Removal of Paramagnetic Ions Prior to Analysis of Organic Reactions in Aqueous Solutions by NMR Spectroscopy

Rio Febrian,† Joseph P. Roddy,‡,† Christine H. Chang,‡ Clinton T. Devall,† and Paul J. Bracher*‡

†Department of Chemistry, Saint Louis University, 3501 Laclede Avenue, St. Louis, Missouri 63103, United States
‡Division of Chemistry & Chemical Engineering, California Institute of Technology, 1200 E. California Blvd., Pasadena, California 91125, United States

Supporting Information

ABSTRACT: This article describes a method for improving 1H NMR spectra of aqueous samples containing paramagnetic metals by precipitation of metal cations with a variety of counteranions. The addition of hydroxide, phosphate, carbonate, and arsenate to solutions of transition metals such as Fe2+ and Mn2+ can reduce line broadening and improve the ability of a spectrometer to lock on the signal of deuterium. The method is most effective under strongly alkaline conditions, and care must be taken to observe whether the organic substrates undergo side reactions or are themselves removed from solution upon addition of the precipitating salts. As a demonstration of the practical value of the method, we show that NMR spectroscopy can be used to monitor the transition-metal-mediated hydrolysis of glycylglycine (Gly2).

INTRODUCTION

NMR spectroscopy is one of the most powerful methods for the characterization of organic compounds and study of organic reactions. Paramagnetic species can render NMR spectroscopy impracticable by broadening spectral peaks and hindering the ability of spectrometers to lock on the signal of deuterium. In this article, we explore the precipitation of paramagnetic cations with a variety of counteranions as a means to improve 1H NMR spectra sufficiently to measure rates of organic reactions in water. We show that—in certain circumstances—the removal of paramagnetic ions by these methods can decrease line broadening in spectra without initiating side reactions or affecting the concentration of the organic solutes present. We demonstrate this method by using it to measure the hydrolysis of a peptide in concentrated (1 M) solutions of various transition metals, for which direct analysis by NMR spectroscopy would otherwise be impossible. We discuss several significant limitations to the method, particularly its requirement for strongly alkaline conditions and the potential for the solid phase to adsorb organic molecules from the mother liquor.

Background. We are interested in studying the kinetics of reactions of biologically relevant organic compounds in water to evaluate theories that propose roles for these molecules in the origin of life. In a previous study, we measured rate constants for the hydrolysis of thioesters using 1H NMR spectroscopy to monitor the change in concentration of reactants over time. The samples in this study were relatively simple solutions in water with a buffer salt (to maintain a constant pH and enable pseudo-first-order kinetics) and an internal standard (to measure accurate concentrations of the thioester). Other studies have shown that transition metal ions can affect the reactivity of peptides and thioesters. Given that the prebiotic ocean was unlikely to have resembled a clean system as water with a mild pH buffer, we wished to explore the effect of various dissolved metals on reactions of prospective prebiotic relevance.

The universal dependence of modern biology on water suggests that life originated in an aqueous environment. Scientists disagree over the composition of the prebiotic ocean, but it is widely assumed that several aqueous metal ions were present. In addition to diamagnetic metals such as Na+, K+, Mg2+, and Ca2+, paramagnetic metals such as Fe2+, Mn2+, and Co2+ are also conjectured to have been present in Earth’s ancient ocean. Many theories and experiments regarding prebiotic chemistry invoke conditions where reaction mixtures are subjected to wet–dry cycles of hydration (e.g., by rain) and evaporation. The process of evaporation of a trapped body of water introduces the potential for significantly higher concentrations of metal ions than in the prebiotic ocean itself—presumably approaching their limit of solubility with whatever counteranions are present in the system.

Paramagnetic ions can present a considerable challenge to analysis by NMR spectroscopy. The broadening of peaks in samples containing paramagnetic species has been studied extensively. As the magnetic susceptibility (χ) of an aqua...
ion increases, the broadening of peaks in the NMR spectrum of a sample also increases. This broadening can complicate spectra by causing peaks to overlap that would otherwise resolve cleanly. Figure 1 documents how increasing the concentration of Mn$^{2+}$ from 0 to 10 mM broadens the signals in $^1$H NMR spectra of the simple peptide Ala$_2$, whereas further increases in concentration make the spectra unintelligible. Millimolar concentrations of paramagnetic ions often make it difficult for spectrometers to attain a stable lock because of the broadening of the deuterium signal. The broadened signal for water can overrun signals of the analyte. To monitor the kinetics of reactions in the presence of large concentrations of paramagnetic species, a method to remove or quench these species prior to analysis is necessary.

