1 **NMR spectroscopic study of organic phosphate esters coprecipitated with calcite**

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ABSTRACT

Organic phosphorus incorporated in calcite during laboratory precipitation experiments and in natural cave deposits was investigated by solid-state NMR spectroscopy. For calcite precipitated in the presence of organic phosphoesters of varying size and functionality, solid-state $^{31}\text{P}\{^1\text{H}\}$ CP/MAS NMR shows that the phosphoesters were incorporated intact into the solid. Systematic changes in the ^{31}P NMR chemical shift of the phosphate group were observed between the solid phosphoester and that incorporated in the solid precipitate, yielding ^{31}P NMR chemical shifts of the coprecipitates in the range of +1.8 to -2.2 ppm. These chemical shifts are distinct from that of similarly prepared calcite coprecipitated with inorganic phosphate, 3.5 ppm. Only minor changes were noted in the phosphoester ^{31}P chemical shift anisotropy (CSA) which suggests no significant change in the local structure of the phosphate group, which is dominated by C-O-P bonding. Close spatial proximity of the organic phosphate group to calcite structural components was revealed by $^{31}\text{P}/^{13}\text{C}$ rotational echo double resonance (REDOR) experiments for coprecipitates prepared with ^{13}C -labeled carbonate. All coprecipitates showed significant ^{31}P dephasing effects upon ^{13}C -irradiation, signaling atomic-scale proximity to carbonate carbon. The dephasing rate for smaller organophosphate molecules is similar to that observed for inorganic phosphate, whereas much slower dephasing was observed for larger molecules having long and/or bulky side-chains. This result suggests that small organic molecules can be tightly enclosed within the calcite structure, whereas significant structural disruption required to accommodate the larger organic molecules leads to longer phosphate-carbonate distances. Comparison of ^{31}P NMR spectroscopic data from the synthetic coprecipitates with those from calcite moonmilk speleothems indicates that phosphorus occurs mainly as inorganic orthophosphate in the natural deposits, although small signals occur with characteristics consistent with phosphate monoesters. The results of this study indicate that trace- to minor concentrations of dissolved organic molecules can be effectively taken up during calcite precipitation and incorporated in the structure, leaving a resilient record of materials present during crystallization.

1.0 INTRODUCTION

As potential recorders of paleoenvironmental information and natural laboratories for understanding control of impurity and trace element distribution during crystallization, calcium carbonate minerals such as calcite continue to be widely studied. During calcite crystal growth, many components of the aqueous fluid adsorb to the mineral surface and can be entrapped or incorporated into the mineral at trace to minor concentrations (Watson, 2004) in such a way as to potentially provide a record of the environmental conditions and solution composition at the time of crystallization (e.g., Borsato et al., 2007; Blythe et al. 2008; Fairchild and Treble, 2009; Perrette et al., 2005). Among the impurities in calcite precipitated in low-temperature environments are large organic molecules, such as humic and fulvic acids (Neuweiler et al., 2000; Smailer and White, 2013), lipids (Pearson et al., 2005; Blyth et al., 2011), and amino acids (Walton, 1998; Penkman et al., 2008), that cannot be incorporated in calcite by simple substitution. Such organic molecules could be useful for extracting paleoenvironmental information if they are incorporated in the calcite crystals at the time of deposition and remain impervious to removal or replacement over geological timescales. Release of organic molecules by digestion of calcite after prolonged exposure to strongly oxidizing solutions is generally accepted as evidence for the presence of intracrystalline organic matter in calcite from biominerals (Berman et al., 1990; Albeck et al. 1993; Aizenberg et al., 1994; Marin et al., 2005; Pokroy et al., 2006a; Nudelman et al., 2007; Okumura et al., 2013) and of abiotic origin (Lauritzen et al., 1994; Sykes et al., 1995; Rousseau et al., 1995; Neuweiler et al., 1999, 2000; Pearson et al., 2005; Huang et al., 2008). For example, from SEM observations Ramseyer et al. (1997) concluded that during abiotic precipitation organic molecules adsorbed to the mineral surface are incorporated into the mineral structure during crystal growth. Such “intracrystalline” organic matter could be a faithful recorder of crystallization conditions if it can be ascertained that the organic molecules do indeed occupy structural positions in the mineral.

Owing to the conformational and size mismatch between such organic molecules and calcite structural components, the relationship between occluded or intracrystalline organic matter and the calcite

structure is not clear. An understanding of this structural relationship is crucial for evaluating the probability that impurities of interest were deposited coevally with the calcite and not altered by subsequent external processes. Occurrence as large structural defects would offer promise that the impurities have been preserved since the time of crystallization and that some information about the composition of the fluid can be inferred from abundances and nature of organic molecules and other trace elements adsorbed to them (Borsato et al., 2007; Fairchild and Treble, 2009). On the other hand, occurrence primarily as inclusions, particulate matter, or as intragranular coatings would offer more challenging prospects of developing robust interpretations from ancient deposits. Distinguishing among these (and potentially other, unimagined) possibilities remains a difficult problem because of the typically low concentrations, heterogeneous nature of the organic material and sub-microscopic scale of the relevant features.

Among the few experimental techniques available to investigate at the atomic scale the relationship between calcite structural components and occluded organic molecules, NMR spectroscopy can provide information on elementally specific spatial proximities in a manner helpful for distinguishing structural components from inclusions and other potential locations of organic matter. In addition to observation of NMR parameters, such as chemical shift, to fingerprint chemical or structural environments, more complex NMR double resonance methods enable interatomic distances to be sensed through measurement of dipolar couplings. The magnetic dipole-dipole interaction between NMR-active nuclei varies with the inverse-cube of the internuclear distance, making it effective for detecting atomic-scale spatial relationships among atoms separated by distances of less than about 5 Å. However, investigation of organic matter in calcite with such methods is challenging because the predominant naturally occurring isotopes of calcite (^{40}Ca , ^{12}C , ^{16}O) are NMR-inactive, the typical concentrations of organic matter approach the practical detection limit for NMR, and the most easily accessed NMR-active nuclide in calcite, ^{13}C , also occurs in the organic molecules. In addition, exploiting potentially useful heteronuclear interactions between ^1H of organic molecules and carbonate carbon is complicated by the

common occurrence of structural water and other forms of H in calcite as an additional source of ^1H signal (Gaffey, 1995; Feng et al., 2006). In a previous study, Phillips et al. (2005) circumvented this problem by preparing citrate/calcite coprecipitates in D_2O solutions, leaving the occluded citrate as the dominant source of ^1H signal. In this case it was possible to detect the presence of the citrate methylene H near carbonate carbon atoms, which had been enriched in ^{13}C in the synthesis, showing that the citrate molecules occur in the calcite structure.

Another potential NMR-based experimental approach is to investigate the proximity of carbonate carbon to other components of organic molecules such as nitrogen and phosphorus (P), using $^{13}\text{C}/^{14}\text{N}$, ^{15}N or $^{13}\text{C}/^{31}\text{P}$ NMR double resonance techniques. For example, in an investigation of the amorphous calcium carbonate (ACC) component of crayfish gastroliths, Akiva-Tal et al. (2011) observed ^{13}C resonances for chitin as well as for small metabolites such as citrate and phosphoenolpyruvate (PEP). The signals for these organic molecules were not present in spectra taken after demineralization, suggesting that they occur in the ACC. In $^{13}\text{C}/^{31}\text{P}$ double resonance experiments (heteronuclear correlation), clear cross-peaks were observed between the ACC carbonate ^{13}C peak and the ^{31}P signals for inorganic phosphate and PEP. This result indicates that both inorganic phosphate and PEP occur in the ACC structure with atomic-scale proximity to carbonate groups. A similar approach was taken by Gertman et al. (2008) to study the nature of impurities in calcite coccoliths. In this case, algae were grown in a medium isotopically enriched in ^{13}C and ^{15}N , which enabled application of NMR double resonance experiments to explore the structural relationship among components of the calcite biomineral. Although a variety ^{15}N and ^{31}P resonances were apparent in spectra of the coccoliths, including several from organic components, these signals were much reduced or absent from samples treated in NaOCl solutions. This result implied that the signals from organic components did not arise from intracrystalline molecules. For the intact samples, double-resonance experiments gave evidence for N- and P-containing molecules associated with the calcite structure, through a REDOR (rotational echo double resonance) dephasing effect on the calcite ^{13}C carbonate peak upon excitation of ^{15}N and ^{31}P resonances. These N- and P-containing molecules detected

in the proximity of the carbonate group could not be unambiguously identified but were attributed to small inorganic molecules because of their proximity to apparently well-ordered regions of the calcite structure.

