthesis is parsimonious.

Due to the poor solubility and reactivity of orthophosphates, their availability on early Earth has long been considered a problem for prebiotic chemistry (Pasek, 2008). Recent work by Whicher *et al.* (2018) has shown that the prebiotic phosphorylating agent acetyl phosphate (AcP) can be formed under alkaline hydrothermal conditions and is capable of phosphorylating ribose under those same conditions. AcP remains the fulcrum between thioester and phosphate metabolism in bacteria and archaea, being the (bound or unbound) intermediate in the substrate-level phosphorylation of ADP to ATP from acetyl CoA (Thauer *et al.,* 1977; Ferry and House, 2006; Schonheit *et al.,* 2016). AcP is immediately proximal to the acetyl-CoA pathway, one key intermediate of which is formaldehyde, albeit as a methylene group bound to a cofactor (H_4F or H_4MPT in acetogens and methanogens, respectively). This raises the possibility that in an alkaline environment, AcP might have been able to selectively funnel the formose reaction towards biologically relevant sugars in a manner like that proposed by Mellersh and Smith. We explore that possibility here by adding AcP to a formose reaction under simulated alkaline hydrothermal vent conditions using a mixed homogeneous/heterogeneous catalyst composed of $Ca(OH)_2$ and $CaCO_3$.

2. Materials and Methods

2.1. Materials

Reactants (formaldehyde, 16%; lithium potassium acetylphosphate, 85%; potassium phosphate dibasic, 99%) were purchased from Sigma-Aldrich. Catalysts ($CaCO_3$, 99% and

$Ca(OH)_2$, 95%) were purchased from Sigma-Aldrich. Sugar/sugar phosphates standards (glyceraldehyde, ≥98%; dihydroxyacetone, ≥98%, USP reference standard; D-ribose, 99%; D-arabinose, ≥98%; D-lyxose, 99%; D-xylose, ≥99%; D-fructose, ≥99%; 2-deoxy-D-ribose, 97%; D-erythrose 4-phosphate, ≥98%; D-glyceraldehyde 3-phosphate, ≥97%; D-glucose 6-phosphate, ≥98%; and D-ribulose, ≥97%) were purchased from Sigma-Aldrich. Derivatization reagents 3-amino-9-ethylcarbazole (AEC), 95%; sodium cyanoborohydride, 95%; and glacial acetic acid were purchased from Sigma-Aldrich, Acros Organics, and Fischer Scientific, respectively. High performance liquid chromatography (HPLC) grade solvents and additives ammonium acetate 99%, water, dichloromethane (DCM), hexane, acetonitrile, and methanol were all purchased from Fischer Scientific.

2.2. Formose reaction experiments

Formaldehyde, $Ca(OH)_2$, and $CaCO_3$ were diluted in HPLC water to achieve a final reaction volume of 2 mL. The initial reaction concentrations were 0.5 M for formaldehyde and 0.167 M for both calcium salts. When applicable, lithium potassium acetyl-phosphate or potassium phosphate dibasic were added to the formose reactions (400 mM) to study its effect. NaOH (5 M) was used to correct any changes in pH on addition of these additives, and equivalent volumes of HPLC water were added to controls to maintain equivalent concentrations. Reactions were carried out at 60°C in glass vials in a metallic block heater. Samples of 100 µL were taken at 0.5, 1, 2, 3, and 5 h and immediately frozen at −80°C.

2.3. Derivatization of sugars and sugar phosphates

Before derivatization, samples were dried under anhydrous N_2 gas (95%, BOC) to remove any unreacted formaldehyde and subsequently resuspended in HPLC water to the initial volume. For derivatization, the AEC method was used as developed by Han *et al.* (2013). A volume of 50 µL of formose reaction sample was mixed with 100 µL of 238 mM AEC, 50 µL of 476 mM sodium cyanoborohydride, and 20 µL of glacial acetic acid, and incubated in metallic block heaters at 70°C for 1 h. Immediately after, reaction samples were cooled on ice for 1 min.

2.4. Liquid-liquid extraction

A volume of 300 µL of water and of 300 µL of 2:1 DCM and hexane were added to the 220 µL derivatized reaction,

