

# PYROPHOSPHATE FORMATION FROM PHOSPHO(ENOL)PYRUVATE ADSORBED ONTO PRECIPITATED ORTHOPHOSPHATE: A MODEL FOR PREBIOTIC CATALYSIS OF TRANSPHOSPHORYLATIONS

MARCELO HERMES-LIMA and ADALBERTO VIEYRA\*

*Departamento de Bioquímica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro,  
21.910, Rio de Janeiro, RJ, Brazil*

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**Abstract.** It has been postulated that adsorption and surface catalysis, as well as repeated drying and wetting cycles, were essential in the synthesis, interconversion and co-evolution of phosphorylated molecules, including 'energy-rich' compounds. We have investigated the formation of pyrophosphate from phospho(enol)pyruvate and orthophosphate. The reaction occurred within hours at 37 °C, required the adsorption of phospho(enol)pyruvate onto sedimented phosphate, exhibited Michaelian-like behavior and showed positive cooperativity with respect to divalent cation concentration. Thus in 'mild', near-equilibrium conditions, the de-solvated surfaces of phosphate crystals can catalyze the formation of pyrophosphate with a kinetic behavior similar to that found in contemporary enzymes. The experimental system we describe may represent a model for the prebiotic catalysis of transphosphorylations.

## 1. Introduction

Bernal (1951) first suggested that adsorption of biomolecules and surface catalysis played an important role in chemical evolution. Subsequently several authors (Miller and Parris, 1964; Lohrmann and Orgel, 1971; Rao *et al.*, 1980; Acevedo and Orgel, 1986) developed models showing that energy donor molecules and self-replicating systems must have formed, interconverted and evolved by passing through repeated drying and wetting cycles (Lahav and Chang, 1976; Lahav *et al.*, 1978). On the other hand, it has been postulated that in early epochs pyrophosphate (PPi) may have acted as a phosphorylating energy acceptor before the advent of ATP-forming reactions (Kulaev *et al.*, 1980; Baltscheffsky *et al.*, 1986). This PPi formation could have taken place on the surface of phosphate minerals, the energy of the phosphoanhydride linkage being derived from suitable organic compounds previously formed far from equilibrium by condensation of simpler molecules after capture of radiant energy (Miller and Parris, 1964; Miller *et al.*, 1976). It has been suggested that these prebiotic phosphorylating and condensing agents capable of forming PPi were preserved as key metabolic intermediates, one likely example being carbamylphosphate (Miller and Parris, 1964; Clarke and Elsdén, 1980).

We recently studied the formation of PPi from orthophosphate (Pi) using as condensing agent acetylphosphate, the simplest chemical analog of the acylphosphate

\* To whom correspondence should be addressed.

group formed in the catalytic cycle of the P-ATPases (Pickart and Jencks, 1984), under conditions in which the reactants became de-solvated and therefore precipitated (Vieyra *et al.*, 1985; Meyer-Fernandes and Vieyra, 1988). In this work we report the transphosphorylation from phospho(enol)pyruvate (P-enolpyruvate) to Pi to form PPi and pyruvate as a model for phosphoryl transfer reactions with high yields at low temperature (37 °C). The experiments were designed: (a) to investigate the kinetic properties of precipitates capable of catalyzing transphosphorylations under conditions of reduced water availability such as might occur during drying periods in prebiotic eras (Lahav and Chang, 1976; Lahav *et al.*, 1978); and (b) to establish a correlation between adsorption of a high-energy phosphorylated compound onto solid Pi and kinetics of its transphosphorylation to PPi.

## 2. Material and Methods\*

Buffers, P-enolpyruvate, ADP, NADH, EGTA, inorganic pyrophosphate, and the coupled enzymes pyruvate kinase/lactate dehydrogenase were purchased from Sigma chemical Co. The radioactive phosphate ( $^{32}\text{Pi}$ ) was obtained from the Brazilian Institute of Atomic Energy, purified using the method of Boyer and Bryan (1967) as modified by de Meis (1984). Dimethyl sulfoxide ( $\text{Me}_2\text{SO}$ ) and poly(ethyleneimine)cellulose (PEI-cellulose) sheets were purchased from Merck (Darmstadt, West Germany). Other chemicals were analytical reagent grade.