The purpose of the present study is to investigate the potential structural relationship between calcite and a variety of dissolved organic molecules present during precipitation, which differ in size and functionality. By choosing for the coprecipitation experiments organic molecules that contain phosphate groups (phosphate mono- and diesters), the NMR-active ^{31}P nucleus can be employed to probe the location of the organic molecules with respect to the carbon atom of calcite carbonate groups. Through measurement of the dipolar interaction between the phosphorus atom of an organic molecule and carbon atoms of the carbonate group in calcite, NMR spectroscopy provides information on the relative proximity of the organic molecule to the calcite structural components and on the size of the defects needed to accommodate the impurities. The results indicate that even the largest organic molecules occur in close proximity to the calcite structure and provide support for the occurrence of “intracrystalline” organic molecules in natural calcite deposits.

An additional motivation for the present study is to advance knowledge on the occurrence and form of phosphorus in calcite, such as in secondary mineral deposits in caves (speleothems). Calcite of low-temperature origin can contain 50 to $>1000 \mu\text{g P g}^{-1}$ calcite (e.g., Danen-Louwerse et al., 1995; Fairchild et al., 2001; Huang et al., 2001; Treble et al., 2003; Baldini et al., 2002; Rodriguez et al., 2008; Frisia et al., 2012). Based on cave monitoring data, annual lamina chemostratigraphy, and principal component analysis of trace elements known to be transported into the cave during high infiltration periods, it has been proposed by some scientists that the phosphorus content of cave deposits might serve as a proxy for hydrological conditions (Huang et al., 2001; Fairchild et al., 2001; Borsato et al., 2007) or nutrient infiltration (Treble et al. 2003). However, this interpretation is not unequivocal. Jones (2011) claimed that P in speleothems might be associated with microbes colonizing the surface during relatively dry periods, which is the opposite of its classical interpretation. The significance of P concentration in

speleothems and other calcite deposits depends on whether it is incorporated structurally, and whether it forms other phases. Both processes can impact the partitioning of P, the meaning of the partition coefficient, and the paleoclimatic significance of this trace element. As for organic matter discussed above, both the form of the phosphorus and its relationship to the calcite structure are difficult to determine, and generally unavailable from microchemical analyses. Recently, by combining microscopy with analyses by micro-X-ray fluorescence and laser-ablation ICPMS, Frisia et al. (2012) identified phosphate-containing phases such as ardealite in the P-rich layers of a calcite speleothem containing unusually high P concentration (up to 8000 $\mu\text{g g}^{-1}$). Frisia et al. (2012) also documented P in speleothem layers that resemble microbial mats and may be of microbial origin, supporting the interpretation of Jones (2011). In a previous study, ^{31}P NMR results for calcitic speleothems deposited in a temperate setting were interpreted to indicate presence of phosphorus mainly as inorganic phosphate, based on spectral similarities with laboratory-grown calcite/phosphate coprecipitates, although the spectra also included peaks assigned to monetite and a second unidentified mineral (Mason et al., 2007). It becomes, thus, clear that a distinction between organic- and inorganic phosphorus in speleothems has the potential to elucidate the significance of this trace element as a paleoproxy, which, most likely, varies in relation to specific environmental settings.

Organic phosphorus (e.g., phosphonates, mono- and diesters) and polyphosphate are known to occur in marine sediments and soils, where ^{31}P NMR spectroscopy has been applied for their identification (e.g., Ingall et al., 1990, Clark et al., 1999; Kolowitz et al., 2001; Sannigrahi and Ingall, 2005; Cade-Menun et al. 2002), chiefly by utilizing extraction techniques. To date, the spectral signature of organic phosphate groups in calcite has not been investigated *in situ*. By examining calcite coprecipitated with phosphate mono- and diesters phosphates we provide NMR spectral characteristics for such coprecipitated organic phosphorus in calcite. Our results demonstrate that large organic molecules can be structurally incorporated in calcite and that organic phosphorus-containing molecules can be

distinguished from inorganic orthophosphate by solid-state NMR spectroscopy if present at sufficient concentration.

2.0 EXPERIMENTAL METHODS

2.1 Materials

Organophosphates: A series of commercially available organic phosphate esters of varying size, structure, and functionality were chosen to investigate uptake during calcite precipitation. Structural diagrams of the molecules are presented in Figure 1, along with abbreviations assigned as described below. These molecules contain several different types of functional groups in addition to phosphate that could interact favorably with the calcite surface to promote adsorption during crystal growth, such as hydroxyl and carboxyl groups.

- Phosphoenolpyruvate (PEP) was obtained as a sodium salt, phosphoenolpyruvic acid trisodium salt heptahydrate, from Alfa Aesar. As shown in Figure 1, PEP is a phosphate monoester bearing a phosphate group and an acrylic acid group with a carboxyl. PEP is a metabolite that has been identified in biogenic amorphous calcium carbonate (Akiva-Tal et al., 2011).
- β -glycerolphosphate (BGP) was obtained as its sodium salt, β -glycerol phosphate disodium pentahydrate ($C_3H_7Na_2O_6P$), from Pfaltz & Bauer and consists of a phosphate monoester attached to a glycerol group. As shown in Figure 1, the functional groups that may contribute to adsorption are the phosphate groups, two sodium-bonded oxygens (-ONa), and the glycerol group with two hydroxyls.
- Lipoteichoic acid (LTA), a bacterial cell wall polymer extracted from a Gram-positive bacterium *Staphylococcus aureus*, was obtained from InvivoGen. As shown in Figure 1, LTA is an amphiphilic molecule consists of a lipid tail connected to a disaccharide D-glucose head group and a negatively charged, hydrophilic teichoic acid chain. The teichoic acid chain is a polyphosphodiester with repeated units containing D-alanine, glucosamine, and hydroxyl groups.

These D-alanine functional groups enable LTA to adhere to a variety of materials including metal oxide surfaces (Wickham and Rice, 2008). The lipid tail with two fatty acid chains acts as the hydrophobic head, while other functional components of LTA including the disaccharide head group and the teichoic acid chain attached comprise a hydrophilic tail enabling bilayer formation in aqueous solutions.

- Diposphoglycerate (DPG) was obtained as its sodium salt (2,3-Diphospho-D-glyceric acid pentasodium salt) from Sigma-Aldrich with the formula $C_4H_5Na_5O_{10}P_2$. As shown in Figure 1, DPG is a phosphate-monoester that consists of two phosphates connected via a glycerate group. The functional groups for DPG adsorption are two phosphate groups each with two sodium bonded oxygens ($-ONa$) and a glycerate group with sodium carboxyl group ($-COONa$).
- Distearoyl phosphoglycerol (DSPG) was obtained as its sodium salt of 1,2-Distearoyl-sn-glycero-3-phospho-rac-(1-glycerol) (Sigma-Aldrich) with the formula $C_{42}H_{82}NaO_{10}P$. As shown in Figure 1, DSPG is a phosphate-diester that has a long lipid tail and a phosphoglycerol group. DSPG contains a lipid tail with two stearyl groups, one phosphate group bonded to sodium ($-ONa$), and one glycerol group with two hydroxyls ($-OH$). The stearyl groups have an 18-carbon chain of saturated fatty acid, giving DSPG a relatively large molecular weight, 801 g/mol, over two times larger than the other organophosphates studied. It is expected that DSPG has relatively low solubility. To ensure no ester precipitated prior to the coprecipitation, the amount of DSPG used in the synthesis was much less than for the other coprecipitation experiments.
- 3-phosphoglycerate (3PG) was obtained as its sodium salt: D-(-)-3-Phosphoglyceric acid disodium salt (Sigma-Aldrich). As shown in Figure 1, the phosphate-monoester is the sodium salt of 3-phosphoglyceric acid. 3PG consists of a phosphate group and a glycerate group bearing a hydroxyl ($-OH$) and carboxyl ($-COONa$). Presence of 3PG in biogenic ACC was reported by Sato et al. (2011).