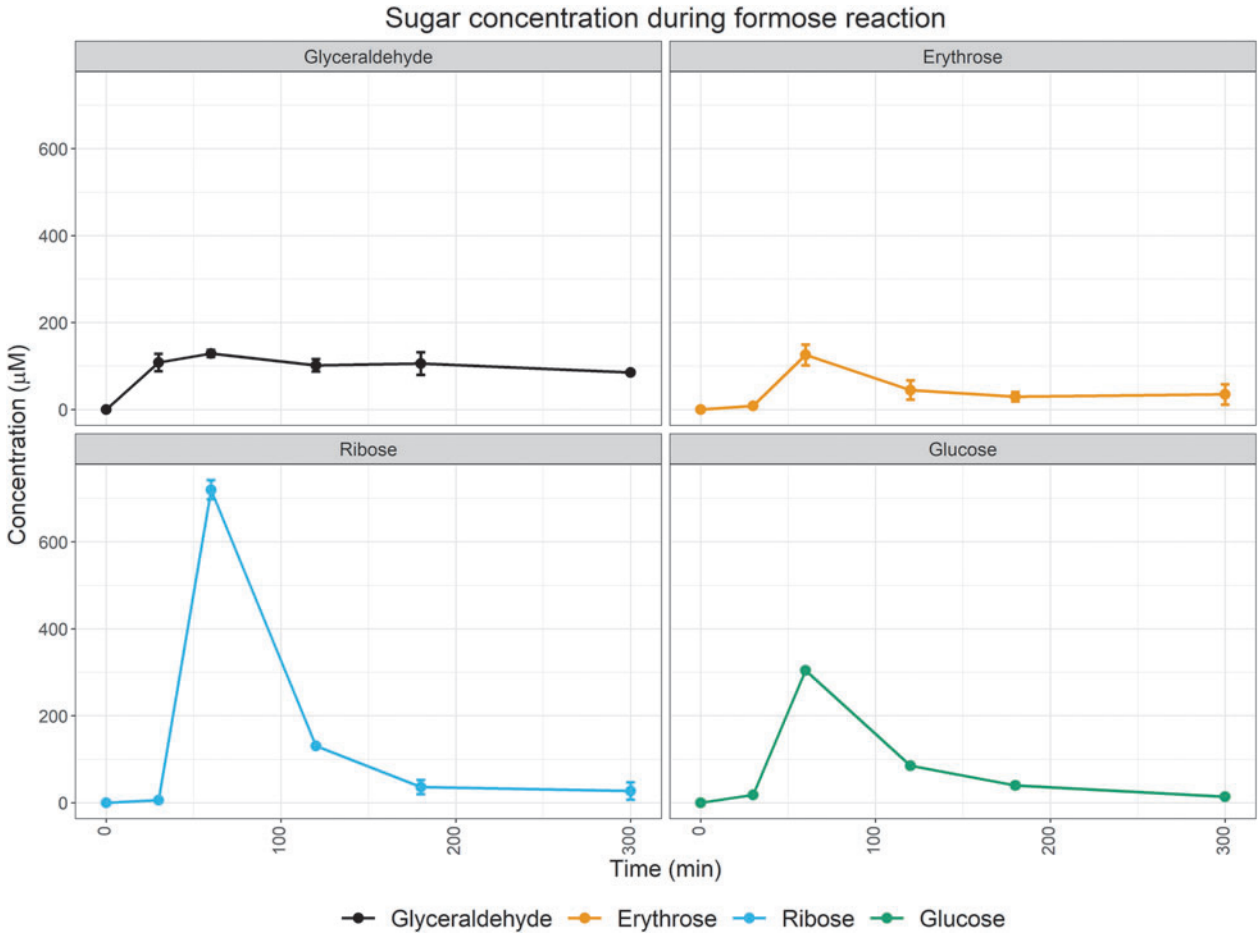

FIG. 2. Sugar concentrations throughout the formose reaction. Time course of sugar concentrations as determined after 3-amino-9-ethylcarbazole (AEC) derivatization, HPLC separation and UV detection. Sugar species have been color coded: black = glyceraldehyde (3C), yellow = erythrose (4C), blue = ribose (5C), and green = glucose (6C). Sugars (together with their chromatographically equivalent epimers) were identified based on their retention time relative to standards. The reaction pH was maintained at 11.5 with 5 M NaOH as required. $N = 3 \pm$ SD.

vortexed and centrifuged at 10,621 rcf for 5 min. A volume of 450 µL of the upper aqueous phase was collected into a separate Eppendorf tube. The extraction step was then repeated two subsequent times with 250 and 300 µL collected, respectively. A volume of 500 µL 2:1 DCM:hexane was then added to this 1 mL volume and was vortexed and centrifuged again. A volume of 750 µL of the upper phase was collected and used for solid phase extraction.

2.5. Solid phase extraction

Residual calcium ions were removed to prevent HPLC instrument damage. Thermo-scientific Hypersep 500 mg 2.8 mL C-18 cartridges were conditioned with 3 mL of methanol, followed by 3 mL of water. The derivatized sample mixture was passed through the matrix. The columns were washed with 1 mL of HPLC water. The derivatized sugars were eluted with 3 mL of 80% aqueous methanol.

2.6. High performance liquid chromatography–ultraviolet (HPLC-UV) analysis

Sugar quantification was achieved using high performance liquid chromatography–ultraviolet (HPLC-UV) analysis. Samples were analyzed using an Agilent 1260 Infinity II LC system with UV detection. Derivatized sugars were separated on Agilent InfinityLab Poroshell 120 EC-C18 (4 mm, 150×4.6 mm) with a 5 mm guard column. Samples were analyzed using a method adapted from Han *et al.* (2013). Elution occurred under isocratic conditions, 75% mobile phase A (0.1 M ammonium acetate pH 4.5) and 25% mobile phase B (acetonitrile) with runs of 60 min. The flow rate was 0.5 mL/min, column was heated to 40°C, the injection volume was of 10 µL, and the UV detector was set to 254 nm.

2.7. Liquid chromatography–mass spectrometry (LC-MS) analysis

Liquid chromatography–mass spectrometry (LC-MS) assays were performed on formose reaction samples in order to provide an unequivocal peak assignation for each sugar and as secondary verification. These analyses were performed using a Thermo HPLC Accela 600 pump and autosampler LC system connected to a Thermo Finnigan LTQ mass spectrometer. The mobile phases were A: 0.05 M ammonium acetate in water (pH 4.5) and B: pure acetonitrile. We used a Thermo Hypersil Gold C18 (1.9 mm, 150×2.1 mm) column at 40°C. The flow rate was adjusted to 0.18 mL/min, and the mobile phase was kept isocratic at 75% A with a run time of