Samples containing P-enolpyruvate incubated in the presence of  $^{32}\text{Pi}$  and various concentrations of  $\text{CaCl}_2$  or  $\text{MgCl}_2$  were assayed for: (a) precipitated  $^{32}\text{Pi}$ ; (b) P-enolpyruvate adsorbed onto Pi sediments, and (c)  $^{32}\text{PPi}$  formed during the initial phase (3 hr).  $^{32}\text{PPi}$  formed was also measured at longer intervals until the reaction approached a plateau (14 days).

Procedures for measuring  $^{32}\text{PPi}$  formation and  $^{32}\text{Pi}$  precipitation were as previously described (Vieyra *et al.*, 1985; Meyer-Fernandes and Vieyra, 1988). The composition of assay media is indicated in the figure legends. To avoid pH shifts during the course of the reaction the assay media contained high concentrations of buffers, at the pH values shown in the figure legends. The determinations of pH in  $\text{Me}_2\text{SO}$  were done as previously described (Vieyra *et al.*, 1985). The radioproduct ( $^{32}\text{PPi}$ ) was identified using enzymatic hydrolysis with inorganic pyrophosphatase and autoradiography of PEI-cellulose thin-layer chromatograms.

The calcium phosphate phase was formed by rapid mixing of  $\text{CaCl}_2$  and Pi. TRIS in buffered assay media. The Ca/P molar ratio in water was calculated using  $^{45}\text{CaCl}_2$  (with unlabelled Pi) and  $^{32}\text{Pi}$ . TRIS (with unlabelled Ca) as previously described (Meyer-Fernandes and Vieyra, 1988), in experiments performed in parallel and repeated at least five times.

\* Abbreviations: PPi, pyrophosphate; Pi, orthophosphate; P-enolpyruvate, phospho(enol)pyruvate;  $\text{Me}_2\text{SO}$ , dimethyl sulfoxide; EGTA, ethyleneglycol-bis-( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid; MES, 2-[N-morpholino]ethanesulfonic acid; MOPS, 3-[N-morpholino]propanesulfonic acid; TRIS, tris-(hydroxymethyl)aminometane.  $\text{PPi}_\infty$ , asymptotical value of PPi formation measured after 14 days.

Adsorption of P-enolpyruvate was measured after prior precipitation of ortho-phosphate-divalent cation complexes for 10 min at room temperature (28 °C). Then, P-enolpyruvate was added and the tubes were centrifuged at 4000 × g. The adsorption equilibrium was attained rapidly (not shown). Adsorbed P-enolpyruvate was measured after dissolving the solid phase with EGTA, using the coupled system of pyruvate kinase, ADP, NADH, and lactate dehydrogenase.

The entropy of activation ( $\Delta S^\ddagger$ ) was calculated according to the absolute rate theory (Glasstone *et al.*, 1941) for temperatures ranging from 35 to 59.5 °C in completely aqueous media and from 30 to 60 °C in media containing 11.3 M Me<sub>2</sub>SO. The period of incubation was 100 min.

### 3. Results and Discussion

Experiments performed in aqueous suspension with a nearly constant amount of a solid matrix of calcium phosphate formed at pH 8.0 (2.5 μmol of phosphorus in the form of insoluble structures in one ml of incubation media) show that adsorption of P-enolpyruvate on the surface of Pi crystals follows a saturable function with respect to initial P-enolpyruvate concentration (Figure 1). This behavior can be described by a Langmuir isotherm (Inset to Figure 1). No P-enolpyruvate