Calcite moonmilk: Two samples of calcite moonmilk, a cave deposit (speleothem) formed by calcite crystals and water, were examined as part of this study to determine how P is incorporated in a climate-sensitive natural deposit known to be associated with bacterial lipids (Blyth and Frisia, 2008). Moonmilk, which consists of an aggregate of nearly defectless fibers and composite rods (Borsato et al., 2000) provides an appropriate example for comparing laboratory and naturally grown crystals incorporating P. Moonmilk calcite grows in a relatively stable environment from waters of low ionic strength and calcite saturation index. Both samples were collected from the top of active stalactites in Coel Zelà, Italy (Borsato et al., 2000). The cave is located at an elevation of 1740 m a.s.l. and characterized by a constant T throughout the year of 5.5 °C. The passage whence moonmilk was removed is entirely developed in pure limestones, which results in Ca and bicarbonate being the dominant dissolved species, with Mg less than 1 mg/l (Borsato et al., 2000). The P concentration was not analyzed for the studied drips. However, data from drips in a cave at mid-altitude in the same region, suggest that the range of P could vary seasonally from 1 up to 4 µg/l (Borsato et al., 2007). Sample CZ-3 was collected in a completely dark area, 80 m from the cave entrance. The second specimen, CZ-12, was collected in a twilight zone which is characterized by annual fluctuations of temperature and relative humidity, 30 m away from the cave entrance. Calcite moonmilk formed in Coel Zelà has morphological characteristics similar to those of the bio-mediated speleothems observed to form above the present timberline in the caves of the Paganella massif. It is thus similar to the samples analyzed by Blyth and Frisia (2008). Both samples were bleached by suspending in sodium hypochlorite solution to remove extra-crystalline organic matter, then rinsed thoroughly and dried. Only reflections arising from calcite were identified by powder XRD.

2.2 Coprecipitation experiments

Coprecipitates of calcite with organic phosphate esters were synthesized according to a modified seeded constant-addition method (Reeder et al., 2000; Zhong and Mucci, 1993; Tesoriero and Pankow, 1996). An initial growth solution was prepared in the reaction vessel (1L Pyrex beakers) consisting of

700 mL deionized water, 0.8401 g ^{13}C -enriched NaHCO_3 (Aldrich), and 1.5 mL of a 60 g/L pre-equilibrated calcite suspension as nucleation seeds, prepared from 5 μm calcite particles (Alfa Aesar). Into this constantly stirred initial solution was pumped simultaneously from separate syringes a 0.1 mol/L CaCl_2 solution and a solution 0.1 mol/L NaHCO_3 (99 at.% ^{13}C) containing also the phosphate ester. For most coprecipitates, the concentration of the organic phosphate was chosen to yield an approximately 1000:1 Ca:P ratio in the two syringe solutions, except for BGP (200:1) and DSPG (7800:1). The solubility of DSPG is relatively low, yielding a cloudy suspension at higher concentrations. Prior to the coprecipitation experiment the pH of all solutions was adjusted to 8.2 by manual addition of NaOH or HCl. The syringe solutions were pumped into the seeded growth solution at a rate of 150 $\mu\text{L}/\text{min}$ through check valves for a total of nine hours. The resulting precipitate was collected through a 0.2 μm membrane filter and rinsed by deionized water. Once collected, the solid was air-dried and stored at -18°C to resist any biodegradation. Calcite was the only phase detected by powder X-ray diffraction. Assuming that all of the organophosphate was incorporated in the solid would result in P contents of approximately 300 $\mu\text{g P g}^{-1}$ calcite for most samples, except for the higher content of BGP (1550 $\mu\text{g P g}^{-1}$) and a lower value for DSPG (40 $\mu\text{g P g}^{-1}$). For comparison, a calcite coprecipitate with inorganic orthophosphate was prepared in a similar manner, with approximately 200 $\mu\text{g P g}^{-1}$ calcite

2.3 NMR spectroscopy

Most $^{31}\text{P}\{^1\text{H}\}$ CP/ MAS experiments were conducted with a 400 MHz (9.4 T) Varian Inova spectrometer operating at 161.87 MHz for ^{31}P and 399.8 MHz for ^1H . Spectra were taken at spinning rates of 3 kHz and 5 kHz with the samples contained in 7.5 mm (o.d.) rotors, and 8 kHz packed in 3.2 mm (o.d.) rotors. The CP/ MAS experiments were generally conducted with 2 ms $^1\text{H} \rightarrow ^{31}\text{P}$ contact time using a linear ramp of the ^{31}P transverse field. The chemical shift anisotropy (CSA) for resonances in the $^{31}\text{P}\{^1\text{H}\}$ CP/MAS spectra were determined from analysis of spinning sideband intensities at spinning rates of 3 and 5 kHz using the HBA program (Eichele and Wasylishen, 2000). The standard (IUPAC) notation

is adopted here, defining the tensor elements such that $\delta_{11} \geq \delta_{22} \geq \delta_{33}$. The isotropic chemical shifts ($\delta_{\text{iso}} = 1/3(\delta_{11} + \delta_{22} + \delta_{33})$) were taken from spectra acquired at 8 kHz and may differ somewhat from the mean of the tensor values obtained from analysis of the spectra acquired at 3 and 5 kHz as a consequence of experimental uncertainty owing to the modest signal-to-noise ratios. The anisotropy of the chemical shift tensor, $\Delta\delta = \delta_{zz} - 1/2 \cdot (\delta_{xx} + \delta_{yy})$, is a useful measure of the symmetry of the phosphate bonding environment and is also reported, where the three principal tensor components are defined such that $|\delta_{zz} - \delta_{\text{iso}}| > |\delta_{yy} - \delta_{\text{iso}}| > |\delta_{xx} - \delta_{\text{iso}}|$.

The $^1\text{H} \rightarrow ^{31}\text{P}\{^{13}\text{C}\}$ CP/REDOR data were obtained with a 500 MHz (11.7 T) Varian Infinity-plus spectrometer using a three-channel Varian/Chemagnetics T3-type probe assembly configured for 4 mm (o.d.) rotors. The REDOR experiments used $^1\text{H} \rightarrow ^{31}\text{P}$ CP (2 ms contact time) to generate the initial ^{31}P signal, and then applied ^{13}C dephasing pulses for periods ranging from 0.25 to 64 ms. The operating frequencies were 125.7, 202.3, and 499.8 MHz for ^{13}C , ^{31}P , and ^1H respectively, and the spinning rate was 8 kHz for all experiments. Typical acquisition conditions include 5 μs ^1H 90° pulse, 2 ms $^1\text{H} \rightarrow ^{31}\text{P}$ contact time, 8 μs ^{13}C 180° dephasing pulses, 10 μs ^{31}P re-focusing pulses, and 1 s relaxation delay for 20,000 – 80,000 acquisitions.

3.0 RESULTS

3.1 Coprecipitate characterization

The calcite/organophosphate coprecipitates were examined by X-ray diffraction, scanning electron microscopy, and BET surface area analysis. In all cases, XRD indicated calcite as the only crystalline phase present. The morphology of calcite precipitated with and without organic phosphate was examined by scanning electron microscopy (SEM). SEM micrographs of several samples including a control calcite/blank precipitate, calcite/BGP coprecipitate, and calcite/LTA coprecipitate are shown in Figure 2. To quantify the elements present in the calcite surface energy dispersive x-ray spectroscopy (EDX) was carried out along with the SEM analysis.

The SEM micrographs show calcium carbonate precipitated as crystalline aggregates with a morphology and shape that were modified slightly by the presence of phosphate esters (Fig. 2). All samples appear to comprise calcite rhombohedra in a wide range of sizes. Minor morphological changes were found in coprecipitate samples. The calcite/blank experiment yielded pure calcite as rhombohedral crystals shown in Figure 2A-B, which exhibit smooth faces and sharp edges. The SEM image of the calcite/BGP coprecipitate in Figure 2 C-D appears to show calcite rhombohedra with smooth faces but with slightly jagged edges. The calcite/LTA coprecipitate in Figure 2 E-D, however, has a more “ragged” appearance: with jagged edges and coarse faces that appear dark in the images. The sizes of aggregated particles in the LTA/calcite sample appears to have a wider range but generally somewhat smaller than both the blank/calcite and GPA/calcite sample.