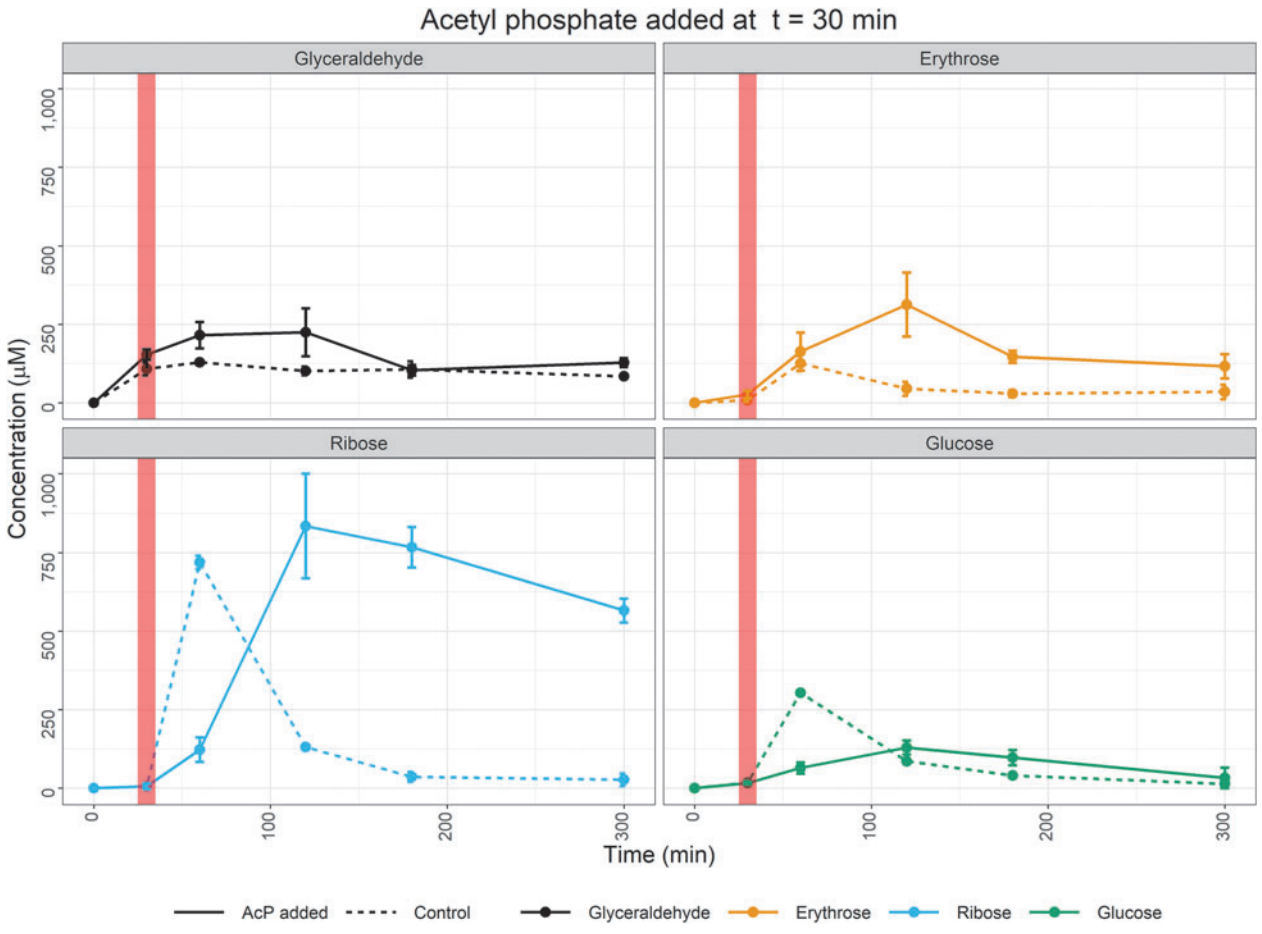

FIG. 3. Addition of acetyl phosphate to formose reaction at $t = 30$ min. Time course of sugar concentrations as determined after AEC derivatization, HPLC separation and UV detection. Solid lines indicate the reaction with acetyl phosphate added; dashed lines indicate control experiments for comparison (shown in Fig. 2). The red vertical bar indicates the point acetyl phosphate was added to the reaction mixture. The reaction pH was maintained at 11.5 with 5 M NaOH as required. $N = 3 \pm$ SD.

50 min. The injection volume was 10 μL, and the ion source was ESI+, with a normalized collision energy of 35. The masses (in *m/z*) monitored were 375.4 (glucose), 455.4 (glucose 6-phosphate), 345.2 (ribose), 425.2 (ribose 5-phosphate), 315.3 (erythrose), 395.1 (erythrose 4-phosphate), 285.1 (glyceraldehyde), 365 (glyceraldehyde 3-phosphate), and 359.2 (fucose; used as external standard).

3. Results

3.1. Formose reaction topology

The topology of the formose reaction network varies depending on the physicochemical conditions under which it is performed. To understand the effect AcP addition had, a basal understanding of the network under the conditions tested was required. Figure 2 shows the standard reaction kinetics for the formose reaction under our catalytic conditions. Maximum sugar concentration occurred close to 1 h into the formose reaction. As such, control reactions were performed alongside all reactions. Smaller sugars increase in concentration before the heavier ones, which is in line with the known formose reaction mechanisms. After 1 h, the concentration of all sugars decreased, and a yellow coloration appeared. This is indicative of formaldehyde exhaustion and sugar caramelization.

3.2. Addition of acetyl phosphate

The addition of AcP at the start of the reaction prevented the formose reaction network from forming. No AEC-derivatized species were detectable by LC-MS or HPLC-UV. This was initially postulated to be due to interference from AcP in the early stages, preventing progression to a reaction network. Addition of AcP after 30 min provided enough time for the network to initiate, so its effect on the reaction became observable. Figure 3 shows that on addition of AcP to the reaction, all sugars presented an improved stability persisting in the system for longer relative to the control. Pentoses such as ribose appeared to be particularly stabilized, remaining at high levels until the end of the reaction. The reaction kinetics appear to have undergone a shift with the peak sugar concentration occurring at $t = 120$ min compared to the control ($t = 60$ min).

