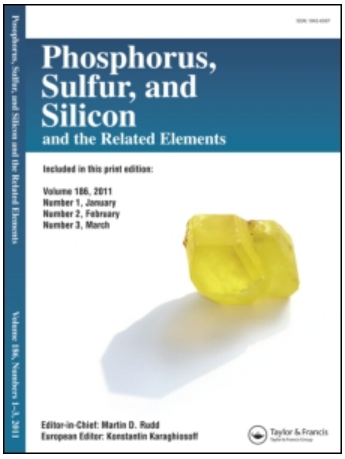

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The Phosphoenolpyruvate Phosphorylation: A Self-Organized Mechanism with Implications to Understand the RNA Transformations

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THE PHOSPHOENOLPYRUVATE PHOSPHORYLATION: A SELF-ORGANIZED MECHANISM WITH IMPLICATIONS TO UNDERSTAND THE RNA TRANSFORMATIONS

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In this article, we present a study about the non-enzymatic hydrolysis of phosphoenolpyruvate (PEP) by following the reaction course through ^{31}P NMR spectroscopy. We have demonstrated that PEP in water exists mainly as a very stable cyclic pentacoordinate phosphorus compound in equilibrium with other cyclic forms. This explains the PEP stability in water. In contrast, after addition of an alcohol to a PEP aqueous solution, other very unstable cyclic pentacoordinated intermediates are formed, which immediately collapse, giving a feasible phosphorylation of the alcohol. It is known that cyclic pentacoordinated phosphorus intermediates are favored over the corresponding acyclic intermediates by a factor of 10^6–10^8, and this preference, found also in this study, might be the "driver mechanism" able to overcome the clutter of abiotic chemistry, thus permitting formation of pre-RNA molecules probably with a "self-organized process."

Keywords Phosphoenolpyruvate; phosphorylation; ^{31}P NMR spectroscopy

INTRODUCTION

How life began on Earth is one of the great scientific mysteries. However, the question of how simple organic molecules go towards the life is largely unanswered, even though there are many hypotheses. Some of these postulate the early appearance of nucleic acids ("replication first"),[1] whereas others postulate the evolution of simple chemical reactions and pathways first ("metabolism first").[2] We think that a model that combines aspects of both could be the correct way but in sequential manner: first "metabolism" and then "replication." It is probably that from simple abiotic and then prebiotic reactions we could arrive at simple pre-RNA molecules. However, we believe that the chief obstacle to understanding the metabolic origin of life or RNA-based life is to identify a plausible mechanism for overcoming the clutter wrought by abiotic chemistry.[1] This yet unknown, but possible, "self-organized, or autocatalytic mechanism, or driver reaction"[2] might be active also in controlling the easy formation and activity of small molecules such as phosphoenolpyruvate

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(PEP) or others related to simple phosphorus compounds, and also in explaining their high performance in the process of phosphorylation, which is essential in the chemical evolution of life.

The scope of this article is to understand why PEP is a very powerful phosphorylating agent in metabolic processes and to see if the mechanism found for PEP may be applied also to the non-enzymatic cleavage or elongation of RNA molecules.

Most of the reactions occurring through organophosphorus intermediates are driven by the ability of the phosphorus to form "hypercoordinate" species, mainly penta- and hexacoordinates,[3,4] which are fluxional species because they may undergo positional changes among substituents. For example, phosphoryl transfer reactions, which are basic biological processes, are generally assumed to involve pentacoordinated intermediates that influence the outcome of the reactions.[5] The trigonal bipyramidal (TBP) geometry represents the most common structure of pentacoordinated phosphorus intermediates.

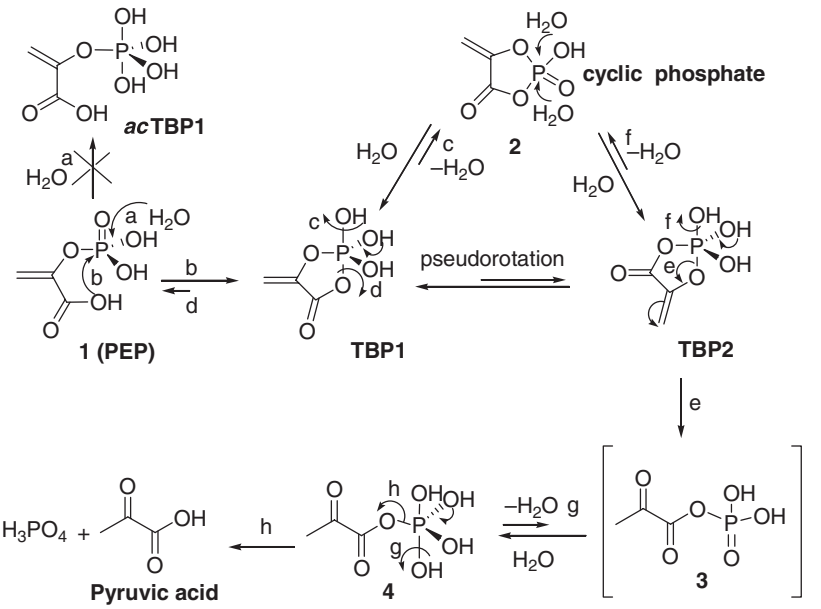
Sufficiently long-lived pentacoordinated intermediates can undergo stereomutation or positional interchange of the substituents at pentacoordinated phosphorus by a turnstile rotation[6] (TR) or a resulting equivalent Berry pseudorotation[7] (BPR), which are very rapid processes, since the energy barriers of pseudorotation are usually relatively low.[8] The relative position of the substituents in pentacoordinated compounds depends on their steric hindrance and apicophilicity. Apicophilicity is the relative preference of substituents to occupy the apical positions as opposed to the equatorial positions in TBP structures: a number of experimental results and theoretical calculations have indicated a general propensity of the more electronegative substituents to prefer the apical positions; in addition, bulky ligands prefer the equatorial positions.[9] Their stability strongly depends on their structure; in particular, when it is possible, the formation of a cycle around the pentacoordinate phosphorus atom is favored over that of the corresponding acyclic intermediate by a factor of 10^6–10^8, as reported by Westheimer.[10] In this way any other possible collateral reaction in which the phosphorus atom belongs to an acyclic pentacoordinate intermediate is practically minimized or annulled.