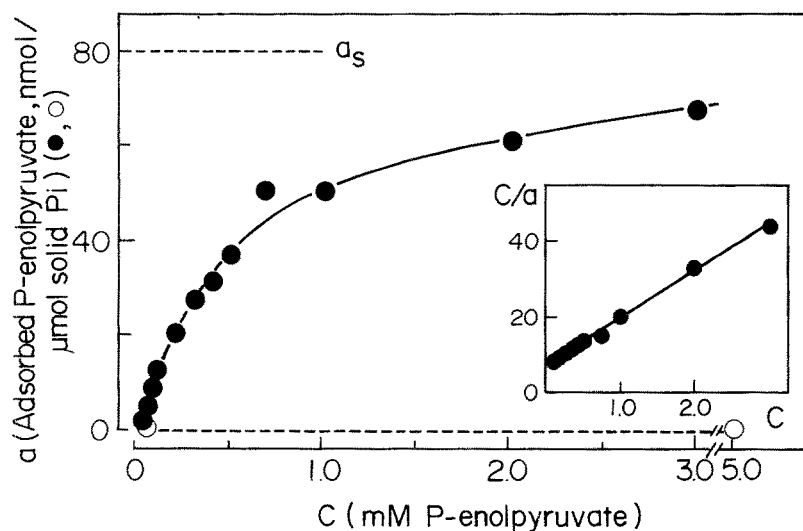


Fig. 1. Adsorption of P-enolpyruvate onto solid Pi. Dependence on P-enolpyruvate concentration. Samples contained buffer MES/MOPS/TRIS 50:50:50 (in millimolar) adjusted to pH 8.0 with HCl, variable concentrations of P-enolpyruvate (shown on the abscissa), 5 μmol Pi and 5 μmol CaCl<sub>2</sub> in a final volume of 1 ml. Open circles indicate that no P-enolpyruvate sedimented in the absence of Pi. *Inset*: graphic representation of Langmuir adsorption equation (Langmuir, 1918)  $C/a = C/a_s + 1/a_{ad} K'$  (reciprocal value of the adsorption coefficient of P-enolpyruvate,  $K_{ad}$ ) and  $a_s$  (asymptotic value of adsorbed P-enolpyruvate in nmol per μmol of solid Pi) were calculated by the least-squares method from the x-intercept and from the slope, respectively, of the function  $C/a$  vs  $C$  ( $r=0.998$ ,  $n=10$ ,  $P<0.001$ ).

precipitated in the absence of Pi (open circles in Figure 1). The reciprocal value of the coefficient for adsorption of P-enolpyruvate on Pi ( $K'$ ) obtained from the x-intercept of the regression line shown in the inset of Figure 1 was 0.55 mM. When the rate of PPi formation was measured under the same conditions as a function of initial P-enolpyruvate concentration in the suspension, it revealed a Michaelian-like behavior. A rectangular hyperbola adjusted to the data (Figure 2) showed that half-maximal velocity of phosphorolysis was attained at 0.88 mM. This value is close to the  $K'$  value for P-enolpyruvate adsorption onto precipitated calcium phosphate (Figure 1). The yield of PPi after 3 h ranged from 3.5 to 4.6% of the adsorbed P-enolpyruvate (Figure 2). The concentration dependence of the rate of reaction resembles that commonly encountered for enzymatic reactions that follow classical Michaelis-Menten kinetics.

Another set of experiments showed that adsorption of P-enolpyruvate onto the Pi surface exhibits a complex dependence on the concentration of  $\text{CaCl}_2$  originally added (Figure 3). In addition, the rate of PPi formation at each  $\text{CaCl}_2$  concentration increased strictly in parallel with both the amount of Pi precipitated and the amount of P-enolpyruvate adsorbed to these solid structures. The Ca/P molar ratio of the solid structures formed in the experimental conditions of Figure 3 varied between 1.40 and 1.67, confirming previous results (Meyer-Fernandes and Vieyra, 1988). This suggests that the calcium phosphate phase is formed by mixed crystals (van Kemenade and Bruyn, 1987).