This effect raises the question of whether AcP promoted sugar synthesis by interfering in the aldol reactions producing sugars, or by selectively stabilizing the sugars once formed. AcP was added at $t = 60$ min to investigate this. At this time point, most of the starting formaldehyde should have been consumed, so later stage reactions were expected to be taking place. The addition of AcP at 1 h showed selective stabilization of pentoses and hexoses with maximal

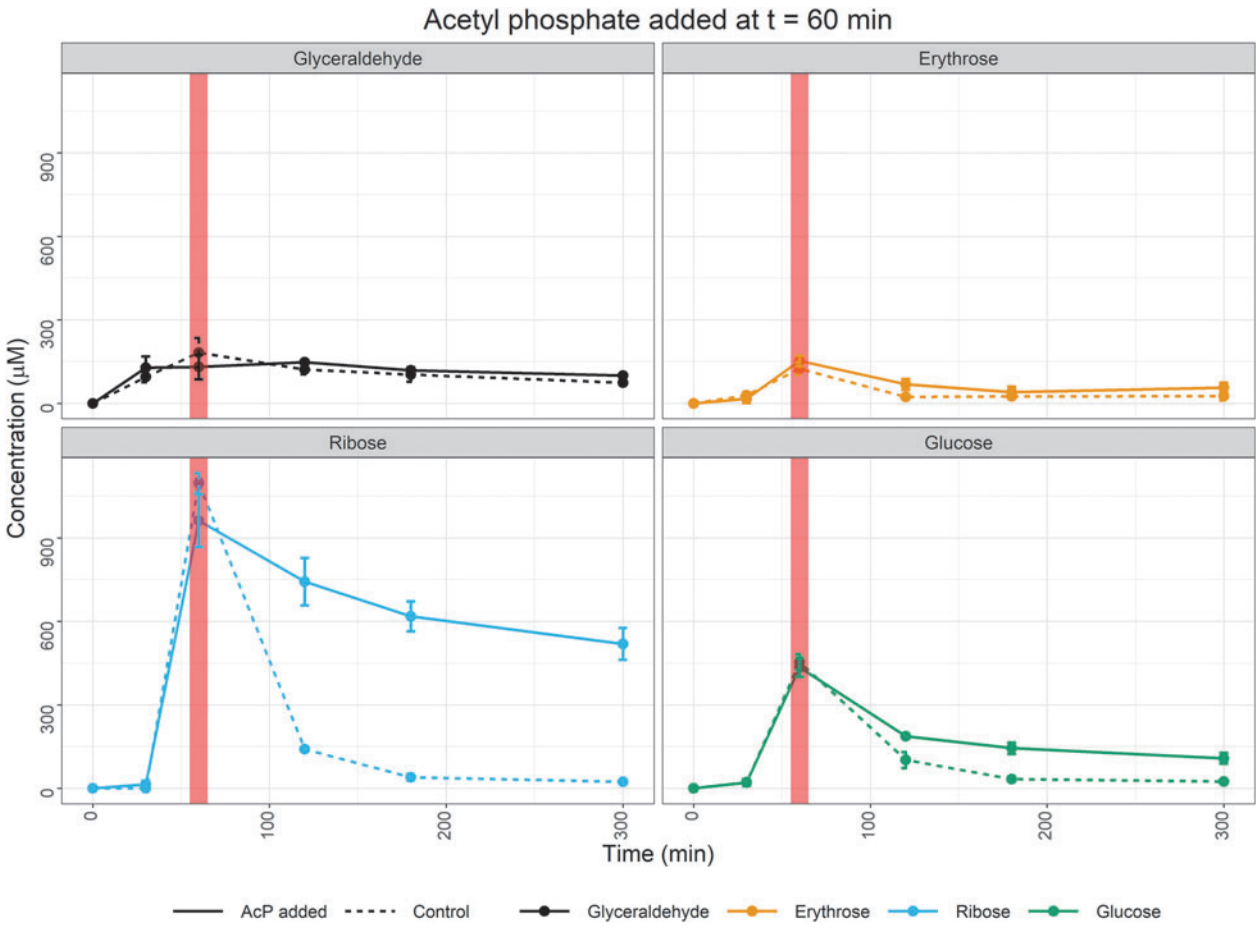

FIG. 4. Addition of acetyl phosphate to formose reaction at $t = 60$ min. Time course of sugar concentrations as determined after AEC derivatization, HPLC separation and UV detection. Solid lines indicate the reaction with acetyl phosphate added; dashed lines indicate control experiments for comparison. The red vertical bar indicates the point acetyl phosphate was added to the reaction mixture. The reaction pH was maintained at 11.5 with 5 M NaOH as required. $N = 3 \pm$ SD.

yield of all species again at $t = 60$ min (Fig. 4). These results suggest AcP contributes to the increased stability of sugars.

3.3. The role of acetyl phosphate

The previous results indicate that the addition of AcP influenced the late stages of the formose reaction, slowing down the degradation and potentially delaying the point of maximal sugar production. AcP did not increase the yield of any sugars relative to the corresponding point in the controls. AcP could have exerted this stabilizing effect through multiple mechanisms, for example directly by the phosphorylation or acetylation of sugars, but also by simply disturbing the pH of the reaction.

