

Nucleoside Phosphorylation by Phosphate Minerals*

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In the presence of formamide, crystal phosphate minerals may act as phosphate donors to nucleosides, yielding both 5'- and, to a lesser extent, 3'-phosphorylated forms. With the mineral Libethenite the formation of 5'-AMP can be as high as 6% of the adenosine input and last for at least 10³ h. At high concentrations, soluble non-mineral phosphate donors (KH₂PO₄ or 5'-CMP) afford 2'- and 2':3'-cyclic AMP in addition to 5'- and 3'-AMP. The phosphate minerals analyzed were Herderite Ca[BePO₄F], Hureaulite Mn²⁺₅(PO₃(OH)₂(PO₄)₂(H₂O)₄, Libethenite Cu²⁺₂(PO₄)(OH), Pyromorphite Pb₅(PO₄)₃Cl, Turquoise Cu²⁺Al₆(PO₄)₄(OH)₈(H₂O)₄, Fluorapatite Ca₅(PO₄)₃F, Hydroxylapatite Ca₅(PO₄)₃OH, Vivianite Fe²⁺₃(PO₄)₂(H₂O)₈, Cornetite Cu²⁺₃(PO₄)(OH)₃, Pseudomalachite Cu²⁺₅(PO₄)₂(OH)₄, Reichenbachite Cu²⁺₅(PO₄)₂(OH)₄, and Ludjibaite Cu²⁺₅(PO₄)₂(OH)₄. Based on their behavior in the formamide-driven nucleoside phosphorylation reaction, these minerals can be characterized as: 1) inactive, 2) low level phosphorylating agents, or 3) active phosphorylating agents. Instances were detected (Libethenite and Hydroxylapatite) in which phosphorylation occurs on the mineral surface, followed by release of the phosphorylated compounds. Libethenite and Cornetite markedly protect the β-glycosidic bond. Thus, activated nucleic monomers can form in a liquid non-aqueous environment in conditions compatible with the thermodynamics of polymerization, providing a solution to the standard-state Gibbs free energy change (ΔG°) problem, the major obstacle for polymerizations in the liquid phase in plausible prebiotic scenarios.

In prebiotic scenarios biopolymers can be thought of as condensation products of abiotically formed monomers. Polymers (polysaccharides, peptides, and polynucleotides) will not spontaneously form in an aqueous solution from their monomers because of the standard-state Gibbs free-energy change (ΔG°), as critically reviewed in Ref. (1). Thermodynamic considerations impose that amino acid polymerization or the formation of phosphodiester or glycosidic linkages will be spontaneous only under highly dehydrating conditions. Thus, either (i) life

did not arise in aqueous environments, or (ii) pre-genetic polymerizations required activated monomers.

In the polymerization process of nucleic acids extant organisms activate the monomers by converting them to phosphorylated derivatives and then utilize the favorable free energy of phosphate hydrolysis to drive the reaction. Does this present day process mimic spontaneously occurring prebiotic reactions, thus representing a sort of biochemiomimesis descending from ancient pathways, or should it be considered a fully novel cellular invention?

The Source of Phosphate Is a Problem—Early studies on the condensation of water-soluble phosphates to polyphosphates and on the phosphorylation, condensation, or polymerization of biomolecules with polyphosphates have been reviewed (2, 3). Most of the phosphorus in the early Earth would have been in the form of water-insoluble minerals like apatites. Therefore, the origin of the water-soluble (poly)phosphates required for prebiotic evolution has long been a mystery (2). Yamagata *et al.* (3) showed that volcanic activity produces water-soluble phosphates through partial hydrolysis of P₄O₁₀, providing at least a partial solution to their origin. However, as pointed out, phosphates from hydrolysis of polyphosphate would precipitate to the sea bed as insoluble salts.

The phosphorylation of biological molecules has been explored through several different routes. Phosphonic acids have been proposed as a source of biophosphates (4). For the phosphorylation of nucleosides, two early reports described the preparation of uridine phosphates by heating uridine with inorganic phosphates in an aqueous environment (5) and the effects of condensing agents on this reaction (6). However, the inefficiency of the system due to the competition of water with the nucleoside was pointed out by the authors. Additionally, cyclization of uridine 2':3'-phosphate occurred under the same conditions but in much greater yield (6) by efficient intramolecular esterification. A possible solution to abiotic nucleotide formation provided by solid state chemistry was also proposed (7, 8). The alternative offered by phosphorylation in organic solvents, notably in formamide, was described in a series of pioneering studies by Schoffstal (9–12).

Building on these latter observations and on the large availability of phosphates in mineral form, we report the efficient phosphorylation of nucleosides occurring in formamide on numerous phosphate minerals. Consequently, the two alternatives mentioned above are not necessarily in contradiction: activated monomers can form in prebiotic conditions in a liq-

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uid, non-aqueous environment in the presence of phosphate minerals in conditions compatible with the thermodynamics of polymerization.