From these considerations, it can be deduced that the super-activated formation of cyclic pentacoordinate phosphorus intermediates might be a possible candidate for this hypothesized important "self-organized or autocatalytic mechanism" acting either on simple molecules or on complex molecules in processes in which phosphorylation or dephosphorylation reactions are involved—processes that are the centerpiece for the evolution of life. In fact, strongly activated phosphorylation processes involving small molecules, such as the so-called "high-energy" biomolecules (e.g., PEP), might be explained in postulating the very favored formation of cyclic pentacoordinate phosphorus intermediates.[5,11,12]

PEP is a simple three-carbon molecule, containing a phosphoryl group, that occupies a central role in primary metabolism. It is a very strong phosphorylating agent that permits the occurrence of a wide range of metabolic events.[13] It might be one of the first prebiotic molecules, likely originating from glucose in an aqueous puddle of the primitive Earth.[14]

PEP is very stable in aqueous solution. In fact, the non-enzymatic hydrolysis of PEP occurs at high temperatures (60–75°C), while the hydrolytic rate is enhanced at room temperature only in the presence of several metal ions.[15] In contrast, when PEP is in the presence of alcohol, it is very unstable, and the formation of phosphorylation products occurs immediately. What is the reason for these contrasting behaviors?

Benkovic and Schray[16] studied the non-enzymatic hydrolysis of PEP and postulated a mechanism in which the cyclic phosphate **2** (Scheme 1), isolated by Clark and Kirby,[17] is involved as an intermediate. Consequently, they proposed the formation of a cyclic



Scheme 1 Mechanism of non-enzymatic hydrolysis of PEP. When dissolved in water (neutral conditions) at room temperature, PEP (**1**) exists prevalently in the cyclic form **TBP1**, and is stable at least for four months. In acidic (pH∼2) aqueous solution, **1** is completely hydrolyzed, giving H_3PO_4 and pyruvic acid in about three months. In acidic (pH∼2) aqueous solution and at 60°C, **1** is completely hydrolyzed after 6 h. Intermediate **2** is very important in this process.

pentacoordinate phosphorus intermediate or transition state as precursor of the cyclic phosphate **2**.

RESULTS AND DISCUSSION

Hydrolysis of PEP

In this study, we have reinvestigated the non-enzymatic hydrolysis of PEP by following the reaction course through ^{31}P NMR spectroscopy. On the basis of our spectroscopic studies, reported below, and taking into consideration the large number of studies that now exist concerning cyclic hypervalent phosphorus intermediates,[5,18] we have drawn a mechanism that gives a clear explanation of the contrasting behavior of PEP. In other words, we will explain why PEP is very resistant to hydrolysis, while it is, in apparent contrast, a powerful phosphorylating agent of alcohols (e.g., it easily undergoes addition of methanol). This phenomenon can be explained as shown in Scheme 1.

An intramolecular nucleophilic attack, via *b*, by the hydroxyl oxygen atom of the carboxy group to the P═O group of PEP (**1**) could form a cyclic pentacoordinate phosphorus intermediate, such as **TBP1**, which is more stable of a factor of about $10^{6–8}$ with respect to the corresponding acyclic pentacoordinate intermediate ***ac*TBP1**, which is derived from the attack of water, via *a*, on the same P═O group (Scheme 1). As a consequence of this huge rate difference between intra- and intermolecular pathways *a* and *b*, water cannot attack the P═O group of PEP, and this explains the great stability of PEP in water. Decomposition of **TBP1**, via *c*, by elimination of water, gives, in very small amount, the cyclic phosphate

intermediate **2**. Addition of water to **2** could give again intermediate **TBP1** together with **TBP2**. Permutational isomerization of **TBP1** might also give the pentacoordinate intermediate **TBP2**. On the other hand, since the more electron-accepting group tends to prefer the apical positions, the formation of the intermediate **TBP2** is very unfavourable with respect to that of **TBP1**. In fact, the PO−C=O group, in apical position in **TBP1**, is more apicophilic than the PO−C=C− group, in apical position in **TBP2**.