The sigmoidal profiles of Ca concentration dependence may indicate that more than one Ca is involved in the adsorption of P-enolpyruvate at the surface of these calcium phosphate structures, and that more than one Ca participates in the transition

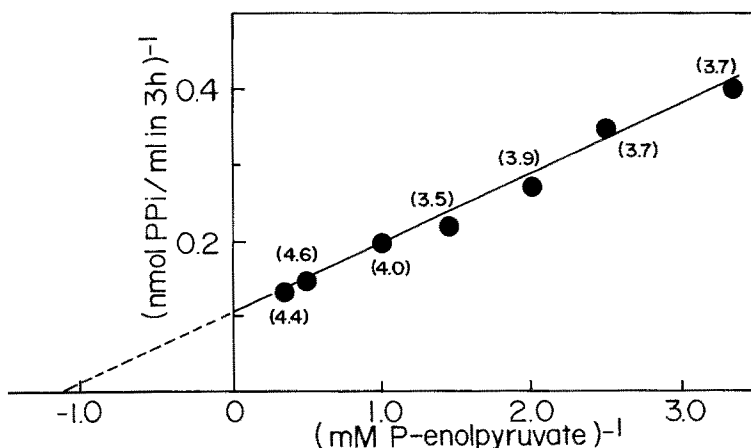


Fig. 2. Dependence of  $^{32}\text{PPi}$  formation on P-enolpyruvate concentration. Samples containing buffer (pH 8.0),  $^{32}\text{Pi}$  and  $\text{CaCl}_2$  as in Figure 1 were supplied with 0.3 to 3.0 mM P-enolpyruvate. After 3 hr (interval in which the reaction proceeded linearly) at 37 °C,  $^{32}\text{PPi}$  was determined as previously described (Vieyra *et al.*, 1985). Numbers in parentheses indicate the corresponding yield of PPi in 3 hr expressed as percent of adsorbed P-enolpyruvate. The line was adjusted by least squares ( $r=0.980$ ,  $n=11$ ,  $P<0.001$ ).

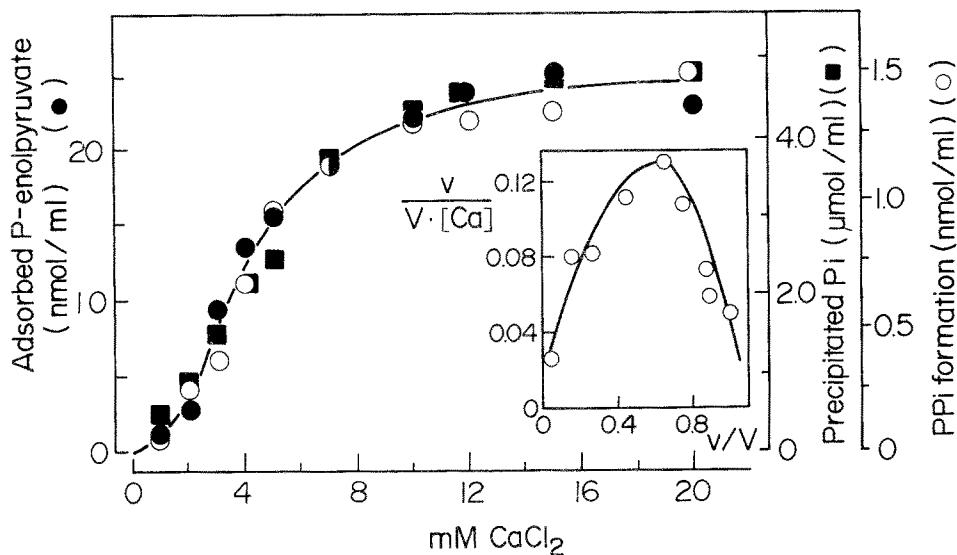


Fig. 3. Precipitation of  $^{32}\text{P}$ i (■), adsorption of P-enolpyruvate (●) and  $^{32}\text{P}$ Pi formation in 3 hr (○). Dependence on  $\text{CaCl}_2$  concentration. Solid  $^{32}\text{P}$ i and P-enolpyruvate were assayed as indicated in Figure 1, except that  $\text{CaCl}_2$  varied from 0 to 20 mM and P-enolpyruvate was 0.05 mM. Inset: Eadie-Scatchard representation of relative rate of PPI formation at different  $\text{CaCl}_2$  concentrations.