Similar EDX spectra were obtained from all the samples including the pure calcite (Figure 2G) and calcite coprecipitated with phosphate monoester BGP (Figure 2H) and diester LTA (Figure 2I). The pure calcite sample yielded a small signal for phosphorus (Figure 2G) which arises from the electrically conductive tape. Somewhat larger phosphorus signals were observed for the coprecipitates although the differences appear minor. This result indicates slightly elevated phosphorus content in the coprecipitates consistent with the similarity of the estimated concentration of 1400 ppm for the calcite/BGP sample and the 1000 ppm estimated detection limit for the EDX. These EDX spectra, although qualitative in nature, are consistent with the presence of a small amount of phosphate in the coprecipitates, but suggest that the organic phosphates are not concentrated on the external crystal surfaces but dispersed within the crystals.

Two samples were tested to obtain their specific surface area by BET analysis: a calcite/BGP coprecipitate sample and a calcite/blank precipitate sample prepared with the same experimental method as the coprecipitate but without the presence of phosphate ester in the anion syringe. The result shows specific surface areas of $0.96 \text{ m}^2/\text{g}$ with correlation coefficient $R = 0.994$ for the calcite/BGP coprecipitate and $1.1 \text{ m}^2/\text{g}$ with $R = 0.998$ for pure calcite sample, which indicates no significant difference with the specific surface area of calcite coprecipitated with the BGP phosphate ester.

3.2 ^{31}P CP/MAS NMR of organophosphates and coprecipitates

The $^{31}\text{P}\{^1\text{H}\}$ CP/MAS spectra of the as-received phosphate ester salts and their corresponding calcite coprecipitates are collected in Figure 3. The chemical shift (δ_{iso}) and CSA data are summarized in Table 1. Notably, significant ^{31}P CP/MAS NMR signal was observed for all of the coprecipitates indicating that in each case a substantial amount of the phosphate ester was incorporated in the solid. All of the samples give spectra containing broad spinning sideband envelopes, spanning approximately 200 ppm, as expected for the asymmetric bonding configuration of the phosphate esters in a rigid environment. For most phosphate ester salts and coprecipitates a single resonance was apparent in the spectra, consisting of a center band at the isotropic chemical shift and a series of spinning sidebands. The spinning sidebands were distinguished from the center band by comparing spectra taken at different spinning rates. For DSPG, the Na-salt gave two relatively narrow centerbands, at +5.2 and +0.8 ppm. The origin of the second peak is unknown, but the similar spinning sideband patterns of these two resonances suggests they correspond to phosphate with similar bonding geometry. For the corresponding DSPG/calcite coprecipitate only a single ^{31}P resonance was resolved although the large peak width could accommodate two resonances with a chemical shift difference similar to that of the Na-salt. For the PEP/calcite coprecipitate, a small shoulder is observed near +4 ppm in addition to the main resonance at -2.1 ppm. This minor peak exhibits little spinning sideband intensity, indicating that it could arise from inorganic orthophosphate produced from minor hydrolysis during the coprecipitation experiment.

The observed ^{31}P δ_{iso} values fall within the range reported for phosphate esters (e.g., Cademenu, 2005) and show some systematic trends between the phosphate esters and the corresponding calcite coprecipitate (Table 1). For those organophosphates obtained as the Na salt (BGP, DPG, PEP, and DSPG) the chemical shift of the coprecipitate is lower than that of the as-received material by 3.3 to 5.6 ppm. In contrast, the calcite coprecipitate for the diester LTA gives a chemical shift that is 2.6 ppm higher than for the as-received acid form in which the phosphate groups are protonated. 3PG represents the intermediate case of a monoester in which the phosphate groups of the as-received Na-salt are mono-

protonated. In this case a smaller difference of 1.1 ppm was noted between the phosphate ester and its coprecipitate. These trends in δ_{iso} parallel those of inorganic orthophosphates (Turner et al., 1986) in which the ^{31}P chemical shift becomes lower with increasing electronegativity of the coordinating cation in the order $\text{Na}^+ > \text{Ca}^{2+} > \text{H}^+$. Thus, the observed changes in isotropic chemical shift between the as-received organophosphates and their corresponding coprecipitates are consistent with change in the bonding of phosphate to Na^+ and/or H^+ in the as-received salts to coordination to primarily Ca^{2+} in the coprecipitates.

In contrast to the isotropic chemical shifts, the SSB intensity patterns and the CSA tensor values derived from them show no clear trend between the phosphate ester salts and corresponding coprecipitates, but instead are similar for all pairs of samples except for 3PG, which is protonated in the as-received material. The chemical shift anisotropy (CSA) is a tensor that can be represented in its diagonalized form by three principal components, the average value of which is numerically equivalent to δ_{iso} . For ^{31}P NMR of phosphate groups, the anisotropy parameter ($\Delta\delta$) is a useful indicator of chemical environment (Hartmann et al., 1994) and is defined as the largest separation between a principal CSA value and δ_{iso} . The ^{31}P CSA is sensitive to the symmetry of the structural environment (Duncan and Douglass, 1984; Un and Klein, 1989). For instance, hydrogen phosphates exhibit larger ^{31}P $\Delta\delta$ values than orthophosphate because of the distortion of the electronic environment and deviation from tetrahedral symmetry (Hartmann et al., 1994). The three principal tensor components (δ_{ii} , $i = 1, 2, 3$) were calculated from the intensity of the spinning sidebands by the method of Herzfeld and Berger (1980). The spinning sideband intensities used for the analysis were obtained from least-squares fits of the spectra to a sum of Gaussian curves with the constraint that for each phosphorus site the width of the peaks fit to the sidebands equal that of the center band. This constraint reduced the number of fitting parameters, allowing the lower intensity sidebands to be included in the fits while ensuring physically meaningful fitted intensity values.

The $^{31}\text{P}\{^1\text{H}\}$ CP/MAS spectra of all the samples, including salts of the phosphate esters and their calcite coprecipitates, exhibit broad sideband envelopes (Fig. 3) indicating significant ^{31}P CSA. The experimentally obtained spectral data are listed in Table 1. The CSA values for the phosphoester salts are in broad agreement with previous work that showed systematic differences between non-protonated monoesters and diesters, with the diesters exhibiting more negative δ_{33} values, by ca. 30-50 ppm (Kohler and Klein, 1976; Terao et al., 1977; Herzfeld et al., 1978; Vogel et al., 1982). The more negative δ_{33} values result in a change in sign of $\Delta\delta$ from positive for monoesters to negative for diesters. The one exception is the ^{31}P CSA of the acid form of the 3PG monoester which also exhibits a negative value of $\Delta\delta$, owing to the large effect of protonation on the electronic environment of the phosphate group. Similar negative values for $\Delta\delta$ have been reported previously for protonated monoesters (Terao et al., 1977).

Most significantly, we observe relatively minor differences in the CSA between the organophosphate Na-salts and their corresponding calcite coprecipitates. For the monoesters BGP, DPG, and PEP the differences in the tensor values are of the order 1-3 ppm for δ_{11} , 3-13 ppm for δ_{22} , and 8-12 ppm for δ_{33} , yielding changes in $\Delta\delta$ and span ($\Omega = \delta_{11} - \delta_{33}$) of 5-12 ppm, generally less than 10 percent (Table 1). Such minor differences are expected if coprecipitation results mainly in a change of the cation coordinated to the phosphate group, e.g. from Na to Ca, but retention of the stronger C-O-P linkage which has a larger influence on the CSA. The differences we observe between the Na-salts and coprecipitates can be attributed to the difference in electronegativity between Ca and Na and perhaps conformational changes in the organophosphate molecule when incorporated in the solid coprecipitate. Hydrolysis of the monoesters during coprecipitation would likely yield orthophosphate, which as a coprecipitate in calcite exhibits much smaller $\Delta\delta$ and Ω , by a factor of more than two (Table 1, see below). The small shoulder on the high-frequency side of the center band of the PEP/calcite coprecipitate appears to have little SSB intensity and a chemical shift consistent with coprecipitated orthophosphate

that could have resulted from minor hydrolysis. This shoulder represents much less than one percent of the phosphorus in the sample however.

Larger CSA differences between the pure organophosphate and coprecipitate are observed for the 3PG-based samples, primarily owing to the protonation of the phosphate group in the acid form (Terao et al., 1977), which causes decreased δ_{33} and large negative value for $\Delta\delta$ (−126 ppm). For the corresponding coprecipitate however, the CSA principal values, $\Delta\delta$, and Ω fall well within the range for the other monoester coprecipitates, consistent with a change from protonated phosphate in the acid to coordination by Ca in the coprecipitate.