Then, the decomposition of the intermediate **TBP2**, via *e*, gives pyruvoyl dihydrogen phosphate (**3**), which can undergo easily an attack by water. In this manner, pentacoordinate phosphorus compound 1-oxo-1-[(tetrahydroxyphosphoranyl)oxy]acetone (**4**) is formed, which immediately collapses to pyruvic acid and phosphoric acid. From Scheme 1, it is evident that hydrolysis comes only from the intermediate **TBP2**, derived principally from **2**. A small portion of **TBP2** could derive from **TBP1**, but this process might be very unfavorable in normal reaction conditions (room temperature and absence of metal ions), where **TBP1** is presumably largely favored over **TBP2** because the PO−C=O group is more apicophilic than the PO−C=C− group.

With these considerations in mind, we carried out the hydrolysis of PEP in different experimental conditions and the reaction course was followed through ^{31}P NMR spectroscopy. When PEP was dissolved at room temperature in neutral aqueous solution, we noted only the formation of an intermediate whose spectroscopic data (Figure 1) are in agreement with the structure of **TBP1** (Scheme 1).

This compound gives a signal, in the ^{31}P NMR spectrum, at $\delta = -3.8$ ppm. It is also stable after several months, and unlike intermediates such as **2**, **TBP2**, and **4**, no trace of phosphoric acid is found after four months. It should be noted that the ^{31}P NMR chemical shift of PEP in water it has been reported[19] to be $\delta = -3.61$ ppm, in agreement with our findings.

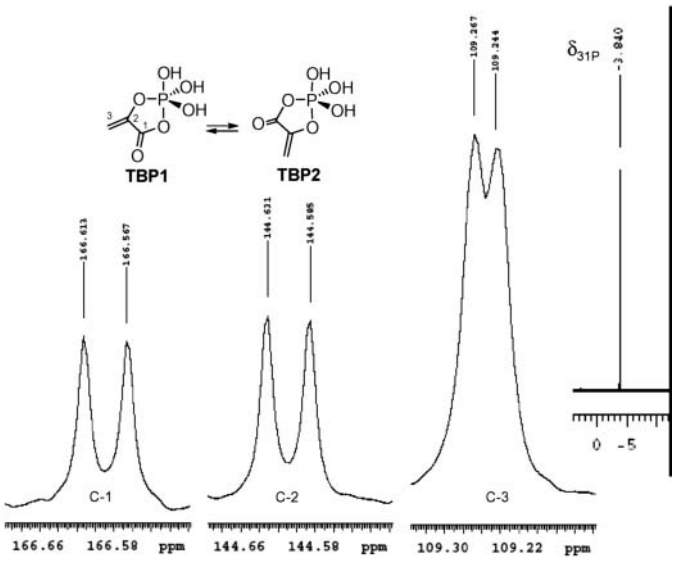


Figure 1 Expanded views of ^{31}P NMR (right, $\delta_{31P} = -3.8$ ppm) and ^{13}C NMR spectra (left) of PEP dissolved in D_2O: $\delta_{13C} = 166.6$ (d, $J = 6.9$ Hz), 144.6 (d, $J = 7.1$ Hz), 109.2 ppm (d, $J = 3.8$ Hz).

It is important to note that ^{31}P NMR signals of pentacoordinated phosphorus species can be found in a very wide range of chemical shifts (from −1 to −70 ppm, and, sometimes, also in the positive zone).[5,20] The ^{31}P chemical shift depends on many factors, such as the presence of particular substituents on the phosphorus, as well as the solvent used and the sample concentration. Sometimes in the negative zone, signals of tricoordinate phosphorus compounds such as phosphines or of tetracoordinated ones such as phosphates can be found. It is possible that the same monophosphate dissolved in organic solvent can have a negative chemical shift, but while in water it can have a positive chemical shift. For these reasons, the structure of a compound (in our case TBP1) cannot be ascertained only by its ^{31}P NMR chemical shift.

In present case, however, the hypothesis that PEP in aqueous solution is in a cyclic form, and consequently with the phosphorus atom in a pentacoordinate state, was confirmed by ^{13}C NMR analysis (Figure 1). The obtained spectral data, $\delta_{13C} = 166.6$ (d, $J = 6.9$ Hz), 144.6 (d, $J = 7.1$ Hz), 109.2 ppm (d, $J = 3.8$ Hz), are consistent with a cyclic structure for **TBP1**. Particularly diagnostic are the very close values of the two coupling constants of the signals belonging to the carboxyl carbon atom (C-1) and to the vinylic carbon atom (C-2), which indicate that they are members of a cyclic structure. In fact, in the acyclic structure **1**, these coupling constant values can not expected to be so similar, because vinylic carbon atom is characterized by a $^2J_{P–C}$ coupling constant, while for the carboxyl carbon atom, a smaller $^3J_{P–C}$ must be found. These considerations are supported also by the fact that similar values of the $^2J_{P–C}$ coupling constant were found for the cyclic compound **2**, prepared as reported in the literature[18] (see the Experimental section). These data demonstrate that PEP in aqueous solution is very stable, and, surprisingly, it is prevalently in a cyclic form in which its phosphorus atom is pentacoordinate, as is well described by the structure of **TBP1**. It should be noted that X-ray diffraction analysis of PEP in solid state revealed that it is an acyclic compound.[21]

When the hydrolysis of PEP was carried out in acidic conditions (pH∼2) at room temperature, we observed again the immediate formation of **TBP1** but also the slow formation of the final product of hydrolysis (phosphoric acid, $\delta_{31P} = 0.5$ ppm). The reaction reached the end point after about three months.