EXPERIMENTAL PROCEDURES

Materials

The minerals studied were: Herderite $\text{Ca}[\text{BePO}_4\text{F}]$, Hureaulite $\text{Mn}^{2+}_5(\text{PO}_3(\text{OH})_2(\text{PO}_4)_2(\text{H}_2\text{O})_4)$, Libethenite $\text{Cu}^{2+}_2(\text{PO}_4)(\text{OH})$, Pyromorphite $\text{Pb}_5(\text{PO}_4)_3\text{Cl}$, Turquoise $\text{Cu}^{2+}_2\text{Al}_6(\text{PO}_4)_4(\text{OH})_8(\text{H}_2\text{O})_4$, Fluorapatite $\text{Ca}_5(\text{PO}_4)_3\text{F}$, Hydroxylapatite $\text{Ca}_5(\text{PO}_4)_3\text{OH}$, Vivianite $\text{Fe}^{2+}_3(\text{PO}_4)_2(\text{H}_2\text{O})_8$, Cornetite $\text{Cu}^{2+}_3(\text{PO}_4)(\text{OH})_3$, Pseudomalachite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$, Reichenbachite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$, and Ludjibaite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$. The minerals were provided by Ezio Curti, former provider and consultant of the Collection of Minerals of the Department of Mineralogy (University La Sapienza, Rome). Pure crystals were isolated (except for Hydroxylapatite) under the microscope, washed twice, first with ethanol and then with analytical grade distilled water, air dried, and manually ground in a ceramic mortar. "High Resolution" grade Hydroxylapatite was obtained from Fluka.

Adenine, adenosine, cytosine, cytidine, and their monophosphorylated forms (5'-AMP, 3'-AMP, 2'-AMP, 2':3'-cyclic AMP, 5'-CMP, 3'-CMP, 2'-CMP, 2':3'-cyclic CMP, and 3':5'-cyclic CMP) were from Sigma Aldrich, analytical grade. Formamide ($\geq 99.5\%$, $\text{H}_2\text{O} \leq 0.1\%$) was from Fluka, and KH_2PO_4 was from Merck.

Methods

Phosphorylation Procedure and Analysis—Adenosine was dissolved in formamide and reacted at the concentration of 0.025 M in 1.5-ml Eppendorf tubes, final volume 1 ml. The reaction was carried out in formamide at 90 °C in the presence of the indicated phosphate donor: KH_2PO_4 (final concentration 0.05 M) or 5'-CMP (final concentration 0.05 M) or one of the indicated phosphate minerals (10 mg/ml, ground as indicated). Phosphates were added to adenosine from concentrated solution in formamide. Where indicated the mineral was pretreated at 130 °C for 72 h in formamide. After the indicated reaction time, the samples were analyzed by high pressure liquid chromatography (HPLC).²

HPLC Analysis—8- μl aliquots of the reaction mixtures were diluted with an equal volume of water to a final concentration of 50% formamide and injected into a SupelcosilTM LC 18-S 5- μm HPLC column (Supelco) 15 cm \times 4.6 mm. Elution was performed at a flow rate of 2 ml/min at room temperature with methanol:30 mM ammonium phosphate, pH 5.3 (2.5:97.5), UV irradiation 254 nm, on a HPLC Beckman System Gold instrument. Identification of the peaks was performed by comparison with standards. In this HPLC system 3':5'-cyclic AMP migrates very close to the large unreacted adenosine peak, thus preventing its precise evaluation. As for the cytosine system, four peaks are resolved, consisting of cytosine, cytidine, (5'-CMP + 3'-CMP + 2'-CMP + 2':3'-cyclic CMP), and 3':5'-cyclic CMP. Thus, the meaning of the assays on cytidine phosphorylation is

limited to the evaluation of the overall phosphorylation of the nucleoside.

Measurement of Phosphate Release in Formamide—The crystal minerals were ground to a fine powder in a ceramic mortar. A suspension of the powder was diluted (1 mg/ml) in formamide, washed, and centrifuged four times. Samples were incubated at 130 °C for the indicated times, and then 100- μl aliquots were evaporated in an oven at 250 °C for 20 min, resuspended in 100 μl of ultrapure water, and analyzed for phosphate by the molybdenum blue-method, which detects only orthophosphate (PO_4^{3-}) (13).

RESULTS

Phosphorylation of Adenosine by KH_2PO_4 or 5'-CMP in Formamide—The phosphorylation of adenosine by two different phosphate donors was analyzed for the sake of comparison with previously reported nucleoside phosphorylation systems (5–12, 14) and to define the kinetics and the product regioselectivity of the phosphate reactions in formamide.