Similarly minor CSA differences between as-received solid and coprecipitate are observed for the two large diesters examined. Both LTA and the main resonance for DSPG exhibit large, negative values for $\Delta\delta$ (−156 and −174 ppm, respectively) and larger spans (186 and 206 ppm) than for the monoesters. These values are the same within uncertainty for the DSPG coprecipitate (−174 vs. for −178 ppm for $\Delta\delta$; 211 vs. 206 ppm for Ω) and about 10 percent lower for the LTA coprecipitate compared to its acid form. The larger change for the LTA samples can be attributed to deprotonation of the phosphate groups of the acid form to coordination by Ca in the coprecipitate. The CSA's for the diester coprecipitates remain distinct from those for the monoesters in both the sign of $\Delta\delta$ and the larger Ω value. The difference in the former is qualitatively apparent in the shape of the SSB patterns (Fig. 3), which show the +1 spinning sideband (near +30 ppm) larger than the −1 SSB (near -30 ppm) for the diesters and their coprecipitates, and the reverse for the monoester coprecipitates and Na-salts (except the acid form of 3PG). Taken together these observations of the CSA suggest that, except for a minor amount of hydrolysis of PEP, the organophosphates retain the nature of the C-O-P bonding environment upon incorporation in the coprecipitates.

3.3 ^{31}P CP/MAS NMR of orthophosphate/calcite coprecipitate

For comparison, we include here ^{31}P NMR results for a calcite/phosphate coprecipitate prepared in a manner similar to that for the organophosphate samples. In essential aspects the results closely resemble those reported in a previous study (Mason et al., 2007), with the CP/MAS spectra showing a broad peak (3.2 ppm FWHM) at +3.6 ppm, assigned to phosphate defects in calcite, and a narrower peak centered near -1 ppm for monetite (Fig. 4). This peak at -1 ppm is a poorly resolved doublet, detail which cannot be discerned at the scale of Figure 4, but consistent with previous reports for monetite (Aue et al., 1984; Belton et al., 1988). The direct excitation (single-pulse) spectrum, which is not shown, includes also a narrow peak at +3.1 ppm for a second, unidentified crystalline phase. (This unidentified phase apparently lacks H as a major component and hence yields no detectable signal in the CP/MAS experiment.) The CSA values for the broad orthophosphate peak were obtained as described above for the organophosphates and are also reported in Table 1. The width of the SSB envelope for the coprecipitated orthophosphate is much smaller than for the organophosphates (cf. Fig. 3), reflecting the more symmetric bonding environment in the absence of C-O-P bonds. Analysis of the SSB intensities yields values for $\Delta\delta$ and Ω (+50 and 51 ppm, respectively) that are smaller by a factor of two compared to the phosphate monoesters. The span is similar to that reported for crystalline Ca-orthophosphates (Pourpoint et al., 2007, Rothwell et al., 1980), but significantly smaller than that for the protonated phosphate of brushite (Hartmann et al., 1994; Pourpoint et al., 2007). The positive value of $\Delta\delta$ differs from the generally negative values for other Ca-orthophosphates, but is significantly smaller than $\Delta\delta$ for the hydrogen phosphate group in brushite (+95 ppm; Pourpoint et al., 2007). These results are inconclusive with regards to the nature of orthophosphate in the calcite coprecipitate, but could indicate the presence of a heterogeneous distribution of phosphate environments that includes a contribution from protonated phosphate or more distorted orthophosphate groups. For present purposes, the salient feature is the clear distinction in the ^{31}P isotropic chemical shift and CSA values of coprecipitated orthophosphate from those of the organophosphate coprecipitates.

3.4 $^{31}\text{P}\{^{13}\text{C}\}$ REDOR

To investigate the relationship of the coprecipitated organophosphates to the calcite structure we performed $^{31}\text{P}\{^{13}\text{C}\}$ rotational echo double resonance (REDOR) experiments on samples prepared with ^{13}C -labelled carbonate. By providing a measure of the ^{31}P - ^{13}C dipolar coupling, the REDOR experiment yields information on the spatial relationship of the phosphate and carbonate groups in the samples. The dipolar coupling is proportional to a sum over the inverse cubed distances from P to neighboring C atoms, with a practical cutoff of about 5 Å to yield a measureable effect; ^{13}C nuclei located at longer distances do not contribute significantly to the REDOR signal. For the present study, distances to the carbon atom of carbonate groups were specifically targeted by preparing the calcite coprecipitates with 99 atom percent ^{13}C -carbonate but natural abundance (1% ^{13}C) organophosphates. The $^{31}\text{P}\{^{13}\text{C}\}$ REDOR experiments consist of two steps. First, a reference ^{31}P spectrum containing signal from all phosphorus environments (S_0) is obtained, in which the ^{13}C - ^{31}P dipolar couplings are effectively removed by MAS. Then a recoupled spectrum (S) is obtained with dipolar coupling to ^{13}C nuclei reintroduced by applying 180° pulses to the ^{13}C nuclei (Gullion and Schaefer, 1989). In the second step, peaks arising from phosphorus close to ^{13}C nuclei (within about 5 Å) are attenuated (dephased) compared to the reference spectrum S_0 . The amount of attenuation depends on the strength of the dipolar coupling and the length of the dephasing period, and is expected to approach 100% at long dephasing time for phosphorus with several ^{13}C nuclei nearby. Phosphate groups not associated with the ^{13}C -labelled carbonate should yield no more than a few percent attenuation owing to the low natural ^{13}C abundance of the organophosphate molecules. The difference spectrum ($S_0 - S$) yields directly the ^{31}P spectral signature of the phosphorus associated with carbonate groups. The attenuation of ^{31}P spectral peaks in the ^{13}C -recoupled spectrum is quantified by the REDOR fraction $\Delta S/S_0 = (S_0 - S)/S_0$. The dipolar coupling, which increases with the number and proximity of nearby carbonate groups, is reflected in the rate of increase of the REDOR fraction with dephasing time.

We obtained several REDOR data sets for each coprecipitate sample, varying the length of the REDOR dephasing time in even multiples of the rotor period (0.125 ms) at a constant MAS rate of 8 kHz. Centerbands for typical REDOR sets are shown in Figure 5, one for a monoester coprecipitate (DGP/calcite; Fig. 5A) and another for a coprecipitate of a large diester (LTA/calcite; Fig. 5B) at intermediate dephasing times. These examples illustrate results obtained for all samples in that significant REDOR attenuation was observed indicating intimate association of all the organophosphates with carbonate groups in the coprecipitates. In addition, no significant difference was noted in the peak shapes between the control spectrum (S_0), representing all phosphorus, and the REDOR difference ($S_0 - S$) containing signal only from ^{31}P associated with ^{13}C -carbonate. This result indicates absence of any spectrally resolved population of phosphate not associated with carbonate groups.

REDOR attenuation curves for each organophosphate coprecipitate are plotted in Figure 6, as the increase in REDOR fraction with increasing dephasing time (T). Included in Figure 6 for comparison is the attenuation curve for the orthophosphate/calcite coprecipitate described above. The results show that all of the monoester coprecipitates exhibit a rapid rise in attenuation with dephasing time that reaches nearly 100% by 8 ms, similar to the result observed for orthophosphate. The rate of attenuation increase appears somewhat lower for the larger DPG monoester, suggesting fewer carbonate groups or longer distances to them compared to the organophosphates containing a single phosphate group. The coprecipitates of the larger diesters LTA and DSPG also show significant attenuation, but the increase with increasing T is much reduced compared to the monoesters and orthophosphate. Nonetheless, the attenuation for LTA/calcite reaches 80% by 8 ms, appearing to approach 100%, and that for DSPG is approximately 60% by $T = 8$ ms. Loss of signal related to spin-spin relaxation prevented us from obtaining results for significantly longer T values.