Furthermore, when this reaction was carried out at 60°C, after 1 h we observed again a high intensity signal of **TBP1**, a very small and transient signal probably belonging to compound **2** ($\delta_{31P} = +2.4$ ppm), a low intensity signal ascribed to **4** ($\delta_{31P} = -10.0$ ppm), and a consistent signal of phosphoric acid. At the end of the reaction, after about 6 h, only the signal of phosphoric acid was present in the ^{31}P NMR spectrum.

Hydrolysis of Cyclic Phosphate 2

It is probably that the true powerful phosphorylating agent in this mixture of intermediates is the cyclic phosphate **2**. A demonstration of this statement comes from the hydrolysis of compound **2**. In fact, when we carried out, at room temperature, the hydrolysis on pure compound **2**, synthesized by using the procedure reported by Clark and Kirby,[17] we observed (by following the reaction course through ^{31}P NMR spectroscopy) the immediate appearance of a broad signal at $\delta_{31P} = -3.7$ ppm, ascribed to **TBP1** and **TBP2**, probably in equilibrium. After a few minutes, a sharp signal of **TBP1** ($\delta_{31P} = -3.8$ ppm) and a high-intensity signal of phosphoric acid ($\delta_{31P} = +0.7$ ppm), together with a low-intensity signal of **4**, appeared (see Scheme 1). When, at this point, the reaction temperature was

raised to 60°C we observed, after two additional hours, the total disappearance of **TBP1** and the complete formation of phosphoric acid (see the Experimental section).

This can be explained in the following manner. Addition of water to **2** is very fast because it is carried out on a phosphoryl group of a cyclic compound, which is more activated of about 10^{6-8}-fold with respect to a correspondent acyclic compound. In this case, the addition of water can occur in two directions with the same probability. The one opposite to the bond PO-CO- gives pentacoordinate intermediate **TBP1**, and the other opposite to the bond POC=C− gives pentacoordinate intermediate **TBP2**.

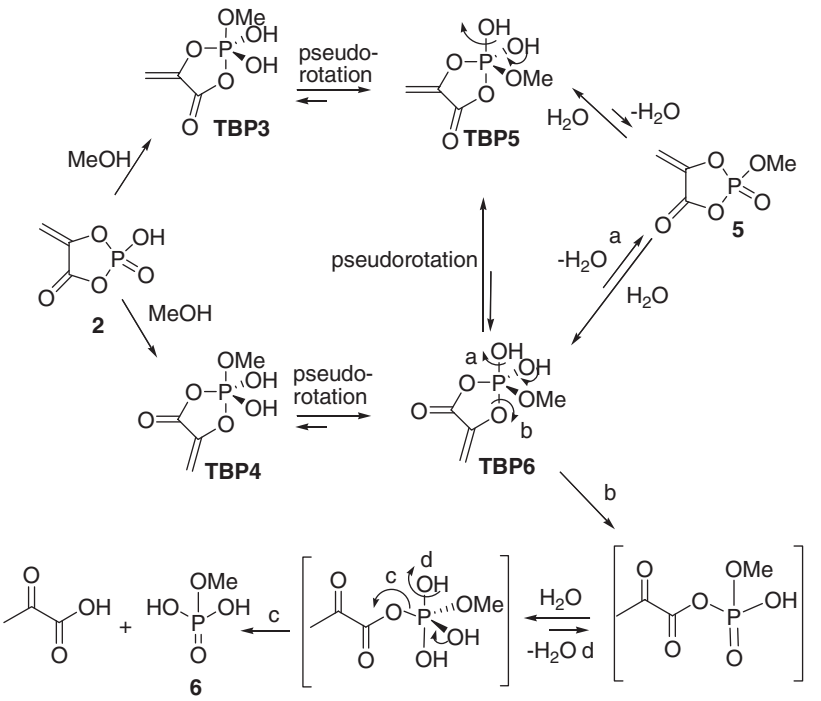
Intermediate **TBP2**, which, in the first instance, is 50% of the two intermediates, immediately gives pyruvic acid and phosphoric acid, probably via the pyruvoyl dihydrogen phosphate (**3**), and, after addition of water, via intermediate **4**, which immediately collapses to pyruvic acid and phosphoric acid. A small part of **TBP2** also might be trasformed into its isomer **TBP1**. The intermediate **TBP1** is very stable, and then it can interconvert to isomer **TBP2** or, when the reaction is carried out at 60°C, it can be transformed into compound **2**. In this manner starting compound **2** is totally hydrolyzed after about two additional hours.