Adenosine was reacted in water or in formamide at 90 °C for increasing times in the presence of the appropriate phosphate donor (KH_2PO_4 or 5'-CMP). The products were analyzed by HPLC. In water no phosphorylated forms were observed (data not shown), and the only reaction that took place was the cleavage of the β -glycosidic bond and the consequent release of adenine. As reported (15), the half-life of this bond at 90 °C in water is 4.5×10^2 h.

Fig. 1A shows the formation of different nucleotides in KH_2PO_4 -containing formamide. The half-life of adenosine under this condition is $\sim 10^3$ h, with its degradation following first order kinetics (15) (data not shown). After 330 h 17% of the adenosine starting material was transformed into phosphorylated products. Of the products, 16% were identified as 5'-AMP, 3'-AMP, 2'-AMP, 2':3'-cyclic AMP, and adenine (in order of appearance). Only traces of additional unidentified compounds were observed. Thus, by default the amount of the unidentifiable (see "Methods") 3':5'-cyclic AMP formed could not have been higher than 1.0%.

Fig. 1B shows the same reaction in 5'-CMP-containing formamide. The overall behavior is quite similar to that observed with KH_2PO_4 . The values are given as the percentage of each AMP nucleotide relative to the adenosine input. The only difference between the KH_2PO_4 - and the 5'-CMP-fed phosphorylation is the initial rate of formation of AMPs, as shown by the comparison of the formation of 5'-AMP from KH_2PO_4 or from 5'-CMP (Fig. 1C). The lag in the 5'-CMP-fed reaction corresponds to the time necessary to make the phosphate moiety available by releasing it from the 5'-CMP donor. The kinetics of nucleotide dephosphorylation in these conditions is reported in Ref. (15) and is in full kinetic agreement. The half-lives of the components of the reaction and of its products in formamide at 90 °C were separately measured by HPLC (data not shown) and were (min): adenine $> 10^5$, 5'-AMP 1.10×10^3 , 3'-AMP 0.40×10^3 , 2'-AMP 0.21×10^3 , 2':3'-cyclic AMP 7.0×10^4 .

These data show that adenosine is phosphorylated in different positions and that the order of appearance of each species is governed by its rate of formation, with the last to form being the 2':3'-cyclic species. The differential rates of degradation (2'-

² The abbreviation used is: HPLC, high pressure liquid chromatography.

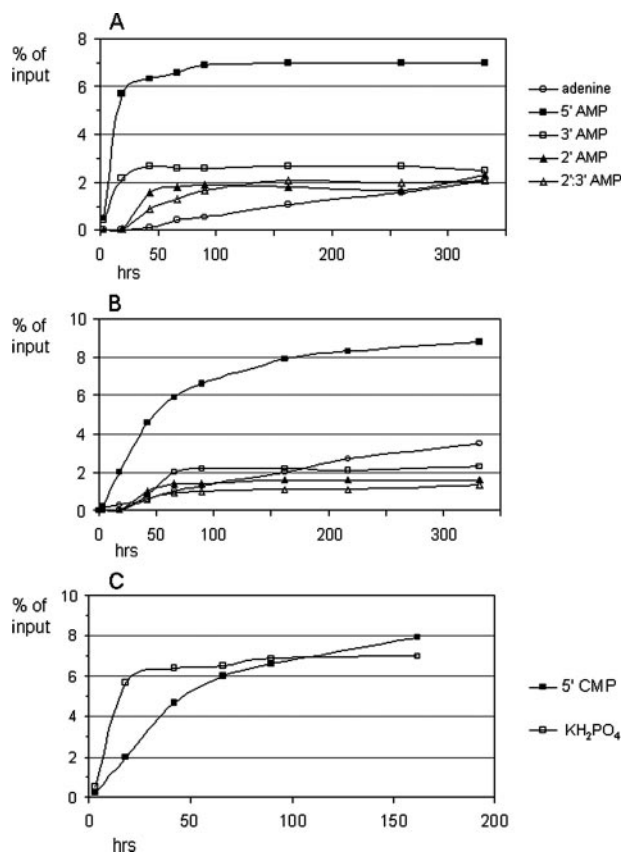


FIGURE 1. Adenosine phosphorylations by KH_2PO_4 or by 5'-CMP. Adenosine was reacted (formamide, 90 °C, as detailed under "Methods") with KH_2PO_4 (A) or 5'-CMP (B) for the indicated times (*abscissa*) and analyzed by HPLC. The afforded products are indicated on the *right side* of panel A. Ordinate, percentage of the product relative to adenosine input. Panel C compares the kinetics of production of 5'-AMP by KH_2PO_4 or 5'-CMP, as indicated.

AMP > 3'-AMP > 5'-AMP > 2':3'-cyclic AMP), measured on the order of minutes, and the species-specific rates of rephosphorylation, measured on the order of hours, determine the steady state equilibrium of the pool of phosphorylated nucleosides.