Addition of AcP caused a rapid drop in the pH, as we did not buffer our experiments, and AcP is acidic relative to the alkaline reaction conditions. We added NaOH to maintain the reaction pH at that of a control formose reaction. However, there was a transient drop in reaction pH to ~7 which lasted <30 s. To verify whether this transient pH drop was responsible for the observed stabilization, HCl was added instead of AcP to mimic the transient pH drop. Figure 5 shows that the addition HCl and its transient neutralization of the reaction solution did slightly delay the degradation/usage as fuel of ribose, but this did not fully explain the observed effects from the addition of AcP.

3.4. Addition of acetyl phosphate versus orthophosphate

The selective stabilization of sugars, particularly of pentoses, was the primary effect observed on the addition of AcP. Since this was not due to pH changes, the observed effect was due to either the reactivity of AcP itself or the presence of its phosphate and/or acetate moieties in the solution after its hydrolysis. Given that the most significant effect was for pentoses, the following study focused on ribose only.

The addition of disodium hydrogen phosphate to the reaction mixture showed nearly identical stabilization phenomena when compared to the addition of AcP (Fig. 6), strongly suggesting that the phosphate moiety of AcP is the major contributor to the observed stabilization. The reaction kinetics for the two reactions were not significantly different, with complete overlap of error bars throughout the reaction. These results suggest that AcP-mediated phosphorylation does not play a role in the observed stabilization of pentose sugars under hydrothermal formose conditions.

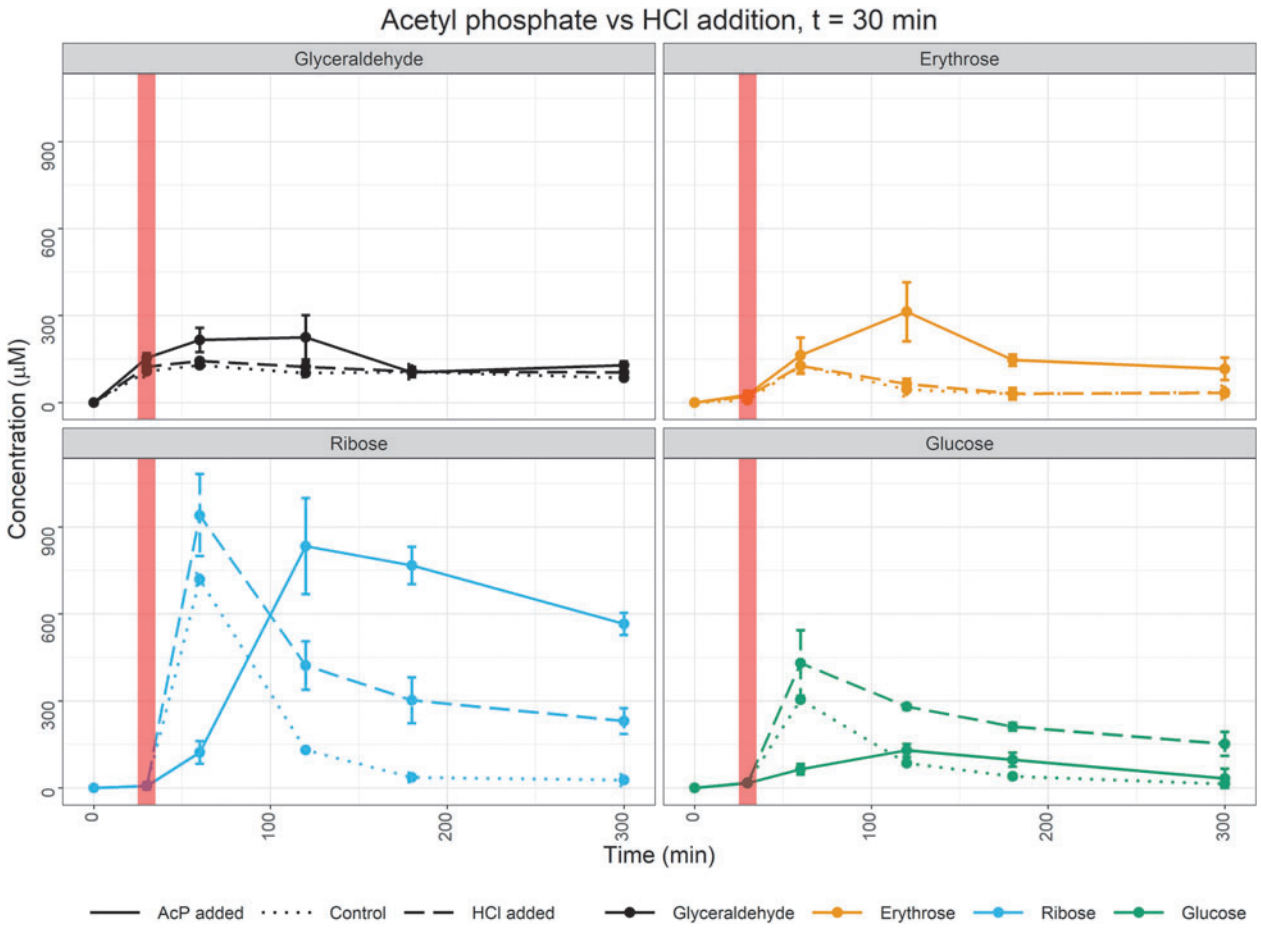

FIG. 5. Addition of acetyl phosphate versus transient HCl addition. Time course of sugar concentrations as determined after AEC derivatization, HPLC separation and UV detection. Solid lines indicate the reaction with acetyl phosphate added, long-dashed lines indicate reaction with HCl addition, and short-dashed lines indicate control experiments. The red vertical bar indicates the point acetyl phosphate was added to the reaction mixture. The reaction pH was maintained at 11.5 with 5 M NaOH as required. $N = 3 \pm$ SD.