Addition of Methanol to 2 and to PEP

First, we will discuss our results on the phosphorylation of an alcohol (methanol) starting from compound **2**, then we will report our findings on the case of PEP.

Theoretically, the dissolution of compound **2** in dry methanol should produce, as first intermediates, **TBP3** and **TBP4** (Scheme 2). But these intermediates, having in apical positions an OMe group, prefer to permutate to the corresponding isomers **TBP5** and **TBP6**, where in the apical position there is an OH group, which is more apicophilic than OMe. In fact, OMe is bulkier than OH. If the reaction is carried out with an alcohol larger than methanol, this preference would be greater than with methanol. In this manner, having only intermediates **TBP5** and **TBP6** in which the OMe group is in the equatorial position, it is not possible to have elimination of MeOH with reformation of **2** (It should be remembered that in a TBP pentacoordinate intermediate, the departure of a group can occur only when it is in apical positions.).[22,23] On the contrary, in the case of addition of water to **2** (above experiment, Scheme 1), we always have intermediate **TBP1** or **TBP2** in which it is possible to eliminate water with reformation of **2**, and for this reason the total rate of the hydrolysis is very slow.

These considerations should explain why PEP prefers to phosphorylate an alcohol rather than water. Experimentally we found a confirmation of this hypothesis. When compound **2** was treated with dry methanol at room temperature and the course of reaction was followed by ^{31}P NMR spectroscopy, we observed the immediate appearance of a signal at ^{31}P NMR ($\delta_{31P} = -2.6$ ppm) ascribed to **TBP5** and **TBP6**, probably in rapid equilibrium, as was found for **TBP1** and **TBP2** in the case of PEP. After a few minutes the formation of methyl cyclic phosphate **5** ($\delta_{31P} = +2.4$ ppm) occurred with concomitant decrease of signal of **TBP5** and **TBP6**. After about 3 h, the prevalent presence of **5** was detected. By the addition of water to the methanolic solution of **5**, we observed, after 1 h, in ^{1}H and ^{31}P MMR spectra, the formation of methyl phosphate **6** ($\delta_{31P} = +1.5$ ppm) and pyruvic acid. Subsequently, these results were confirmed when we dissolved PEP (**1**) in a solution of dry methanol at 60°C. In this case we found similar results (see the Experimental section).



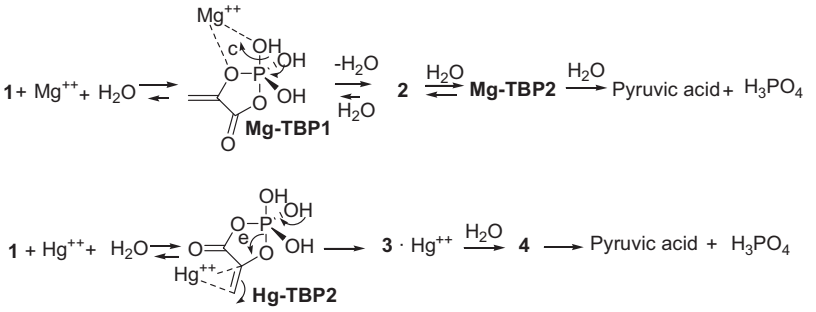
Scheme 2 Phosphorylation of methanol starting from pure **2**.

Hydrolysis of PEP in the Presence of Metal Ions

When the hydrolysis of PEP is carried out in the presence of some metal ions such as Mg^{++} or Hg^{++}, the hydrolytic rate is enhanced. Benkovic and Schray[15] studied the catalytic activity of these metal ions on the hydrolysis of PEP via kinetic measurements. He found for Mg^{++} a good activity, but surprisingly for Hg^{++}, he observed an increase of the hydrolysis rate of a factor of about 10^6, similar to that of the enzymatic hydrolysis. Now, with our knowledge about the factors that influence the apicophilicity of a group, we could also explain this catalytic activity.

It is well known[24] that mercury ions form labile interactions with olefin bonds. In an analogous manner, the POC=C− group in **TBP2** is coordinated with Hg^{++}, and consequently this group becomes more apicophilic than PO−C=O, and so stabilizing the intermediate **TBP2**. In this manner (Scheme 3), the equilibrium is totally shifted towards **Hg-TBP2** in which the POC=C−Hg group, owing to its high electron-withdrawing power, is the most apicophilic and thus the best leaving group. This causes the immediate apical departure of this substituent and the formation of the acyclic pyruvoyl dihydrogen phosphate (**3**), which immediately undergoes hydrolysis.

Actually, when the hydrolysis of PEP was carried at room temperature in the presence Hg^{++} ions and the reaction course was followed by ^{31}P NMR spectroscopy, we observed the immediate appearance of a signal at $\delta_{31P} = -4.1$ ppm, probably belonging to **Hg-TBP2**, and the concomitant appearance of the signal of phosphoric acid. The end of the reaction occurred after about 4 days at room temperature. When the same reaction was carried out at 60°C, it appeared to be complete after a few minutes. When the hydrolysis



Scheme 3 Metal ions catalysis in PEP hydrolysis.