The cleavage of the β -glycosidic bond causes the progressive increase of adenine, deriving both from adenosine and from the phosphorylated forms. The 10-fold protective effect on the β -glycosidic bond exerted by the presence of a phosphate moiety bound at 5' was reported (15). Thus, adenine is mostly formed from unreacted, unphosphorylated adenosine. At longer times the 2':3'-cyclic species prevails. Eventually ($\sim 2 \times 10^3$ h) only adenine will remain (data not shown). A similar overall behavior is observed in the specular system of cytidine phosphorylation by KH_2PO_4 or by 5'-AMP. The primary difference is the higher total amount of phosphorylated forms in the cytosine system (21%) relative to the adenine system (16%). Thus, the overall rate of phosphorylation is determined more by the acceptor than by the donor. For either system, no phosphorylated forms are observed in water. Thus, the active component of the phosphorylation reaction is formamide.

Release of Phosphate by Formamide—As a prerequisite to the analysis of the phosphorylation (or otherwise) of adenosine by phosphate minerals, the kinetics of free phosphate release (or its absence) were determined. The molybdenum-blue color-

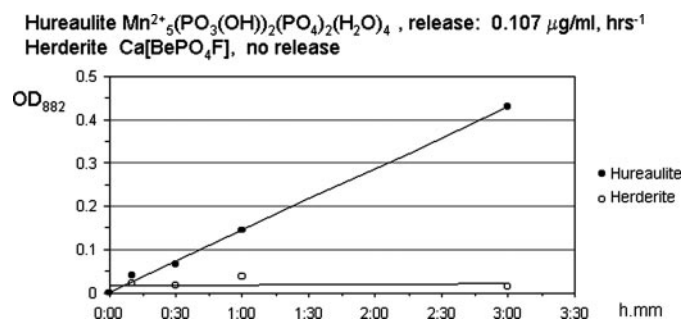


FIGURE 2. Release of phosphate by two phosphate minerals. Herderite $\text{Ca}[\text{BePO}_4\text{F}]$ and Hureaulite $\text{Mn}^{2+}_5(\text{PO}_3(\text{OH})_2(\text{PO}_4)_2(\text{H}_2\text{O})_4)$ (10 mg/ml in formamide) were treated at 130 °C for the indicated times. Samples were analyzed by the molybdenum-blue method (see "Methods"). Translation factor, $\text{OD}_{882} \times 1.7 = \mu\text{g/ml}$ phosphate.

metric assay allowing only the detection of orthophosphate (13) was the selected method. Given that all of the phosphate minerals studied contain single phosphorus units, the method is adequate.

The ground mineral material was reacted at 130 °C in formamide, and samples were analyzed at various time intervals. Fig. 2 shows examples of the two typical different behaviors: Herderite $\text{Ca}[\text{BePO}_4\text{F}]$, which did not release phosphate, and Hureaulite $\text{Mn}^{2+}_5(\text{PO}_3(\text{OH})_2(\text{PO}_4)_2(\text{H}_2\text{O})_4)$, which steadily released phosphate as a function of time. The phosphate release by the other 10 minerals analyzed is listed below. The amount of phosphate released refers to assays containing 1 mg/ml of mineral and is given as $\mu\text{g/ml}, \text{h}^{-1}$. The number in square brackets refers to the number of hours in which the release of phosphate proceeded linearly before progressively reaching a plateau, which is interpreted as being due to exhaustion and/or clogging of the reactive surfaces by reaction products. Minerals are ordered according to increasing elemental complexity. The minerals release as follows: Herderite $\text{Ca}[\text{BePO}_4\text{F}]$, no release; Hureaulite $\text{Mn}^{2+}_5(\text{PO}_3(\text{OH})_2(\text{PO}_4)_2(\text{H}_2\text{O})_4)$, 0.255 [5]; Libethenite $\text{Cu}^{2+}_2(\text{PO}_4)(\text{OH})$, 0.306 [2.5]; Pyromorphite $\text{Pb}_5(\text{PO}_4)_3\text{Cl}$, no release; Turquoise $\text{Cu}^{2+}_2\text{Al}_6(\text{PO}_4)_4(\text{OH})_8(\text{H}_2\text{O})_4$, 0.01 [1]; Fluorapatite $\text{Ca}_5(\text{PO}_4)_3\text{F}$, no release; Hydroxylapatite $\text{Ca}_5(\text{PO}_4)_3\text{OH}$, 0.102 [5]; Vivianite $\text{Fe}^{2+}_3(\text{PO}_4)_2(\text{H}_2\text{O})_8$, no release; Cornetite $\text{Cu}^{2+}_3(\text{PO}_4)(\text{OH})_3$, 0.81 [3]; Pseudomalachite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$, 0.170 [2]; Reichenbachite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$, 0.765 [0.5]; Ludjibaite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$, 0.72 [0.5].