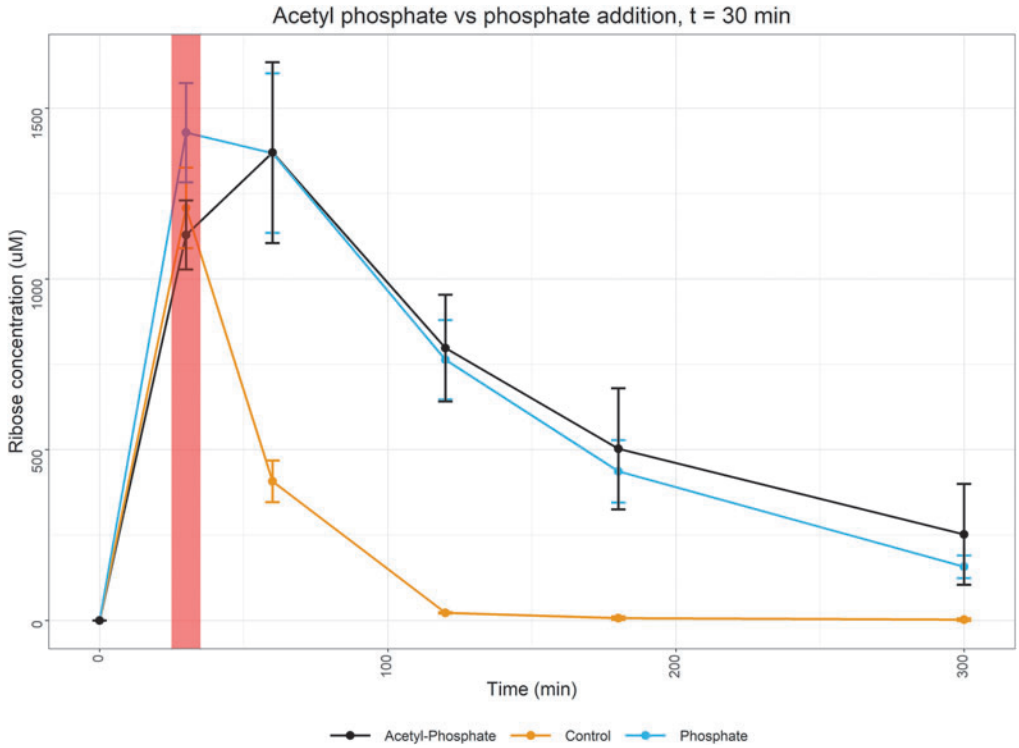

FIG. 6. Ribose concentrations during the formose reaction with acetyl phosphate or phosphate additions. Time course diagram of ribose concentrations as determined after AEC derivatization and HPLC-UV separation. The red vertical bar indicates the addition of acetyl phosphate, phosphate, or water at $t = 30$; pH was adjusted back to 11 with 5 M NaOH. $N = 3 \pm$ SD.

3.5. Mass spectrometry analyses

Even though phosphorylation did not seem to be the cause of pentose stabilization, phosphorylated or acetylated sugars may still form in the reaction media in limited amounts (Whicher *et al.,* 2018) and could still be relevant to the origins of life. Mass spectrometry techniques were therefore used to analyze derivatized formose samples from both control and AcP experiments to explore changes in product spectra. Samples were taken from a 90 min time point (from reactions shown in Fig. 4) to ensure similar concentration of sugar species.

Figure 7 shows that the product distribution between the control and the AcP experiments did not change, indicating that there was no change in the dynamics of the formose network. There was no significant covalent modification of sugars in either reaction. Acetylated sugars could not be identified in either spectrum, and while phosphorylated sugars were present in the AcP sample, their concentration was below the limit of quantification for the analytical method used.

Deoxysugars were present in both samples, which is in line with the observations by Kopetzki and Antonietti (2011) in their hydrothermal formose experiments. Their concentration may increase on AcP addition, but without appropriate standards it was difficult to investigate further.

3.6. Changes in the heterogeneous catalyst

These data show that the stabilization of pentoses depends on the time at which AcP or orthophosphate was added to the reaction. Addition at the start of the reaction inhibited the formation of sugars. AcP addition prior to the yellowing point (Kopetzki and Antonietti, 2011) ensured that the yellowing point was never reached (Fig. 8A), while addition during or after the yellowing point (30–60 min in) stabilized pentoses. This implies that the addition of phosphate groups inhibits the chemistry of the formose reaction preventing both aldol and caramelization reactions from taking place.

When AcP or orthophosphate was added to mixtures of the catalyst (without formaldehyde), the nature of the solid catalyst changed—an increase in volume and a slight color shift to a more brilliant white (Fig. 8B). This strongly suggests the precipitation of soluble calcium, likely as a mixture of basic calcium phosphates ($Ca_2(PO_4)_3$) and hydroxyapatites ($Ca_5(PO_4)_3OH$). This conclusion is supported by the K_{sp} (solubility product constants) for calcium phosphate (2.07×10^{-33}) and hydroxyapatite (2.91×10^{-58}) compared with calcium hydroxide or calcium carbonate (5.02×10^{-6} and 3.36×10^{-9}, respectively) (Bell *et al.,* 1978; Lide, 2007).

4. Discussion

In line with the current understanding of the formose reaction, our results show that under submarine alkaline hydrothermal conditions a wide range of monomeric sugars, including target molecules such as ribose, are produced (Fig. 2).