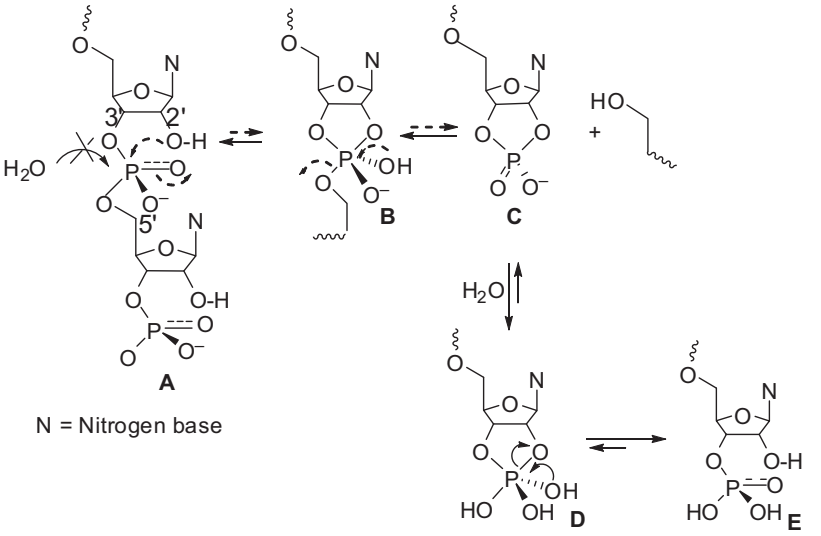
of PEP was carried at room temperature in the presence of Mg^{++} ions, we observed after 20 min a signal at $\delta_{31P} = -4.3$ ppm, indicating the presence of a complex with Mg^{++} (**Mg-TBP1** in Scheme 3) together with the signal of phosphoric acid. At the end of the reaction, after about 4 months, we observed only the signal of phosphoric acid. When this reaction was carried out at 60°C, its end point occurred after 4 h. In this case, it is likely that the coordination of Mg with the two oxygen atoms, as depicted in Scheme 3, favors the departure of the OH group in apical position, causing the formation of compound **2**, which is the true phosphorylating agent.

In all these mechanisms, the formation of hexacoordinated species must not be excluded, but they are probably very unstable because they have only one cycle around the P atom.[3,4] For this reason we did not detect suitable signals in the ^{31}P NMR spectrum.

Correlation with RNA

Next, we will see if the mechanism found for PEP may be applied in similar manner to the non-enzymatic cleavage (or elongation) of RNA molecules. The mechanistic details of the non-enzymatic hydrolysis of RNA remain obscure, despite extensive efforts over many years to determine them.[19,25] The emphasis of the recent investigations on RNA hydrolysis focused on the study and the role of the factors that govern the formation, isomerization, and breakdown of the pentacoordinated phosphorus intermediates such as **B** (Scheme 4) that are involved in this process. Now we re-propose this generally accepted mechanism, but in the light of the results obtained on the hydrolysis of PEP. In particular, we will see how the formation of the pentacoordinate intermediate **B** and of the cyclic intermediate **C** occurs, which is very similar to intermediates **TBP1** and **2**, respectively, involved in PEP mechanism depicted in Scheme 1.

As shown in the proposed mechanism (Scheme 4), the 2′-oxygen of the ribose ring first attacks the phosphorus atom (Scheme 4, structure **A**), acting as an internal nucleophile to generate the cyclic pentacoordinate intermediate or transition state **B**. This attack should be activated by a factor of $\sim 10^6$-fold with respect to any other external nucleophilic attack, such as that with water, which would give formation of an unfavored acyclic pentacoordinate transition state. The cyclic phosphodiester **C** can be obtained by collapse of the pentacoordinate intermediate **B** after departure of the group **O-5′**, which is the most apicophilic group in **B**. Now, once **C** is formed, which is very similar to compound **2** in the PEP mechanism, it can easily undergo an attack, activated by its cyclic form, by the



Scheme 4 Proposed mechanism of self-cleavage or hydrolysis of ribozymes with formation of cyclic phosphodiester **C**, causing the 3′-5′ bond cutting of the RNA chain. Structure **A** represents the 3′,5′-phosphodiester linkage in the ground-state configuration. The N group represents any of the four natural nucleotide base moieties. Dashed lines depict the continuation of the RNA chain. It is reported[26] that the 5′-thio RNA was cleaved almost two orders of magnitude more rapidly than the parental 5′-oxy RNA substrate. This is in accord with a possible better coordination of the Mg ion on sulfur than on oxygen.

nucleophile H_2O with formation of the stabilized cyclic pentacoordinate intermediate or transition state **D**, which then collapses, giving the product of hydrolysis **E**.

It should be noted that the **O-2′** group is a more apicophilic and leaving group than **O-3′** in all pentacoordinate cyclic intermediates, such as those shown in Scheme 4. This is due to the presence of the N group (N represents one of the four natural nucleotide base moieties), which is more electron-withdrawing than **C-5′**. In this manner, the almost exclusive ligation of phosphoryl group in **O-3′** position in the RNA chain is explained. These last steps can also explain the facile elongation of RNA, which is the reverse (normal arrows in Scheme 4) of the cleavage reaction (dotted arrows).