Phosphorylation of Adenosine by Phosphate Minerals—Minerals were pretreated (130 °C, 72 h, in formamide), and then the temperature was lowered to 90 °C and adenosine was added. Samples were taken at various times, and the reaction products were analyzed by HPLC. Three different classes of activity were identified.

Class 1: no phosphorylation, as shown by Herderite, Pyromorphite, Turquoise, Fluorapatite, Vivianite. Minerals of Class 1 release no or minimal ($\leq 0.01 \mu\text{g/ml}$) amounts of phosphate.

Class 2: phosphorylation at very low levels, as exemplified by Hureaulite $\text{Mn}^{2+}_5(\text{PO}_3(\text{OH})_2(\text{PO}_4)_2(\text{H}_2\text{O})_4$ (Fig. 3) and Pseudomalachite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$ (data not shown). In this case the products identified are limited to the most abundant 5'-AMP, while only traces of other compounds were observed. Class 2 minerals do not presumably take active part in the phos-

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phorylation process, their function being quite likely limited to a passive release of phosphate into the medium.

Class 3: a group of minerals was identified that are characterized by an active behavior: Libethenite $\text{Cu}^{2+}_2(\text{PO}_4)(\text{OH})$, Ludjibaite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$, Reichenbachite $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$, Cornetite $\text{Cu}^{2+}_3(\text{PO}_4)(\text{OH})_3$, and Hydroxylapatite $\text{Ca}_5(\text{PO}_4)_3\text{OH}$.

Fig. 4 shows the phosphorylation reactions, carried out as for the previous samples, with an additional analytical step. Two parallel samples were treated as follows. Both were pretreated at 130 °C (72 h). One sample was then centrifuged three times to remove the mineral, leaving the released phosphate in the formamide supernatant (dubbed as "solute"), while the other sample still contained 1 mg of the mineral/1 ml of formamide. Adenosine was then added to each sample and reacted as before in the standard conditions (90 °C, formamide). The two samples were analyzed separately. Fig. 4A shows that for Libethenite the mineral-containing fraction affords a major production and release of 5'-AMP, continuous over ~1000 h. At the end of the experiment the mineral was treated with a solution of sodium pyrophosphate to wash out possible surface-bound compounds. The release of adenine (4.0%), 3'-AMP (1.3%), and

2':3'-cyclic AMP (0.92%) was detected. Thus, Libethenite actively promotes phosphorylation, selectively releasing from its surface one of the formed compounds (5'-AMP) and keeping in bound, but releasable, form other products of the reaction. The solute-containing supernatant from Libethenite yields only trace amounts of 5'-AMP, as observed for Class 2 minerals (not shown). Cornetite behaved similarly but was characterized by lower yield and no surface retention (Fig. 4B). Only marginal amounts of adenine were detected in Libethenite and Cornetite, which therefore protected the β -glycosidic bond of adenosine from cleavage.

Ludjibaite and Reichenbachite were analyzed similarly. The mineral-deprived part of the samples behaved like Libethenite (*i.e.* minor production of 5'-AMP, not shown). The products of the mineral-containing samples of Ludjibaite and Reichenbachite are shown in Fig. 4, C and D, respectively. Aggregates started forming after 100 h for Ludjibaite and after 50 h for Reichenbachite. Thus, the reactions could not be analyzed for longer times. Both minerals yield both the 5'- and 3'-forms with initial rates that are roughly comparable with that of Libethenite. Notably, the yield of 5'-AMP by Reichenbachite at 50 h is the highest observed (1.7%), which is twice that by Libethenite and three times that by Ludjibaite. A sodium pyrophosphate wash did not release additional compounds.

The four minerals identified as Class 3 display pseudo-catalytic activity. The mineral must be present for the phosphorylation reaction to occur. Here, mineral surface phenomena are a requirement of the reaction, as shown by the fact that the pre-released phosphates (solute) are almost inert and that the presence of the mineral is necessary for the phosphorylation process to occur at a high pace. However, given that the phosphate component of the mineral is transferred to the recipient adenosine, these minerals cannot be classified as catalysts. The catalytic function is exerted by formamide.

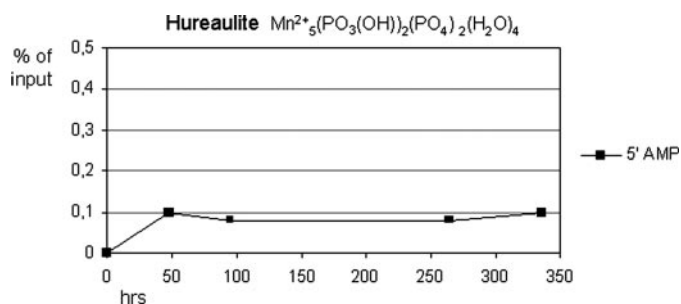


FIGURE 3. Formation of 5'-AMP from adenosine in formamide/Hureaulite at 90 °C for the indicated times (*abscissa*). Hureaulite pretreatment, 72 h at 130 °C.