3.5 ^{31}P NMR of calcite moonmilk

As part of this study, we examined by NMR spectroscopy calcite specimens in a moonmilk speleothem consisting of defect-free, nanometer scale calcite fibers (Borsato et al. 2000). Moonmilk occurs in intimate association with microorganisms and organic material that evidence suggests play a role in its formation and morphology (Blyth and Frisia, 2008; Canaveras et al., 2006; Sanchez-Moral et al., 2012; Bindschedler et al. 2014). Growth of moonmilk calcite occurs in a cold environment, where dripwater enters the cave understaturated, and calcite nucleation and growth is attained only via microbial metabolic processes, as suggested by lipid extraction from the calcite crystals (Blyth and Frisia, 2008). The ^{31}P CP/MAS NMR spectra of both specimens (CZ-3 and CZ-12; Fig. 4) yielded observable, although weak, signal consisting of a broad centerband near +3.5 ppm (5 ppm FWHM) with a weak shoulder. For both spectra this shoulder can be fit with a second, broad peak centered near -2 ppm with a width of 6 ppm (FWHM). Both the main centerband and shoulder have associated spinning sidebands, but those for the shoulder exhibit larger intensity relative to the centerband, giving the spinning sidebands a more asymmetric appearance than the centerband region. To estimate CSA values for comparison with the synthetic coprecipitates, we performed least-squares fits of the spectra to two spinning sideband envelopes assuming that the peak shapes are Gaussian, peaks in each SSB envelope are spaced at the measured spinning rate (3.0 kHz), and that the widths of the SSB's are the same as for the associated centerband. The resulting CSA values are collected in Table 1.

Although the low signal-to-noise ratio and poor resolution, owing to the large peak widths, leads to considerable uncertainty in the estimated CSA values, comparison with the data from the coprecipitates suggests spectral assignments. Both the isotropic chemical shift and CSA values of the more prominent centerband are in reasonable agreement with those from the orthophosphate/calcite coprecipitate (Table 1). The isotropic chemical shifts (+3.5 ppm) are the same within uncertainty, as are the span, Ω . There is some variation in the anisotropy, from +50 ppm for synthetic orthophosphate/calcite to -41 ppm for CZ-3 and +36 ppm for CZ-12, but of these values are relatively small in an absolute sense and much smaller than those for the organophosphate coprecipitates. We note that for these small values of $\Delta\delta$, the

differences in sign result from relatively small differences in the principal values δ_{22} and δ_{33} . The negative value for CZ-3 results from δ_{22} being closer in value to δ_{11} than to δ_{33} . Some variation in the ^{31}P CSA's can be expected from variations in the distribution of phosphate bonding environments. Heterogeneity is indicated by the large width of the ^{31}P peak. Although the small CSA's suggest dominance of non-protonated orthophosphate, the CSA of hydrogen phosphate groups is significantly larger, so that small variations in the concentration of protonated phosphate groups could bring about observable changes in the apparent CSA.

For the small shoulder, it was possible to obtain reasonable fits to the SSB envelope only for sample CZ-3, although the overall spinning sideband pattern for CZ-12 appears similar, particularly in the enhanced intensity of the +1 SSB, near +20 ppm. The fitted CSA parameters for the shoulder near -2 ppm for CZ-3 (Table 1) are remarkably similar to those for the phosphate monoester coprecipitates. The value for the span, $\Omega = 131$ ppm, falls in the middle of the values for the monoesters (121 to 159 ppm), as does the value for the anisotropy, 113 ppm, compare to 105-148 ppm for the monoester coprecipitates. These observations are consistent with assignment of this resonance to phosphate monoesters occluded in calcite. For both CZ-12 and CZ-3 the organophosphate resonance accounts for about $25 \pm 5\%$ of the ^{31}P CP/MAS intensity, indicating that inorganic phosphate is the most prevalent reservoir of phosphorus in the sample. Although the ^{31}P CP/MAS experiment is not strictly quantitative, the signal intensity for the coprecipitates of orthophosphate and the organophosphates reach a maximum value between 1-2 ms and appeared to show minor intensity variation with change of contact time from 1-5 ms suggesting that the intensities approximate population ratios. Unfortunately the signal is much too weak for the CP dynamics measurements that would be required for quantitative interpretation.

Unfortunately, the $^{31}\text{P}/^{13}\text{C}$ REDOR method cannot be usefully applied at normal ^{13}C abundance, so we cannot unambiguously assign the apparent organophosphate ^{31}P resonance to P structurally bound in the calcite. Although the moonmilk samples were treated to remove fluid-accessible organic matter, the possibility remains for organic material to be located on buried interfaces, surfaces of fluid inclusions,

or between crystallites in the fibers. However these results succeed in demonstrating that organic phosphoesters can be resolved from calcite-associated orthophosphate using these NMR spectroscopic methods.

4.0 DISCUSSION

4.1 Structural accommodation of occluded organic molecules

The NMR spectroscopic results presented above for the organophosphate/calcite coprecipitates point strongly to the incorporation of organic molecules in the calcite structure during precipitation. The similarity of the ^{31}P CSA's of the organophosphates in the soluble salts and in the respective calcite coprecipitates indicates that the essential features of the organic carbon-phosphate bonding is retained in the collected solids. That some minor differences in the CSA's were noted suggests slight conformational changes upon incorporation in the precipitated calcite, but no change in the number of C-O-P bonds. Strong attenuation of the ^{31}P NMR signal in the $^{31}\text{P}\{^{13}\text{C}\}$ REDOR experiments requires intimate association, at the atomic scale, of phosphorus with abundant ^{13}C nuclei, the overwhelming source of which is the ^{13}C -carbonate used in the synthesis. Furthermore, the SEM/EDX data show no evidence for phases other than calcite, nor for particles or surface regions with highly elevated concentration of phosphorus, consistent with dispersal of P at a low concentration throughout the samples. The precipitates also exhibit modest BET specific surface areas, too low to accommodate the organophosphate molecules as adsorbates.

Further analysis of the REDOR results provides some indication of the size of the defects that host the organophosphates in the calcite structure. Previous work (Pan et al., 1990) shows that the initial REDOR attenuation follows a parabolic increase with dephasing time (T), and that in the "multi-spin" regime this relationship depends on the second moment of the heteronuclear dipolar coupling between the observed and recoupled nuclei (Bertmer et al., 2000; Chan and Eckert, 2000). The REDOR results of the present study fall in this multi-spin regime in the sense that organic phosphate hosted in the calcite

structure is likely surrounded by several ^{13}C -labeled carbonate groups. In such an environment, the relevant dipolar second moment is between the observed ^{31}P nucleus and adjacent ^{13}C nuclei ($M_2^{\text{P-C}}$). The dipolar second moment depends on physical constants and a sum over the inverse sixth power of distances from the central P to the nearby ^{13}C nuclei, and is easily calculated from a structural model (van Vleck, 1948). Previous results indicate that complications arising from homonuclear dipolar coupling (e.g., between ^{13}C - ^{13}C) and pulse imperfections can be minimized by restricting attention to the early part of the dephasing curve, corresponding to REDOR fractions $\Delta S/S_0 < 0.3$. To quantify the rate of REDOR dephasing for each coprecipitate sample, we fit the initial portion of the attenuation curves shown in Figure 6 to a parabolic function, using the experimental REDOR fractions less than 0.5. From the fitted parameters we obtained an interpolated REDOR time constant, $\tau_{0.3}$, corresponding to the dephasing time, T , at which the REDOR fraction reaches 0.3. These $\tau_{0.3}$ values are collected in Table 1 and range from 1.38 ms for PEP/calcite to 3.3 ms for LTA/calcite.

From an expression given by Bertmer et al. (2000) and assuming as a first approximation that P occupies a C-position in an otherwise ordered calcite crystal containing 100% ^{13}C -carbonate, we estimate a limiting value of $\tau_{0.3} = 1.20$ ms for a simple substitution model in which there is no disruption of the structure. This value is smaller than any of those measured for the coprecipitates (Table 1), as expected since substitution of a phosphate or organophosphate group would likely result in defects with P-C(carbonate) distances longer than the C-C distances of calcite. Longer P- ^{13}C distances and/or fewer nearby ^{13}C nuclei reduces $M_2^{\text{P-C}}$, thereby lengthening $\tau_{0.3}$. To visualize the magnitude of the effect, we consider a simple model in which substitution of an organophosphate molecule yields a defect with a reduced number of nearest carbonate groups. In the calcite structure, the twelve nearest carbon atoms around a carbonate group are expected to have the strongest effect on the REDOR attenuation, six each at 4.047 and 4.988 Å (Markgraf and Reeder, 1985). Assuming that the phosphate P occupies a carbon position, these twelve adjacent carbonate groups together account for 85% of the $M_2^{\text{P-C}}$ owing to the

inverse sixth-power dependence on internuclear distance; the next closest C occur at a distance of 6.374 Å.