The addition of AcP at the start of the experiment inhibited the formation of any sugars, even when controlling for pH changes. The addition of AcP after the reaction network had established itself stabilized most sugars, but with no increase in overall yields (Figs. 3 and 4). This

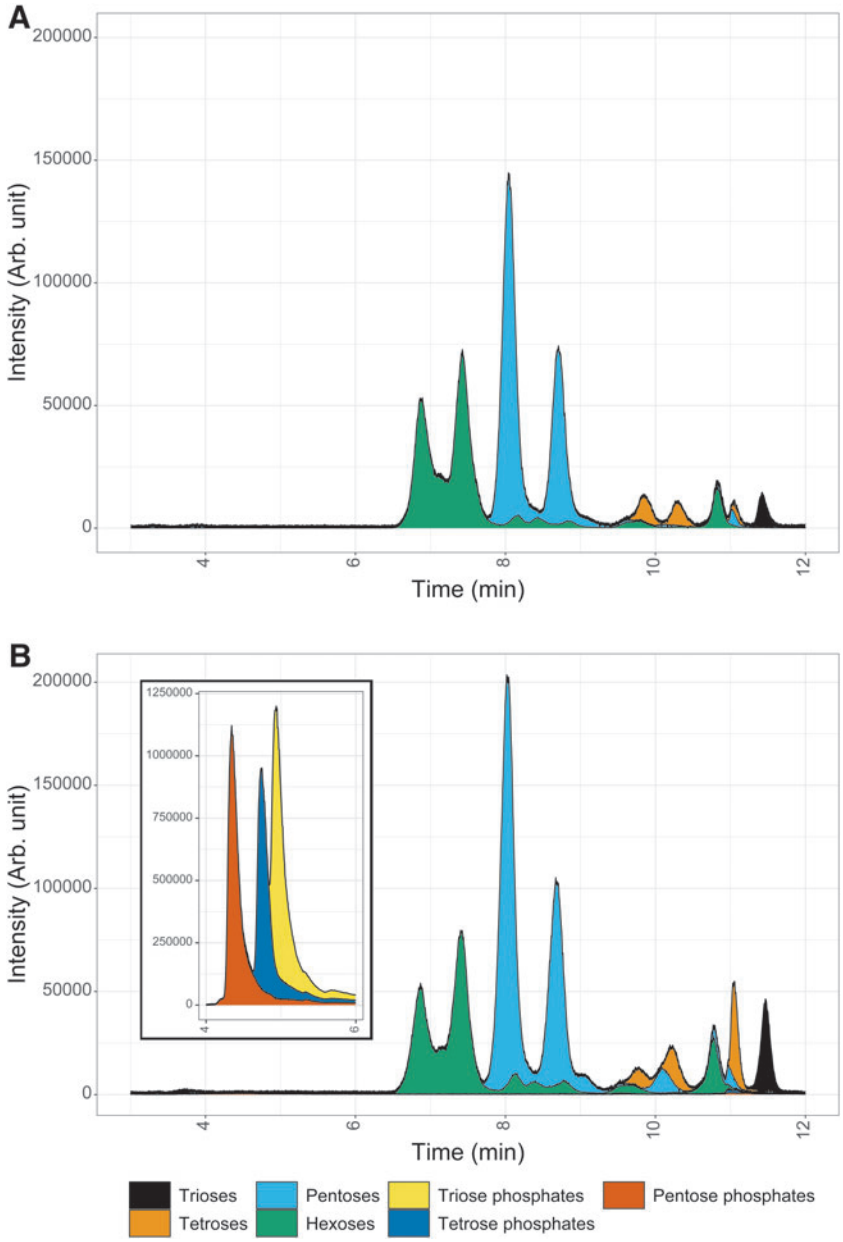

FIG. 7. Representative mass spectra for formose reactions with and without acetyl phosphate. (**A**) Control formose reaction. (**B**) Formose reaction with AcP addition at $t = 60$. Inset in (B) is representative spectra for three sugar phosphate standards: ribose-5-phosphate, erythrose-4-phosphate, and glyceraldehyde-3-phosphate. Both samples were taken from reactions at $t = 90$ min, derivatized using the same AEC method. Data is presented as intensity in arbitrary units from the mass spectrometer detector.

stabilization was most pronounced for pentose sugars. Together these data show that the addition of AcP facilitated a change in the degradation or consumption of sugars in established formose networks. This change in kinetics was not attributable to a transient change in pH (Fig. 5) but to the addition of the phosphate moiety (Fig. 6). Phosphate has known stabilizing effects on ribose (Nitta *et al.,* 2016). But the product spectra, as determined by mass spectroscopy analyses, did not show any change in the product distribution of isomer species (Fig. 7), nor the appearance of significant quantities of covalently modified species (either acetylated or phosphorylated sugars).

Given that calcium phosphate (apatite) and hydroxyapatite both have extremely low K_{sp} values, and that there were visible changes of the heterogenous catalyst (Fig. 8), it is likely that the stabilization of pentoses was simply due to the precipitation of all free Ca^{2+} leading to a near-total inhibition of formose chemistries. Ca^{2+} ions stabilize the enediolate state of sugars, an essential intermediate state in aldol reactions, and their removal heavily impacts formaldehyde condensation. Calcium likely precipitated as an apatite-hydroxyapatite mixture, but we did not analyze the precipitate. In order to obtain a finer picture of the effect the heterogenous catalyst had on the overall reactivity, a proper study of the catalyst composition should be carried out for future work focusing on this—or similar—set of reaction conditions. While calcium minerals such as apatite are capable of formose chemistries (Usami and Okamoto, 2017), this takes place over significantly longer timescales than those explored in this paper.