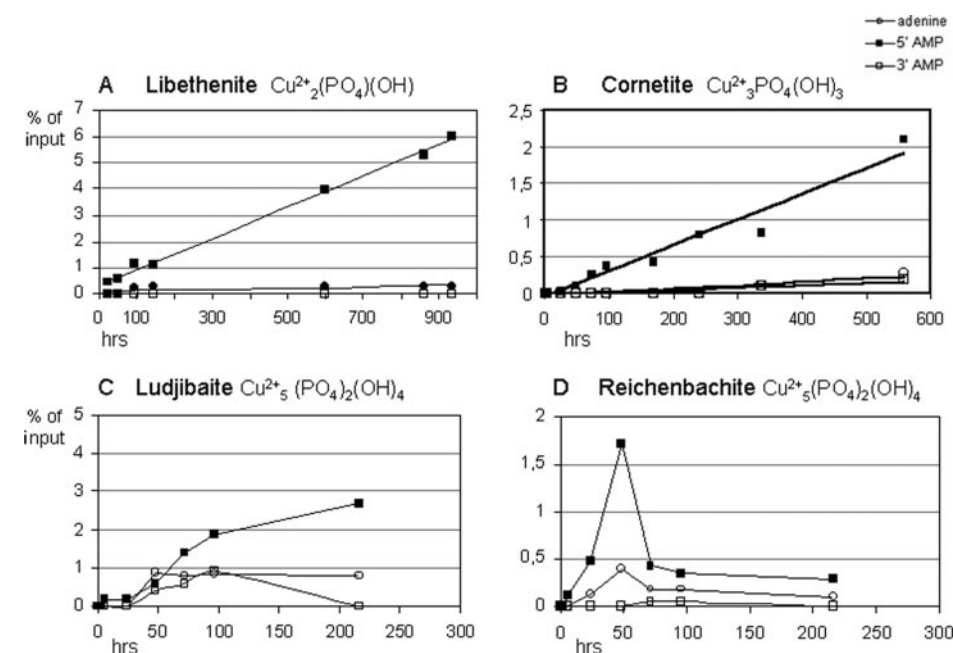


FIGURE 4. Adenosine phosphorylations in formamide/mineral at 90 °C for the indicated times. Mineral pretreatment, 72 h at 130 °C. The panels show the products (adenine, 5'-AMP, 3'-AMP, symbols as indicated in the top right corner) obtained in the presence of the indicated mineral. Note the different scales.

Class 3 minerals are all Cu^{2+} phosphates with very similar chemical compositions. Two of them (Ludjibaite and Reichenbachite) are chemically identical (both $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$). Nevertheless, their effects on the kinetics of the reaction differ. The third chemically identical mineral Pseudomalachite (also $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$) displays, as mentioned above, a totally different behavior characterized by minimal reactivity. The likely explanation and interest of these differences lies in their different crystallographic structures.

The structures of the three polymorphs of $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$ are all based on sheets of octahedra that are linked by (PO_4) tetrahedra. The sheets of octahedra are somewhat unusual in that they are not close-packed octahedra interspersed with vacancies (as is common in this type

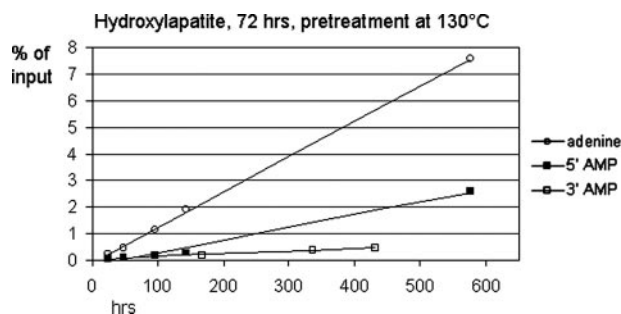


FIGURE 5. Adenosine phosphorylations in formamide/Hydroxylapatite. Procedures as above.

of structure). The structures of the three polymorphs of $\text{Cu}^{2+}_5(\text{PO}_4)_2(\text{OH})_4$ and their differences have been described in detail (19). However, in the absence of direct structural analysis of the interaction of their surfaces with adenosine and formamide, no correlation between differential crystal structure and differential phosphorylation activity is possible.

Hydroxylapatite, analyzed as for the Class 3 minerals, showed an intermediate behavior (Fig. 5): moderate production of 5'-AMP with 2.5% at 580 h on the mineral, 1.0% with the solute fraction; minor production of 3'-AMP; no release by pyrophosphate wash; and enhancement of the β -glycosidic bond cleavage (8% after 580 h) with fast degradation (data not shown) of adenosine.