Figure 7 illustrates how the calculated $\tau_{0.3}$ value increases with omission of successively more of the nearest carbon atoms, in comparison with the values for the coprecipitate samples interpolated from the observed REDOR attenuation curves. This diagram shows that accommodation of the smallest organophosphates disrupts the structure to an extent equivalent to omission of between two and four nearest carbonate groups and is similar to the effect of orthophosphate. Interestingly, there is some evidence from the shorter $\tau_{0.3}$ for PEP and 3PG, that incorporation of these molecules disrupts the calcite structure to a lesser extent than for orthophosphate and might be more readily incorporated, possibly owing to the ability of the carboxyl group to substitute for carbonate. This observation suggests that properties other than size of the molecule contribute to the style of accommodation in the calcite structure, such as ability to engage effective or multidentate binding to calcium. Not surprisingly, the larger DPG molecule, containing two phosphate groups, gives a longer value for $\tau_{0.3}$, equivalent to omission of the six nearest carbonate groups, a radius of about 4 Å. The much larger phosphate esters LTA and DSPG yield REDOR attenuation curves with significantly lower rates, corresponding to $\tau_{0.3}$ values greater than 3 ms which is equivalent to omission of the 12 nearest carbonate groups, out to a radius of approximately 5-6 Å. Systematic experimental errors in the REDOR dephasing curves, such as pulse imperfections and dipolar recoupling inefficiencies should reduce the initial attenuation rate, lengthening the experimental $\tau_{0.3}$. Correcting for such effects would systematically shift the measured values (horizontal lines in Fig. 7) down relative to the calculated theoretical values. Thus the measured $\tau_{0.3}$ should be considered maximum values, and hence maximum extents of structural disruption. However, since the REDOR experiments were conducted under identical conditions with the same instrumentation for all of the samples, such systematic errors in the attenuation rate can be expected to affect all of samples uniformly. For this reason, the relative results for the coprecipitates can be

considered robust, such as the smaller disruption radius for the small organophosphates relative to orthophosphate.

An alternative structural interpretation of these REDOR attenuation rates is by comparison to that expected for adsorption complexes on the calcite surface. In an idealized case, with the phosphate P occupying a C position on the surface, it can be expected that the heteronuclear dipolar second moment M_2^{P-C} would be reduced to one-half its value for the simple substitution discussed above. Reduction of M_2^{P-C} by a factor of two increases the value of $\tau_{0.3}$ by a factor of $\sqrt{2}$, from 1.2 ms for the simple substitution to 1.7 ms for simple adsorption, owing to the parabolic relationship of the initial REDOR attenuation on dephasing time and its linear dependence on M_2^{P-C} . Relaxation of the surface complex into the interfacial region or bonding to the surface through organic functional groups other than phosphate would result in longer P- ^{13}C (carbonate) distances and longer $\tau_{0.3}$ values. The results shown in Figure 7 suggest that the REDOR data for the larger organophosphates DPG, LTA, and DSPG could be consistent with rigid adsorption complexes, or other reduced dimensional relationship of the coprecipitated molecule with the bulk of the calcite crystal such as location at internal interfaces or mosaic boundaries. Those for the smaller molecules however require larger M_2^{P-C} than can be explained by adsorption, and indicate that these molecules are more intimately imbedded in the calcite structure.

4.2 Intracrystalline organic matter in calcite

Our NMR results add to a growing body of evidence that occluded organic molecules in calcite can occur in a truly intracrystalline form, that is, as structural defects in the calcite structure. X-ray diffraction results for both synthetic organic/calcite coprecipitates (Aizenberg et al., 1997; Weber et al., 2014; Borukhin et al., 2012; Okumura et al., 2013; Kim et al., 2011; Pokroy et al., 2006a) and biogenic calcite (Berman et al. 1988, 1990; Pokroy et al., 2006b) show that the crystal lattice is systematically and anisotropically distorted relative to purely inorganic calcite. Such effects have been shown to be concentration dependent (Weber et al., 2014), providing strong evidence that accommodation of the

occluded molecules systematically distorts the calcite structure, altering the average size of the unit cell. Refinement of neutron diffraction data for biogenic calcite yields systematically longer carbonate C-O bonds (Zolotoyabko et al., 2010) than for inorganic calcite. Heating biogenic calcite to oxidize the occluded organic material reduces the average coherent crystal block size (Pokroy et al., 2006b), which was interpreted to indicate that the occluded molecules occur coherently within the calcite structure. A similar conclusion was reached by Berman et al. (1988) in which low mosaicity was observed in biogenic calcite despite minor concentrations of intracrystalline proteins. For biogenic calcite Metzler et al. (2010) observed a decrease in carbon XPS intensity in certain directions that was interpreted to be caused by disorder in the orientation of carbonate C-O bonds in response to the presence of occluded organic molecules. Imaging methods can also provide evidence for structural incorporation, such as the study of Kim et al. (2011) in which TEM images of calcite coprecipitated with copolymer micelles that showed the micelles intimately embedded in the structure with distorted shapes suggesting strong interaction with the calcite structural components.

Although the results of these previous studies are generally accepted as indicating structural incorporation, NMR spectroscopy remains one of the few methods by which a direct atomic-scale spatial interaction between occluded molecules and calcite structural components can be detected. The present results, along with our previous study of citrate/calcite coprecipitates (Feng et al., 2008), are in agreement with and amplify the results of these previous studies, by demonstrating direct, atomic scale proximity between the phosphate group of coprecipitated organic molecules and carbonate carbon in the calcite structure, even for large molecules containing more than sixty carbon atoms.

Our observation in the present study of atomic-scale proximity of coprecipitated organic molecules to the mineral structural components provides strong support that these foreign materials constitute intracrystalline defects. In the absence of pervasive porosity, such fully encased intracrystalline material can be expected to remain inaccessible to fluids or organisms on geological timescales, being released or externally altered only in the event of dissolution or recrystallization of the

encasing calcite crystal (excluding spontaneous alteration processes such as amino acid racemization, heating, or decomposition of unstable molecules). Such intracrystalline materials are appropriate for use in paleoenvironmental and paleoecological studies in that the foreign material can be presumed to have been present in the fluid from which calcite precipitated. However, it can be envisioned that many processes determine the extent to which dissolved molecules are occluded in calcite. Mechanistically, the most likely process of incorporating larger molecules as defects involves adsorption to the calcite surface followed by step bunching and eventual overgrowth by the surrounding crystal. In such a process the strength of bonding to the calcite surface, the rates of surface attachment/detachment, and the rate of crystal growth can all be expected to affect the partitioning into calcite (e.g., Watson, 2004, Thien et al., 2014). With these considerations and the chemical heterogeneity of dissolved organic matter it would seem difficult at this point to envision universal quantitative models for the occlusion of intracrystalline organic molecules in calcite. For example, by AFM Kim et al. (2009) observed that polyvinylalcohol adsorbed on growing calcite crystals, causing step bunching and 2-dimensional island nucleation that suggested the PVA was subsequently overgrown and occluded. Other polymers did not appear to affect step migration and were presumably excluded from the growing calcite crystal. Other studies also point to the possibility of selective coprecipitation, such as Dai et al. (2008) who found that a fluorescence-labeled polyaspartic acid molecule was excluded from growing calcite. These studies suggest that specific interactions between adsorbate molecules and calcite surfaces can affect the efficacy of coprecipitation and the impurity content of calcite, but the results of the present study demonstrate that processes exist that allow growing calcite crystals to encase diverse molecules, providing potential mechanism for long-term preservation.

4.3 Phosphorus in natural calcite deposits

Our study supports the notion that P is incorporated in speleothems through diverse mechanisms and occurs in different forms. The case of moonmilk suggests that even in the presence of biomediation, P is incorporated principally as inorganic phosphate in the calcite structure. Calcite moonmilk forms

from infiltration waters with a low saturation index (SI) for calcite, $SI < 0.2$, and a Ca^{2+} concentration from 0.9 to 1.2 mmol/L which, in the studied mountainous setting, corresponds to the vegetation belt above timberline (Borsato et al., 2000). A rise in temperature would imply an increase in soil CO_2 , higher dissolved Ca^{2+} and increased SI for calcite, which would shift the system to the formation of sparry speleothems (Frisia and Borsato, 2010; Borsato et al., 2016). As moonmilk calcite requires infiltration to form, it is reasonable to infer that its incorporation of P is a reliable proxy of hydrology. This study suggests that even in the case of biomediation, a thermodynamic partition coefficient could describe P incorporation in most speleothems, although the possibility exists for a growth rate dependence (Watson, 2004). It is also suggested that incorporation of P associated with organic molecules from soil provenance is more likely to be the case for speleothem grown in caves under efficient forest soils, at higher temperature and higher SI for calcite. Finally, our results indicate that organically bound P can be occluded in structural defects in moonmilk crystals.