state of the phosphoryl transfer reaction (Vieyra *et al.*, 1985), as in the transphosphorylations that occur in homogeneous solutions (Lowenstein, 1958; Miller and Westheimer, 1966). An Eadie-Scatchard-like plot of rate of PPI formation against Ca concentration (Inset to Figure 3) shows a profile that is typical of positive cooperativity. The apparent  $n$  value ( $n_{\text{app}}$ ) was 2.5, calculated from  $v/V_{\text{max}}$  at the peak of the curve (Inset to Figure 3), since at this point  $v/V_{\text{max}} = (n_{\text{app}} - 1)/n_{\text{app}}$ . This  $n$  value, which may be considered as an index of the degree of interaction between calcium sites involved in the reaction with P-enolpyruvate, is the same as that found to provide the best adjustment of the function  $1/v$  against  $1/[\text{Ca}]^n$  to a straight line (not shown).

To explore further the effect of de-solvation of the reactants, adsorption of P-enolpyruvate on precipitated Pi and phosphorolysis were assayed in the presence of  $\text{MgCl}_2$  and  $\text{Me}_2\text{SO}$ , an agent that promotes de-solvation of anions (Parker, 1962; Miller, 1963) and decreases water activity (de Meis *et al.*, 1985), and thus can simulate the drying processes that could have occurred in primitive eras (Lahav and Chang, 1976). Magnesium was chosen due to its ubiquitous participation in phosphoryl transfer reactions (Knowles, 1980). It is of interest that even after two weeks of incubation, phosphorolysis could not be detected in the presence of Mg in purely aqueous medium where both Pi and P-enolpyruvate remained entirely in soluble form. However, Mg became as effective as Ca when P-enolpyruvate was adsorbed on solid Pi upon addition of  $\text{Me}_2\text{SO}$  (Figure 4). The importance of de-solvation in promoting adsorption of P-enolpyruvate as a preliminary to phosphorolysis

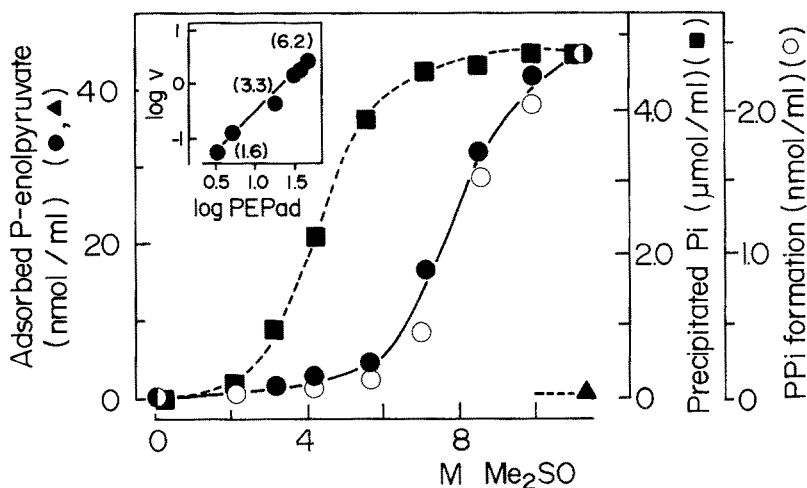


Fig. 4. Precipitation of  $^{32}\text{P}$ i (■), adsorption of P-enolpyruvate (●) and  $^{32}\text{P}$ Pi formation in 3 hr (○) in Mg-containing media. Effects of dimethyl sulfoxide. Assays were as indicated in Figure 1, except that P-enolpyruvate was 0.05 mM, pH was 9.0, an optimal concentration (5 mM) of  $\text{MgCl}_2$  was used instead of  $\text{CaCl}_2$  and  $\text{Me}_2\text{SO}$  was added at concentrations varying between 2.1 and 11.3 M. Solid triangle indicates that no P-enolpyruvate sedimented in the absence of added Pi. *Inset*: Rate of PPI formation as a function of adsorbed P-enolpyruvate ( $\text{PEP}_{ad}$ ) ( $r=0.991$ ,  $n=12$ ,  $P<0.001$ ). Yields of PPI after 3 hr are indicated by the numbers in parentheses.