These findings indicate a serious problem with phosphorylation as a mechanism of refining a hydrothermal formose process towards the formation of biological sugars. While calcium ions are not the only catalyst capable of aldol chemistry, they are one of the most prebiotically relevant (*e.g.,* Estrada *et al.,* 2019) and abundant inorganic catalysts

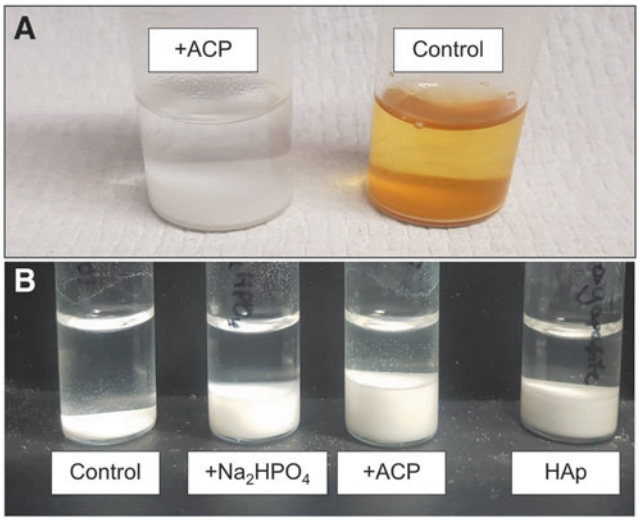

FIG. 8. Changes in catalyst on addition of AcP/Pi. (**A**) Reaction vessels after 5 h of formose reaction, left to cool. When AcP was added to the reaction, the reaction never progressed to the ''yellowing point.'' (**B**) Changes in catalyst volume on addition of AcP/Pi. Addition of AcP or orthophosphate to the $CaOH/CaCO_3$ catalyst caused significant expansion of the heterogeneous phase. Hydroxyapatite (HAp), the likely dominant precipitate, is shown for reference.

in submarine hydrothermal conditions (Kelley *et al.,* 2001). Our results suggest that simple phosphate currencies and a calcium catalyzed formose reaction may be mutually exclusive, and that ''high-energy'' phosphate species are consumed before having the opportunity to phosphorylate species in relevant yields.

These observations are specific to a one-pot reaction setting—wherein all reactions are combined at the start of the reaction and expected to persist until required—and utilize a reaction setup designed to proceed rapidly (5 h from start to finish). Any delayed synthesis with the modified catalyst after 5 h could not be observed in this study. In addition, the reaction conditions used are only of modest geological plausibility, as we focused on proof of principle chemical interactions. The percolating nature of alkaline hydrothermal vent systems (Russell *et al.,* 1994; Martin *et al.,* 2008), much like those proposed by Mellersh and Smith (2010), provide heterogeneous settings that can facilitate the synthesis of different molecules in distinct microenvironments with independent chemistries, while still allowing later mixing. As such, the phosphorylation processes and sugar synthesis may proceed independently before later combination. Nonetheless, these considerations must impose tight limits on the use of phosphorylation inside protocells unless and until Ca^{2+} ions could be excluded. Whether Ca^{2+} influx could be limited by simple bilayer membranes composed of mixed amphiphiles is an interesting question (Jordan *et al.,* 2019a, 2019b). It is also possible that reverse Krebs cycle intermediates such as citrate could chelate Ca^{2+} ions inside protocells, but we have not explored whether chelated Ca^{2+} could still drive the formose reaction without precipitating out as a phosphate mineral. It seems unlikely that this would provide a general solution to the issues identified here.

The stabilization of pentoses relative to other sugars reported here is of no small relevance for prebiotic chemistry. Even with the depletion of calcium, the sugars remain in a highly alkaline solution ~pH 12 and at 60°C. This may seem trivial, but one of the principal criticisms of ribose as a prebiotic sugar is its poor stability (Larralde *et al.,* 1995). Our data clearly show that even under such harsh conditions pentoses like ribose persist relative to other sugars. Work by Usami and Okamoto (2017) suggests that this stabilization could be attributable to the new precipitate formation. These authors show that ribose is the favored product of a formose reaction when hydroxyapatite is the main catalyst. Their reactions were considerably longer than ours (128 vs. 5 h), potentially explaining the lack of rapid sugar formation on early addition of AcP.

5. Conclusions

The formose reaction is a leading hypothesis for the prebiotic synthesis of sugars. Its chemical simplicity, compatibility with early Earth conditions, and efficiency make it an attractive proposal. The primary failing of the formose reaction is its lack of specificity for which isomers are formed. As such, an efficient formose-based synthesis of ribose has become something of a holy grail to the prebiotic chemist (Raos, 2018).

The intriguing proposal by Mellersh and Smith (2010) prompted an investigation into whether a biologically reminiscent phosphorylation of sugar intermediates by a prebiotic phosphorylating agent could channel the formose reaction towards biochemically relevant sugars such as ribose. We observed that adding a phosphorylating species to a classical calcium-dependent formose reaction appears to have an identical effect to the addition of inorganic phosphate, with both causing a rapid precipitation of free calcium and termination (or at least significant slowdown) of the reaction network. An interesting stabilization of pentoses is seen in the remaining alkaline solution, independent of direct phosphorylation, suggesting the possibility of stabilizing chelation—similar to that exerted by borate ions (Ricardo, *et al.,* 2004)—or precipitate surface-mediated stabilization of such essential species. These data present an interesting conundrum: either phosphorylating agents are incompatible with the original prebiotic source of sugars, or the classical formose conditions are not representative of a real Hadean Earth environment—or an alternative pathway for the synthesis of sugars occurred at the origins of life.

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Abbreviations Used

AcP = acetyl phosphate
AEC = 3-amino-9-ethylcarbazole
DCM = dichloromethane
HPLC = high performance liquid chromatography
HPLC-UV = high performance liquid chromatography–ultraviolet
LC-MS = liquid chromatography–mass spectrometry