Typical catalyst behavior was observed for the non-phosphate mineral Malachite $\text{Cu}_2^{2+}(\text{CO}_3)(\text{OH})_2$. At 24 h in the presence of added KH_2PO_4 (assays as in Fig. 1), the yield of 5'-AMP was 7% and that of 3'-AMP was 4.2%. A consistent formation of the 2':3'-cyclic AMP was observed at later times (2.7% at 130 h). Overall degradation started at ~ 200 h (data not shown).

In conclusion, Class 1 and 2 crystal minerals are intrinsically very stable and release, upon the treatments we describe, no or limited amounts of phosphate, respectively. Class 3 are all copper phosphate minerals, with the exception of hydroxylapatite. Interestingly, these compounds are chemically very similar (and in one case identical), yet their crystallographic structure and their behavior in phosphorylation differ. These minerals do at the same time release phosphate and copper ions, as shown by the intense blue coloration of the solution. Copper ions can act as catalysts in the phosphorylation reactions: the phosphorylation of a variety of sugars and structurally related compounds is catalyzed by copper salts (16), and copper ions interact with the phosphate moiety activating it toward nucleophilic additions (17). In addition, copper ions are involved in the nucleoside phosphorylation activity of enzymes as purine nucleoside phosphorylase, a key enzyme in the purine-salvage pathway (18).

In two selected minerals, Hureaulite and Pseudomalachite, the variation of concentration of the mineral (1, 2, and 4 \times) did not cause variation of the slope or of the yield of 5'-AMP (the only recovered product), suggesting that the concentration of the phosphate source is not a crucial parameter for the reaction. These data are confirmed by the analysis of phosphorylation performed with Libethenite, the most efficient mineral in the phosphorylation process, and with Cornetite. In these

instances, in the conversion range studied (6 and 2% conversion of substrate, respectively, after more than 20 days) a linear regression is observed, in agreement with a "zero order" kinetics, independent of the concentration of reagents.

DISCUSSION

The synthesis of non-ionic backbones and the evaluation of their possible biological relevance have been the focus of major efforts, beginning with the pioneering observations by Pitha (20) and Schneider and Benner (21) but concentrated mostly in the last decade (22). However, two properties of anionic polymers are difficult to surrogate: the avoidance of complex folding and the possibility to replace their variable components (*i.e.* the nucleic bases) without losing the dominant genetic character. Why nature chose phosphate has been clearly defined (23). The series of the phosphate negative charges keeps the linear molecule unfolded, facilitating its replication. Given the intrinsic instability of a pre-genetic polymer, replication is largely equivalent to survival. Thus, despite the numerous interesting results on non-phosphate backbones (22, 24, 25), the phosphorylation of nucleosides and their subsequent polymerization into phosphodiester chains, as exists today, should be considered an *ab initio* Darwinian success in genetic evolution, hence the interest of prebiotic spontaneous phosphorylation processes. Here we have described the phosphorylation of nucleosides in abiotic conditions, carried out by numerous phosphate minerals in formamide.

Minerals and their surfaces have long been examined for the roles that they play in the synthesis of polymers. For instance, mineral surfaces have been suggested to provide substrates to support the catalytic assembly of organic and biochemical molecules (26, 27). Their possible active role in origin-of-life processes was soon pointed out (28), and mineral theories for the origin of informational polymers were elaborated (*i.e.* as exemplified by the iron sulfide system 29). Clays, in particular, have been the object of repeated studies (28, 30, 31). With the exception of hydroxylapatite, phosphate minerals attracted less attention. The reports by Winter and Zubay (32) and by Acevedo and Orgel (33) remain isolated. The first describes the binding of adenine and adenine-related compounds to hydroxylapatite, and the second describes a template-directed oligonucleotide ligation on hydroxylapatite. In the dry film urea-based nucleoside phosphorylation system described by Zubay, hydroxylapatite was only $\sim 10\%$ as effective as inorganic orthophosphates (quantitative results being confined to inosine).

Despite the numerous investigations on the roles of minerals in prebiotic chemistry, the identification of phosphate minerals as donors to facilitate the passage from nucleosides to nucleotides was still lacking. The present observation of the efficient phosphorylation of a nucleoside by several phosphate minerals, each mineral being characterized by different kinetic and product regio-selectivity behavior, provides a greater number of plausible prebiotic scenarios based on the self-organization properties of nucleic bases.