Strategies to remove paramagnetic species or reduce their influence during analysis by NMR spectroscopy have been described in the literature.\textsuperscript{24−26} The general methods most commonly reported are chelation and precipitation. Ding and co-workers improved \textsuperscript{31}P NMR spectra of environmental samples that contained paramagnetic metal ions, including Fe$^{3+}$ and Mn$^{2+}$, by 8-hydroxyquinoline-assisted precipitation.\textsuperscript{27} In another approach, Barge et al. removed Fe$^{3+}$ and Fe$^{2+}$ by precipitation with hydroxide prior to analysis by $^1$H NMR spectroscopy.\textsuperscript{28} Other approaches have attempted to improve NMR spectra of paramagnetic samples by altering the parameters of the acquisition experiment. Although helpful for qualitative measurements, these methods are sometimes less effective for experiments that require quantitative accuracy, such as kinetics measurements.\textsuperscript{29,30}

\section*{RESULTS AND DISCUSSION}

In developing a method for the analysis of organic reactions in water containing paramagnetic transition metals, it was clear that any method used to prepare samples for analysis by NMR spectroscopy should: (i) be simple, (ii) be rapid, (iii) be inexpensive, (iv) remove enough of the paramagnetic metal to enable the instrument to lock on the signal of deuterium, (v) remove enough of the metal such that at least one peak in the spectrum of the substrate was resolved from the rest of the spectrum of the reaction mixture, and (vi) not affect the concentration of the organic substrate present in the sample.

In searching for a more straightforward alternative to adding new reagents to our samples, we first attempted to precipitate the paramagnetic species present by the addition of a less polar solvent that was miscible with water (e.g., dimethylsulfoxide or tetrahydrofuran). We found that adding quantities as high as 3:1 (v/v, organic solvent/water) did not remove enough metal to be effective. Methods such as liquid−liquid extraction into an organic phase or evaporation of the solvent followed by solid−liquid extraction into a deuterated organic solvent seemed too impractical to attempt.

Inspired by previous reports,\textsuperscript{27,28} we pursued the idea of precipitating insoluble salts of the paramagnetic metals as a means of preparing aliquots of reaction mixtures for subsequent analysis by NMR spectroscopy. Our primary goal was to find alternatives to the use of hydroxide, as we knew that many substrates of presumed prebiotic importance—similar to thioesters—hydrolyze rapidly in strongly alkaline conditions. For instance, thioesters are not stable at high pH. S-Methylthioacetate, the simplest example of an alkyl thioester, has a half-life of 43 s at pH 13 and a half-life of 7.2 min at pH 12. A quick survey of the solubility product constants ($K_{sp}$) for a variety of inorganic salts of our target metals suggested that carbonate (CO$_3$$^{2−}$), phosphate (PO$_4$$^{3−}$), and arsenate (AsO$_4$$^{3−}$) could be particularly effective (see Table S1 in the Supporting Information). Seeking to extend our previous work,\textsuperscript{3} we selected the transition-metal-mediated hydrolysis of peptides and thioesters as model reactions to monitor by the method.

Figure 2 depicts the basic procedure for the precipitation method. A stock solution of the precipitating agent was dispensed into an aliquot of a reaction mixture, and the resulting solid was isolated by centrifugation. The supernate was mixed with an equal volume of deuterated water (D$_2$O) for analysis by NMR spectroscopy. In general, the method was able to remove enough paramagnetic metal (e.g., Fe$^{3+}$ or Mn$^{2+}$) from the solutions to permit the NMR spectrometer to lock on the signal of deuterium, (v) remove enough of the metal such that at least one peak in the NMR spectrum of the substrate was resolved from the rest of the spectrum of the reaction mixture, and (vi) not affect the concentration of the organic substrate present in the sample.

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observed in a sample to which we added Chelex, a metal−
chelating solid resin, rather than a precipitating anion.