It should be noted that on the basis of these different apicophilicities of OH groups caused by the presence of N-base bonded at **C-1′**, we can deduce that in the natural formation of a ribonucleotide, the RNA building block, the attack on the sugar by the N base must occur before the phosphorylation, which consequently selects the **O-3′** position. In other words, we think that the natural assembling of a ribonucleotide must occur in a "self-organized process" in which the first step should be the attack of the nitrogen base on the sugar.

Obviously, in the mechanism depicted in Scheme 4, metal ions such as Mg^{++} could play a key role in driving the reaction in one direction or its reverse. For example, coordination of the magnesium ion to the 5′-oxygen should favor the apical position of this group and its subsequent departure.

In addition, this mechanism might explain both the difficulty of RNA to undergo hydrolysis and its ability to facilitate chemical transformations, such as its elongation process as well as peptide bond formation and transesterification.[27] We suspect that in these

transformations, the cyclic phosphate **C** is the true "catalyst" of the ribozyme, similar to the cyclic phosphate intermediate **2** of PEP.

CONCLUSIONS

In conclusion, we have explained why a small molecule such as PEP, the so-called "high-energy" biomolecule, is very stable in aqueous solution while it is a powerful phosphorylating agent for alcohols. The mechanisms involved in these processes are well explained by the intervention of cyclic phosphorus pentacoordinate intermediates, which have been identified by spectroscopic data. In addition we have found that PEP in aqueous solution is prevalently in a cyclic form while in solid state is an acyclic compound.[22] The self-organized mechanism found in PEP hydrolysis and phosphorylation may be well applied also to RNA molecules.

Finally, we think that this "self-organized process" found in PEP, driven by cyclic phosphorus intermediates, might also be involved in primordial reactions. For example, very simple phosphorus derivatives, such as P_4O_{10}, a polycyclic compound contained in a primitive puddle of the early Earth, might promote the assembling of pre-RNA molecules. Work is in progress in this direction.

EXPERIMENTAL

NMR spectra were recorded at 300, 400, or 600 MHz for ^{1}H NMR; at 75.45, 100.57, or 150.82 MHz for ^{13}C NMR; and at 161.89 MHz for ^{31}P NMR, with Varian Gemini 300, Varian Mercury 400, or Varian Inova 600 instruments. ^{31}P NMR chemical shifts were referenced to external standard 85% H_3PO_4 aqueous solution; ^{1}H and ^{13}C NMR chemical shifts for samples dissolved in pyridine-d_5 were referenced to solvent (8.72 and 149.5 ppm for the lowest field signal in ^{1}H and ^{13}C spectrum, respectively); for those dissolved in D_2O, to external 3-(trimethylsilyl)propionic acid; and to CD_3CN (1.93 and 1.26 ppm for ^{1}H and ^{13}C spectrum, respectively).^{13}C and ^{31}P NMR spectra were recorded in a ^{1}H broadband decoupling mode. *J* values are given in Hz. All commercially available solvents and reagents were >99.5% pure. 2-(Phosphonooxy)acrylic acid (PEP, **1**) was purchased as potassium or cyclohexylammonium salt from Sigma-Aldrich.

Structure of 2-(Phosphonooxy)acrylic Acid (1, PEP) in Water

Potassium salt of **1** (0.015 g, 0.073 mmol) was dissolved in D_2O (0.7 mL), and the solution was poured in a NMR tube. The ^{31}P NMR spectrum of the solution showed a signal ascribed to compound **TBP1**, stable in solution for at least four months.

2,2,2-Trihydroxy-5-methylene-1,3,2λ^5-dioxaphospholan-4-one (**TBP1**): ^{31}P NMR (161.89 MHz, D_2O): $\delta = -3.8$ ppm; ^{1}H NMR (400 MHz, D_2O): $\delta = 5.74$ (dd, $J =$ 2.5 Hz, $J = 2.5$ Hz, 1H), 5.40 ppm (dd, $J = 2.1$ Hz, $J = 2.5$ Hz, 1H); ^{13}C NMR (150.82 MHz, D_2O): $\delta = 166.6$ (d, $J = 6.9$ Hz), 144.6 (d, $J = 7.1$ Hz), 109.2 ppm (d, $J = 3.8$ Hz).

Behavior of Compound 1 in Acidic Aqueous Medium

Compound **1** (as potassium salt) was dissolved in acidic (DCl) D_2O solution (0.7 mL, pD$\sim$2). Immediately, the ^{31}P NMR spectrum showed a signal ascribed to compound **TBP1** (δ_{31P} −3.8 ppm) (see above). After about 24 h at room temperature, the ^{31}P NMR

spectrum showed the presence of a signal at δ +0.5 ppm, corresponding to that of deuterated phosphoric acid (checked through addition of a small amount of an authentic sample of H_3PO_4). After 40 days, ^{31}P NMR spectrum showed the two signals of D_3PO_4:**TBP1** in 40:60 relative height. The reaction was complete after about three months.