The present results contrast with those of Mason et al. (2007) for stalagmite columnar (macrocrystalline) calcite, which showed ^{31}P NMR evidence for the presence of P as mineral inclusions (monetite and an unidentified phase), in addition to structural inorganic phosphate. Phosphate mineral inclusions were also observed in calcite coprecipitated with inorganic phosphate in laboratory experiments (see also Fig. 4). The absence of P-rich mineral inclusions in calcite moonmilk might reflect the chemistry of the originating drip waters or be a consequence of the growth mechanism of the moonmilk crystals, but further investigation is needed. Mason et al. (2007) postulated that for the Grotta di Ernesto stalagmites, which are overlain by a forest soil, desorption of orthophosphate colloiddally transported onto stalagmites during infiltration events promotes formation of calcium phosphate surface precipitates that are enclosed in the calcite by subsequent crystal growth. Here, we find that P associated with organic molecules can be incorporated as structural defects in calcite, but results for the moonmilk speleothems suggest that this is not the most important source of P in calcite. The different species of P species found in different types of speleothems and the variation observed among samples indicates that

709 multiple processes contribute to P uptake by calcite. Any significance inferred from calcite P content and
710 variations thereof must account for the different forms present, which can only be gleaned from
711 spectroscopic data.

712

713

5. CONCLUSIONS

The results of this study provide evidence for the structural incorporation in calcite of large organic molecules from the solution during calcite precipitation. Atomic-scale spatial proximity of calcite carbonate groups to the phosphate moiety of the investigated organic molecules was observed through NMR double-resonance methods. These results imply that calcite precipitated under natural conditions can accommodate organic molecules as structural defects. Such structurally occluded organic molecules can be expected to remain resilient to alteration provided that the calcite crystals do not recrystallize. The results of this study support the use of inorganically precipitated calcite as a repository for materials present in the solution at the time of precipitation, such as for paleoenvironmental proxies. NMR spectroscopic data for natural calcite moonmilk show that organophosphate can be detected in situ using these methods, but that inorganic orthophosphate is the dominant form of phosphorus in the calcite. Our study also supports the notion that P incorporation in speleothems is complex and varies among different types. Only spectroscopic investigation can elucidate how it is incorporated and in what form, which are important factors affecting the significance of P as a proxy of environmental or climate processes.

ACKNOWLEDGEMENTS

This work was supported by the US NSF (EAR0819838) and conducted with NMR instrumentation provided by NSF grants CHE0321001 and CHE0714183.

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FIGURE CAPTIONS

Figure 1. Structure and chemical formulae of organic phosphates coprecipitated with calcite and investigated by NMR spectroscopy in this study.

Figure 2. Scanning electron micrographs (A-F) and energy dispersive X-ray spectra (G-I) of selected sample materials synthesized for this study, including synthetic calcite (top row), calcite/BGP coprecipitate (middle row) and calcite/LTA coprecipitate (bottom row).

Figure 3. $^{31}\text{P}\{^1\text{H}\}$ CP/MAS spectra of solid phosphoester salts and corresponding calcite coprecipitates. All spectra taken at a 5 kHz spinning, 2 ms CP contact time, 1-2 s relaxation delay for 40,000-80,000 acquisitions. See Figure 1 for sample labels. Centerbands lie in the spectral region between the dotted lines. All other features are spinning sidebands.

Figure 4. $^{31}\text{P}\{^1\text{H}\}$ CP/MAS NMR spectra of synthetic phosphate/calcite coprecipitate (top) and calcite moonmilk samples CZ-3 (middle; 1 ms contact time, 2 s relaxation delay, 164000 acquisitions) and CZ-12 (bottom; 2 ms contact time, 1 s relaxation delay, 67000 acquisitions). Asterisks denote spinning sideband positions.

Figure 5. Typical $^{31}\text{P}\{^{13}\text{C}\}$ CP/REDOR NMR spectral sets for phosphoester/calcite coprecipitates. The decreased intensity of the dephased spectra (S) compared to the control spectra (S_0) indicates close spatial proximity of phosphate P to carbonate carbon ($<6\text{\AA}$). Spectra acquired at 11.7 T, 8 kHz spinning rate, 2 ms $^1\text{H}\rightarrow^{13}\text{C}$ CP contact time and 3 ms dephasing time. Spectra within each set are scaled proportional to absolute intensity.

Figure 6. $^{31}\text{P}\{^{13}\text{C}\}$ CP/REDOR dephasing curves for organophosphate/calcite coprecipitate samples.

Monoester and diester coprecipitate samples are denoted by the filled and hollow symbols, respectively.

“X” is for the orthophosphate coprecipitate.

Figure 7. Comparison of $^{31}\text{P}\{^{13}\text{C}\}$ REDOR dephasing time constants $\tau_{0.3}$ (time required to yield a

REDOR fraction $\Delta S/S_0 = 0.3$) for phosphate/calcite coprecipitates (dashed horizontal lines; as labeled in

Table 1), with the estimated variation of time constants with number of nearest C positions vacant (solid

symbols). Calculated values were estimated from the initial multispin REDOR response of Bertmer et al.

(2000), assuming phosphate P occupies a C position in the calcite structure. Dotted line denotes the

estimated $\tau_{0.3}$ value for an idealized adsorbate (see text).

Table 1. NMR chemical shift and REDOR data for phosphate esters, their corresponding calcite coprecipitates, and calcite coprecipitate with inorganic orthophosphate (ortho-P). Isotropic chemical shifts (δ_{iso}) were measured at 8 kHz spinning rate. CSA values are averages of spectra taken at spinning rates of 3 and 5 kHz and include the principal values (δ_{ii} , $i=1-3$), the anisotropy ($\Delta\delta$), and the span ($\Omega = \delta_{11} - \delta_{33}$). REDOR data are represented by the dephasing time to reach a REDOR fraction $\Delta S/S_0 = 0.3$ ($\tau_{0.3}$). “type” signifies monoester (m) or diester (d) and “form” refers to Na-salt (Na) vs. protonated phosphate (H) for as-received organophosphates. Estimated uncertainties are given as the error in the last digit. *n.d.* indicates value could not be determined.

sample	type	form	chemical shift (ppm)						$\tau_{0.3}$ (ms)
			δ_{iso}	δ_{11}	δ_{22}	δ_{33}	$\Delta\delta$	Ω	
BGP	m	Na	+6.5(4)	+72(6)	-22(4)	-30(3)	+97(7)	102(8)	1.56
BGP/calcite			+0.9	+71(5)	-19(4)	-50(2)	+105(7)	121(8)	
DPG	m	Na	+6.4(3)	+84(2)	-19(2)	-46(2)	+117(4)	130(8)	2.0
DPG/calcite			+1.7(3)	+86(4)	-32(3)	-49(8)	+127(6)	135(8)	
3PG	m	Na,H	+2.9(3)	+76(3)	14(3)	-81(3)	-126(5)	157(5)	1.47
3PG/calcite			+1.8(3)	+82(3)	-32(7)	-44(7)	+119(5)	126(6)	
PEP	m	Na	+3.3(5)	+94(5)	-30(3)	-54(3)	+136(7)	148(4)	1.38
PEP/calcite			-2.1(5)	+97(4)	-41(4)	-62(2)	+148(7)	159(4)	
LTA	d	H	-4.1	+78(4)	18(2)	-108(2)	-156(5)	186(4)	3.0
LTA/calcite			-1.5(5)	+73(4)	17(3)	-94(2)	-139(5)	167(4)	
DSPG	d	Na	+5.2(3)	+80(3)	41(2)	-106(1)	-167(4)		3.3
DSPG/calcite			+0.8(3)	+90(2)	28(1)	-116(1)	-174(2)	206(4)	
ortho-P/calcite			-2.5	+90(7)	23(8)	-121(5)	-178(9)	211(9)	1.47
CZ-3			+3.5(3)	+29(5)	+5(5)	-24(5)	-41(4)	53(8)	<i>n.d.</i>
			-2(2)	+74(5)	-22(5)	-57(5)	+113(5)	131(8)	<i>n.d.</i>
CZ-12			+3.5(3)	+28(5)	-2(5)	-15(5)	+36(4)	43(8)	<i>n.d.</i>
			-2(2)	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>

FIGURES

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