To construct a quantitative evaluation of the effect
of phosphate as a precipitating agent, we screened its use against
a variety of paramagnetic metals. In these experiments, 250 μL
of 100 mM K3PO4 was added to 250 μL aliquots of 25 mM
Gly2 with 20 mM MnCl2, CuCl2, FeCl2, FeCl3, CoCl2, or
NiCl2. The resulting NMR spectra appear in Figure S2 in the
Supporting Information. We used inductively coupled plasma−
online emission spectroscopy (ICP−OES) to measure the
concentration of the paramagnetic metals in solution both
before and after precipitation. In general, the samples that had
lower remaining concentrations of metals produced better
spectra (e.g., Mn2+ and Co2+) than the samples that had larger

Table 1. Comparison of 1H NMR Spectral Integration
Following Treatment of a Sample Containing 25 mM Gly2
and 20 mM Mn2+.

| sample | downfield peak integration (A) | upfield peak integration (B) | sum of Gly2 integration | full width half maximum (Hz) |
|--------|-------------------------------|-------------------------------|------------------------|-----------------------------|
| no salt (control) | N/A | N/A | N/A | 334.67 |
| K3PO4 added | 1.01 | 1.08 | 2.09 | 4.03 |
| K2CO3 added | 0.58 | 0.20 | 0.78 | 7.69 |
| Na3HAsO4 added | 0.58 | 0.63 | 1.21 | 5.52 |
| KOH added | 0.70 | 0.85 | 1.55 | 4.94 |
| Chelex added | N/A | N/A | N/A | 51.88 |

The spectrum of the nonprecipitated control sample could not be
integrated because of catastrophic line broadening.

residual concentrations of metal (e.g., Fe3+). This observation
is consistent with both Mn3(PO4)2 and Co3(PO4)2 having Ksp
values over 10 orders of magnitude lower than the Ksp value of
FePO4.

NMR spectra of the same samples with water suppression
made it evident that the method is not without its faults. Some
Figure S4 showed that increasing the concentration of the metal removes. The results summarized in Table S7 and the concentration of Mn²⁺ remaining in the precipitated phosphate stock from 10 to 100 mM resulted in a reduction of the effects of pH on the metal removed. The results summarized in Table S7 and Figure S4 showed that increasing the concentration of the phosphate stock from 10 to 100 mM resulted in a reduction of the concentration of Mn²⁺ remaining in the precipitated sample from 14 mM (at pH 6, giving a poor-quality spectrum) to 0.4 mM (at pH 12, giving a spectrum that could be integrated). Increasing the equivalents of precipitating ions to the metal removes an increasing amount of metal but comes with the cost of raising the pH of the precipitated solution.

Furthermore, we treated a mixture of 25 mM Gly₂ + 28 mM MnCl₂ with 100 mM of K₃PO₄, K₂HPO₄, or KH₂PO₄ to assess the effects of pH on the metal removed. The results are summarized in Table S6 and Figure S3. The pH of the final precipitated solutions were 12, 8, and 5, respectively. The final concentrations of Mn²⁺ were 0.4, 1.2, and 22.3 mM, respectively. Thus, for equal concentrations of phosphates, increasingly alkaline conditions favored removal of more metal and significantly improved NMR spectra.

These results constitute a major limitation of the method: some Ksp values of the paramagnetic salts are not sufficiently low to remove enough of the paramagnetic cation from solution to enable analysis by NMR spectroscopy. The limitation becomes a greater challenge as the pH of the solution becomes increasingly acidic. We found it impossible to use the method to reliably analyze reactions of thioesters because (i) at pH < 12, the precipitation reaction did not remove enough of the paramagnetic metal from solution and (ii) at pH > 12, although we could obtain better spectra, the thioester hydrolyzed significantly during the analysis. Figures S10 and S11 in the Supporting Information show that acetic acid (δ = 1.5–2.0 ppm)—the hydrolysis product of S-methyl thioacetate (SMTA)—forms immediately, following the addition of the alkaline precipitating salts. These observations are consistent with SMTA’s half-life of 7.2 min at pH 12.

For samples tolerant of base, we explored the use of salts of the same counterions as mentioned previously but protonated (e.g., KH₂PO₄ and K₂HPO₄ rather than K₃PO₄) so that they would not render the samples as alkaline, following the precipitation reaction. In these cases, at lower values of pH, the precipitation reactions were not sufficient to enable a deuterium lock on the spectrometer, and no sensible spectra could be obtained. These results are consistent with reports of higher Ksp values for metal salts of basic anions in increasingly acidic solutions.