The Interest of the 2':3'-Cyclic Phosphate Nucleotides—The more stable phosphorylation product in the formamide system described here is the 2':3'-cyclic form. The activated monomer,

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a necessary precursor of spontaneous abiotic polymerization, need not be a polyphosphate, namely a triphosphate. The preferential use of nucleosides phosphorylated at the 5'-position was discussed (34, 35). Nevertheless, the interest of the 2':3'-cyclic form should also be considered. First, the 2':3'-cyclic monophosphate nucleoside has been shown to form in numerous different reactions. It was observed that under neutral or mildly acidic conditions uridylyl (3',5')-uridine (36, 37) and adenylyl (3',5')-adenosine (38) undergo a buffer-catalyzed isomerization to their corresponding 2',5'-dinucleoside monophosphates and a buffer-catalyzed hydrolysis to form 2':3'-cyclic monophosphates with cleavage of the 5'-linked nucleosides. In addition to the formation from dinucleotides, the buffer-independent isomerization and phosphoester hydrolysis of methyl and isopropyl esters of adenosine 2'- and 3'-monophosphates yielding 2':3'-cyclic monophosphates was reported (39). Kinetics and mechanisms of these reactions were also determined (40). Additionally, 2':3'-cyclic monophosphate forms were reported by Lohrmann and Orgel under aqueous conditions (6) (where this was the most abundant product) as well as in dry film urea-based (13) reactions, by Schoffstall (9–12) with formamide chemistry and by Zubay (7) in dry film urea. Nucleoside 3'-monophosphates efficiently form 2':3'-cyclic compounds in evaporates containing trimetaphosphate and Mg^{2+} (8), and 2':3'-cyclic monophosphate extremities are formed upon hydrolysis of ribo-oligomers (41). The large negative standard enthalpy of hydrolysis (42) and high reactivity (43) of 2':3'-cyclic monophosphate nucleosides have also been pointed out.

The facile formation and the reactivity of this specific cyclic form make it particularly interesting in the perspective of polymer chain formation. It was actually shown (44, 45) that 3',5'-linked hexa-adenylic acid with a 2':3'-cyclic phosphate terminus couples on a polyuridylic acid template in the presence of ethylene diamine to form the dodecamer and octadecamer (6). The bond produced was largely that of the 2',5'-isomer, but ~5% of 3',5'-bonds also formed. The same authors observed (44) that upon annealing with a 3',5'-linked complementary poly U strand the stability of the 2',5'-bond becomes ~900-fold lower than that of the 3',5'-bond. This helical conformation-induced selective instability rapidly leads to a majority of the "natural" 3',5'-phosphoester bonds. Considered as a group, these studies support the prebiotic relevance of the formation of 2':3'-cyclic AMP by soluble phosphate donors in formamide and of the orders-of-magnitude larger stability of this monophosphate in formamide as reported above.

In a critical analysis of the prebiotic cytosine (and other nucleic bases) synthesis (46), Shapiro concludes that "the evidence that is available at the present time does not support the idea that RNA (...) was present at the start of life. This conclusion could be reversed if a prebiotic simulation were devised that produced all of the bases in good yield under a single set of conditions, by using a plausible combination of water, atmospheric components, and minerals."

HCN and H_2O are abundant components of interstellar clouds, HCN being the most abundant three-element compound (47) and H_2O the most abundant oxygen-containing

molecule (47). Consequently, they presumably were also abundant components of the early Earth.

Our working hypothesis is that water combined with HCN, affording formamide, thus quenching its reactivity and instability. Based on its wide liquid range (4–210 °C) and limited azeotropic effects, formamide could have easily formed highly concentrated or completely anhydrous solutions.

The plausible presence of varying amounts of water in formamide even at high temperature could be considered a possible cause of rapid degradation of the formed nucleotides. Thus, we have measured the stability of both 3'- and 5'-AMP in different formamide/water ratios (0, 10, 25, 66, and 99.5) at 90 °C. The results showed that the half-life of the phosphoester bonds increases relative to pure water by less than a factor of two at high ($\geq 99.5\%$) formamide/water ratio (to be detailed elsewhere). At temperatures higher than 100 °C, the presence of water would not have posed a long-lasting stability problem.

By simple heating in the presence of common catalysts, formamide yields a complete set of nucleic bases (48–51), acyclonucleosides (52), creates conditions in which phosphoester bonds in polymers are more stable than in the monomers (14, 53), and favors the formation of micelles (54, 55). The novel property of formamide described here, efficient phosphorylation of nucleosides from minerals, provides one more missing link in the identification of a single unifying chemical frame for the self-organization of nucleic polymers in prebiotic conditions.

Keeping in the Shapiro logic, the minerals instrumental to kick start informational polymers could have been any of those previously described (48–52) or combinations thereof, with Montmorillonites affording the highest yields and TiO_2 affording acyclonucleosides. The presence of mineral phosphates (which also catalyze the condensation of formamide into a large set of nucleic precursors) (51) could have allowed, within the same chemical frame, the next step toward polymerization: their phosphorylation.

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