The same experiment was carried out at 60°C in the NMR tube. After 1 h, the ^{31}P NMR spectrum showed presence of the signal of **TBP1**, a transient signal at $\delta = +2.4$ ppm (probably belonging to compound **2**), the signal of 1-oxo-1-[(tetrahydroxyphosphoranyl)oxy]acetone (**4**) ($\delta_{31P} = -10.0$ ppm), and that of deuterated phosphoric acid, in about 100:1:7:15 relative ratio. After 6 h, the only signal detected was that of D_3PO_4.

Behavior of 2-Hydroxy-5-methylene-1,3,2-dioxaphospholan-4-one 2-Oxide (2)

Compound **2** was synthesized in pyridine as previously described.[17] The structure of **2** (or of its cyclohexylammonium salt) was ascertained in non-hydrolytic conditions; its spectral data in different solvents are as follows:

^1H NMR (400 MHz, pyridine-d_5): $\delta = 6.24$ (dd, $J = 2.2$ Hz, $J = 2.2$ Hz, 1H), 5.94 ppm (dd, $J = 1.9$ Hz, $J = 2.2$ Hz, 1H); ^1H NMR (300 MHz, CD_3CN): $\delta = 5.91$ (dd, $J = 2.2$ Hz, $J = 2.2$ Hz, 1H), 5.61 ppm (dd, $J = 2.1$ Hz, $J = 2.2$ Hz, 1H); ^{13}C NMR (75.45 MHz, CD_3CN): $\delta = 163.8$ (d, $J = 6.6$ Hz, C=O), 144.4 (d, $J = 7.2$ Hz, CC=O), 111.3 ppm (d, $J = 4.6$ Hz, CH_2); ^{31}P NMR (161.89 MHz, $CDCl_3$): $\delta = +2.1$ ppm.

Compound **2** (0.010 g, 0.074 mmol) was dissolved in D_2O (0.7 mL) in an NMR tube. In the ^{31}P NMR spectrum, immediately the presence of compounds **TBP1** ($\delta = -3.8$ ppm), D_3PO_4, and **4** in 20:10:1 relative height were observed. At this point, the reaction temperature was raised to 60°C. After 2 h at this temperature, only the presence of deuterated phosphoric acid was detected.

Addition of Methanol at Room Temperature to Pure Compound 2

Compound **2** (0.010 g, 0.074 mmol) was dissolved in anhydrous methanol (0.7 mL) in an NMR tube and kept at room temperature. The ^{31}P NMR spectrum showed a signal corresponding to intermediates **TBP5** and **TBP6** ($\delta_{31P} = -2.6$ ppm). After a few minutes, the signal of **TBP5** and **TBP6** decreased, and concomitantly a signal ($\delta_{31P} = +2.4$ ppm), probably belonging to the methyl cyclic phosphate **5**, appeared. After about 3 h, the prevalent presence of **5** was detected. Water was added to this solution, and, after 1 h, ^1H NMR and ^{31}P NMR spectra showed the presence of methyl dihydrogen phosphate (**6**) ($\delta_{31P} = +1.5$ ppm) and pyruvic acid.

Addition of Methanol at 60°C to PEP (1)

In this case, PEP (**1**) was dissolved in dry methanol at 60°C. We found at first a very low signal ($\delta_{31P} = -3.8$ ppm) belonging to intermediates **TBP1** and **TBP2**; then the signals of intermediates **TBP5** and **TBP6** ($\delta_{31P} = -2.6$ ppm) and that of the very unstable methyl cyclic phosphate **5** ($\delta_{31P} = +2.4$ ppm) appeared. This spectrum also showed two signals at $\delta_{31P} = -2.3$ ppm and δ_{31P} +3.4 ppm corresponding to 2-hydroxy-2,2-dimethoxy-5-methylene-1,3,2λ^5-dioxaphospholan-4-one (derived from a second attack by methanol on intermediate **5**) and to dimethyl pyruvoyl phosphate, respectively. After the addition of

water, we observed immediate formation of methyl phosphate **6** ($\delta_{31P} = +1.5$ ppm) and dimethyl phosphate ($\delta_{31P} = +2.8$ ppm).

Hydrolysis of PEP in the Presence of Hg^{++} Ions

$HgSO_4$ (0.022 g, 0.073 mmol) was added at room temperature to a solution of potassium salt of **1** (0.015 g, 0.073 mmol) in water (2.0 mL). The reaction course was followed by ^{31}P NMR spectroscopy. The immediate appearance of a signal at $\delta_{31P} = -4.1$ ppm, probably belonging to **Hg-TBP2**, and the concomitant appearance of the signal of phosphoric acid were observed. The reaction appeared to be complete after about 4 days. When the same reaction was carried out at 60°C, it appeared to be complete after a few minutes

Hydrolysis of PEP in the Presence of Mg^{++} Ions

$MgCl_2$ (0.007 g, 0.073 mmol) was added at room temperature to a solution of potassium salt of **1** (0.015 g, 0.073 mmol) in water (1.0 mL). The reaction course was followed by ^{31}P NMR spectroscopy. The appearance after about 20 min. of a signal at $\delta_{31P} = -4.3$ ppm, probably belonging to **Mg-TBP2**, was observed, together with the signal of phosphoric acid. The reaction was complete after about 4 months. When this reaction was carried out at 60°C, its end-point occurred after 4 h.

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