We treated a mixture of 25 mM Gly₂ + 28 mM MnCl₂ with increasing concentrations of K₃PO₄ to assess the effects on the metal removed. The results summarized in Table S7 and Figure S4 showed that increasing the concentration of the phosphate stock from 10 to 100 mM resulted in a reduction of the concentration of Mn²⁺ remaining in the precipitated sample from 14 mM (at pH 6, giving a poor-quality spectrum) to 0.4 mM (at pH 12, giving a spectrum that could be integrated). Increasing the equivalents of precipitating ions to the metal removes an increasing amount of metal but comes with the cost of raising the pH of the precipitated solution.

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For samples tolerant of base, the improvement of NMR spectra can be quite compelling. Figure 4 demonstrates the improvement of the ¹H NMR spectrum of Ala₂ obtained when 20 mM of Mn²⁺ is removed by precipitation with potassium phosphate. Here, the Ala₂ is stable to the strongly basic conditions required of the precipitation reaction (we see no new peak for free alanine that would be the product of hydrolysis). Indeed, this method is especially suitable for qualitative characterization where there are no concerns if the precipitation method removes a fraction of the organic compound(s) present from the solution. Of course, we sought to develop a method that would enable the measurement of reaction kinetics with quantitative accuracy, and we found our method to be suitable for monitoring reactions with organic substrates tolerant of basic conditions on short time scales, such as peptides.

Figure 5 summarizes how the method was used to measure rate constants for the hydrolysis of glycylglycine (Gly₂) in aqueous 1 M HCl with 1 M FeCl₂, 1 M MnCl₂, and no transition metal. As the two methylene signals for Gly₂ gradually recede at longer reaction times, a single peak for glycine (Gly) gradually appears. From the integration values of these peaks, we constructed a linear plot corresponding to pseudo-first-order kinetics, from which we obtained observed rate constants for hydrolysis. The observed pseudo-first-order rate constant, kobs, is equal to the slope of the best-fit line. Gly₂ hydrolyzes more quickly in both FeCl₂ or MnCl₂ solutions than in the control sample, where no additional metal is present. The measured rate constants were 0.016, 0.019, and 0.019 h⁻¹ for the standard, FeCl₂, and MnCl₂ samples, respectively.

Guidance to the Reader. This method for removing paramagnetic metals to enable NMR spectroscopy functions best under strongly alkaline conditions where the Ksp values for the metal salts are favorably low. At lower values of pH, enough metal persists in the supernate to broaden peaks in the NMR spectra. At high pH, reactions such as hydrolysis might be more likely to occur. Thus, this precipitation method tends to work best for substrates that are not sensitive to high pH. For applications with substrates that are sensitive to high pH, the use of Chelex can improve spectra but may be accompanied by loss of quantitative precision, as the Chelex resin appears to remove organic substances in addition to the metals.

Although effective in removing Fe²⁺, Co²⁺, and Mn²⁺, we observed some limitations to our method when it is applied to samples with Fe²⁺ and Ni²⁺ (see Figure S2 in the Supporting Information).
Kinetics when [H+] and [Mn2+] are sampled at timed intervals and treated with K3PO4 to precipitate the NMR spectra for monitoring the Mn2+ reaction. Aliquots were drawn at timed intervals and diluted with an equal volume of a 1:1 H2O/D2O as the solvent.

Figure 5. Kinetics of the hydrolysis of Gly2, in the presence of Mn2+ and Fe2+. (A) Hydrolysis of 50 mM Gly2 in 1 M HCl at 70°C with no salt, 1 M Fe2+, and 1 M Mn2+. 1H NMR peak assignments of the starting materials and products are indicated with red arrows. (B) 1H NMR spectra for monitoring the Mn2+ reaction. Aliquots were sampled at timed intervals and treated with K3PO4 to precipitate the paramagnetic metal prior to collection of each spectrum. (C) Plots demonstrating the reactions are governed by pseudo-first-order kinetics when [H+] and [Mn2+] ≫ [Gly2].

EXPERIMENTAL SECTION

Standard Procedure for Sample Preparation. A reaction mixture was prepared for analysis by vortex mixing a vial with a 5 mL solution of known concentration (5, 10, 20 mM, or 1 M) of a metal chloride [metal = manganese(II), iron(II), iron(III), nickel(II), and cobalt(II)], 10 mM of internal standard (t-BuOH), and 50 mM of analyte (a thioester or a peptide) in deionized water. Each standard sample for NMR spectroscopy was prepared by vortex mixing a 125 μL aliquot of the reaction mixture with 375 μL D2O in an NMR tube. Each precipitation sample was prepared by mixing 250 μL of the supernatant of the precipitated mixture and 250 μL D2O. The precipitated mixture contains a 250 μL aliquot of the reaction mixture with 250 μL of the precipitating salt (KOH, K2CO3, or K3PO4) in excess concentration (e.g., 100 mM salt for 20 mM metal samples, 1.5 M salt for 1 M metal samples) relative to the metal ion concentration (or, in the case of Chelex, ~2 g of the resin) in a microcentrifuge tube. Table S3 in the Supporting Information contains a summary of precipitation experiments we conducted.