emerged from the close correlation between the rate of PPI formation and amount of P-enolpyruvate adsorbed on precipitated Pi when water was gradually replaced by  $\text{Me}_2\text{SO}$  in Mg-containing media. In these conditions the yield of PPI in 3 h increased from 1.6 to 6.2% when the  $\text{Me}_2\text{SO}$  concentration was raised from 4.2 M to 11.3 M (Inset to Figure 4). No P-enolpyruvate precipitated in the absence of Pi. In contrast, it was adsorbed onto precipitated magnesium phosphate salts at  $\text{Me}_2\text{SO}$  concentrations higher than 5 M following a Langmuir isotherm in which the asymptotical  $a_s$  value attained 136 nmol P-enolpyruvate adsorbed per  $\mu\text{mol}$  of solid magnesium phosphate and  $K'$  was 0.61 mM (not shown). This value is remarkably similar to that found in purely aqueous medium with calcium phosphate crystals (0.55 mM; Inset to Figure 1).

Another similarity to the reaction occurring in purely aqueous medium was observed when PPI formation was assayed at 11.3 M  $\text{Me}_2\text{SO}$ . Under these conditions, the maximal amount of PPI formed after 3 hr was essentially the same as the value found in water with maximal adsorption of P-enolpyruvate onto a surface of calcium phosphate. A plot of  $v$  against  $v/[\text{Mg}]$  revealed a cooperative profile,  $n_{app}$  being 2.5 as in pure water with Ca (not shown).

In the preceding experiments, initial velocities were measured. To investigate the kinetics of PPI formation under heterogeneous conditions, the time course of the reaction was studied during 14 days. The reaction followed first-order kinetics ( $\text{PPI}_t = \text{PPI}_\infty [1 - e^{-kt}]$ ), and at the end of this period a steady level of PPI was reached.

In completely aqueous media containing of 0.05 mM P-enolpyruvate and the other conditions described in the legend of Figure 2, the yield of PPI attained 43% of initially adsorbed P-enolpyruvate and  $k$  (the first-order rate constant) was  $1.63 \times 10^{-4} \text{ min}^{-1}$ . In the presence of 11.3 M  $\text{Me}_2\text{SO}$  (0.05 mM P-enolpyruvate, pH 9.0, and  $\text{MgCl}_2$  instead of  $\text{CaCl}_2$ ) the yield was 56% of initially adsorbed P-enolpyruvate, and  $k$  was  $1.50 \times 10^{-4} \text{ min}^{-1}$ , i.e. essentially the same as that found with Ca in purely aqueous medium.

The pH dependence of PPI formation in completely aqueous media is shown in Figure 5. A small increase in reaction rate was observed up to pH 7.0, followed by a sharp increase between pH 7.0 and pH 7.8 (open circles). Similar profiles were found in this pH range for calcium phosphate precipitation (Inset) and P-enolpyruvate adsorption (filled circles). Since the predominant ionic form of P-enolpyruvate present in solution between pH 7.0 and 7.8 is the trivalent anion (Benkovic and Schray, 1968), this may be the species that adsorbs onto sedimented Pi and participates in the phosphoryl transfer reaction. As pH increased above 7.8 phosphorolysis gradually decreased, together with adsorption of P-enolpyruvate. The decrease in the amount of adsorbed P-enolpyruvate and in the reaction rate, may be due to modifications in the charge of the surface (Burton *et al.*, 1969) or to competition with hydroxyl anions (Orenberg *et al.*, 1985). Other explanations are also possible.

In the presence of  $\text{Me}_2\text{SO}$ , both adsorption of P-enolpyruvate and reaction rate continuously increased from pH 6.0 with a sigmoidal profile, reaching a maximum at pH 9.0 (not shown). For this reason, the  $\text{Me}_2\text{SO}$  experiments described below were done at pH 9.0. The difference in the pH profiles of both P-enolpyruvate adsorption and reaction rate, when compared with those found in pure water, may