General Conditions for Kinetics Experiments. The reaction mixtures generally contained the peptide or thioester substrate, a soluble salt of a transition metal, hydrochloric acid, and water as the solvent. Aliquots of these mixtures were drawn at timed intervals and diluted with an equal volume of a sufficiently concentrated solution of the precipitating anion (CO32−, PO43−, or AsO43−) in D2O. Thus, the reactions were run in pure H2O, but the samples on which NMR spectra were collected had ~1:1 H2O/D2O as the solvent.

NMR Spectroscopy. Samples were analyzed on a Bruker (Billerica, Massachusetts) Avance III 400 MHz NMR spectrometer with 16 scans and a recycle delay (d1) of 15 s, which is greater than 7 times the T1 relaxation time of the methylene protons of linear glycine peptides under the conditions of the analysis. All spectra were collected using a standard 1-D 1H NMR and a 1-D excitation sculpting pulse programs (specifically, “zg30” and “zgssp” on Bruker’s TopSpin 3.2 software). All spectral data were processed with MestreNova software, version 11.0.3.

Kinetics of Hydrolysis of Gly2. In 20 mL scintillation vials, 10 mL of aqueous solutions were prepared with 0.1 M Gly2, 1.0 M HCl, and one among 1 M MnCl2, 1 M FeCl3, or no metal (as a control). The reaction mixtures were stirred at 70°C for the duration of the experiment. At intervals of 8–12 h, a 250 μL aliquot was removed from each reaction and immediately subjected to precipitation by the addition of 250 μL of 1.5 M K3PO4 in water. After the solid precipitate was spun down by centrifugation at 12 000 rpm (13 500g) for 2 min, 250 μL of the liquid supernate was mixed with 250 μL of D2O in a standard 5 mM NMR tube for analysis by 1H NMR spectroscopy at 400 MHz. Each reaction mixture was sampled at 5 time points, and the relative ratio of the reactant to the hydrolyzed product was determined and used to construct a plot of −ln([Gly2]/[Gly2]0) versus time. From this plot, the rate constant (k) is derived by calculating the slope of the best-

CONCLUSIONS

We report a method for collecting NMR spectra of organic compounds and monitoring their reactions by precipitating any transition metals present prior to analysis. This method for the removal of metals ensures that the presence of paramagnetic metals does not broaden peaks in the spectra. The principal advantages of our system are that it: (i) removes paramagnetic ions, (ii) permits complex mixtures that absorb all over the UV–vis spectrum, (iii) permits the NMR instrument to lock on the deuterated solvent, (iv) allows higher spectral resolution for 1H NMR, and (v) does not add new organic species that could muddle the NMR spectra. Future efforts to identify precipitating anions that function at less alkaline values of pH could lead to a more versatile method.
fit line and dividing by the concentration of acid and salt (here, 1 M for both), according to the integrated pseudo-first-order rate law, as shown in eq S4 of the Supporting Information.

**ASSOCIATED CONTENT**

1. Supporting Information
The Supporting Information is available free of charge at [https://pubs.acs.org/doi/10.1021/acsomega.9b02610](https://pubs.acs.org/doi/10.1021/acsomega.9b02610).

Additional experimental detail of the HPLC method and practical notes for its application, materials, derivation of pseudo-first-order kinetics, NMR spectra of samples referred in this text, ICP–OES data, and additional references (PDF)

**AUTHOR INFORMATION**

Corresponding Author
*E-mail: paul.bracher@slu.edu.

ORCID
Paul J. Bracher: 0000-0001-5769-8364

Author Contributions
P.J.B. conceived the project. C.H.C. conducted the initial experiments at the California Institute of Technology. R.F., J.P.R., and C.T.D. conducted experiments at Saint Louis University. All authors interpreted data. R.F. and P.J.B. primarily wrote the paper with input from all authors.

Notes
The authors declare no competing financial interest.

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