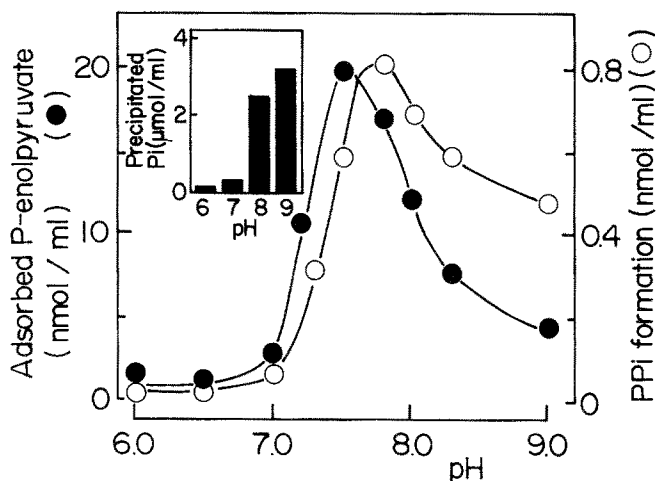


Fig. 5. pH dependence of P-enolpyruvate adsorption (●) and  $^{32}\text{PPI}$  formation in 3 hr (○). Assays were as indicated in Figure 1, except that pH varied from 6.0 to 9.0, and P-enolpyruvate was 0.05 mM. Inset: Precipitation of  $^{32}\text{Pi}$  under the same conditions.

be due to solvent-induced changes in the organization of the sediment structures rather than to a direct influence of cosolvent in the reaction itself. It has been postulated that  $\text{Me}_2\text{SO}$  effects in transphosphorylations catalyzed by energy-transducing systems are mainly due to its effects on the properties of water (de Meis, 1987; Kandpal *et al.*, 1987).

Finally, the temperature dependence of reaction rates showed high and negative  $\Delta S^\ddagger$  values:  $-210 \text{ J K}^{-1}\text{mol}^{-1}$  in water with Ca and  $-144 \text{ J K}^{-1}\text{mol}^{-1}$  in 11.3 M  $\text{Me}_2\text{SO}$  with Mg. These unusual negative values (Schaleger and Long, 1963; Ramirez and Marecek, 1980), which indicate an important effect of electrostriction, are compatible with strong diminution of the solvation shell and immobilization of water molecules surrounding reacting anions (Parker, 1967; Laidler, 1978).

#### 4. Conclusions

We conclude that P-enolpyruvate can transfer its phosphoryl group to Pi to form PPi in the presence of divalent cations such as Ca or Mg, provided the reactants become de-solvated and precipitate. The yields of PPi in both types of media during the initial and linear phase of reaction (3 hr) were in the range of those observed for other transphosphorylations in heterogeneous conditions (Weber, 1982; Lowenstein, 1960; Nelson and Racker, 1973; Saygin and Ellmauerer, 1984). Moreover, the high efficiency of the reaction is demonstrated by the PPi yields attained as the reaction approached a plateau: 43% of the adsorbed P-enolpyruvate in pure water and 56% in 11.3 M  $\text{Me}_2\text{SO}$ . These values are of the same order of magnitude as those found for PPi formation from ADP and ATP (Burley, 1965).

As shown in this report the solid calcium phosphate and magnesium phosphate phases serve as concentrating surfaces for P-enolpyruvate and participate in the phosphoryl transfer reaction of pyrophosphate formation with a behavior similar to that of enzymes as proposed for smectite minerals in the formation of 2',3'-cAMP (Ferris and Hagan, 1986; Ferris *et al.*, 1988). Since PPi formed remained almost entirely in the sediments (Meyer-Fernandes and Vieyra, 1988) it is plausible that it could undergo further transformations in the presence of suitable reactants (Rabinowitz *et al.*, 1969; Neuman *et al.*, 1970; Weber, 1982). The occurrence of this reaction under the conditions described demonstrates that dehydrated surfaces can account for transphosphorylation between compounds of high energy charge in the absence of enzymes and at low temperatures, an essential condition for the stability of organic compounds during longer periods of time (Miller and Bada, 1988). This mechanism could be relevant to primitive earth conditions when phosphorylated compounds first appeared and evolved (Clarke and Elsdon, 1980; Kulaev *et al.*, 1980; Baltscheffsky *et al.*, 1986), and could be taken over by enzyme proteins at a later evolutionary stage (Cairns-Smith, 1982; Ferris *et al.*, 1988).



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