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The Mechanism of Formyl Phosphate Hydrolysis

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The hydrolysis of formyl phosphate was studied in unbuffered and buffered solutions. In the absence of buffer the rate constant (8.8×10^{-3} min^{-1} at 25°C and pH 7) varies little from about pH 5 to 8 and increases dramatically at low and high pH values. At pH 7 the hydrolysis appears to proceed via two mechanisms, nucleophilic attack on the carbonyl carbon and a mechanism involving P–O bond cleavage. This is based on the facts that the reaction proceeds 45% by C–O bond cleavage and 55% by P–O bond cleavage (shown using $H_2{}^{18}O$); there is a solvent isotope effect of 1.6; the $\Delta S^{\ddagger}$ (−11.8 e.u.) is intermediate between that expected for a unimolecular and a bimolecular reaction; the reaction rate is affected by organic solvent and buffer. At pH 1 and 11 the mechanism is entirely nucleophilic substitution at the carbonyl since the reaction proceeds 100% by C–O cleavage, and the $\Delta S^{\ddagger}$ (−21.3 and −25.5 e.u.) is that expected for a bimolecular reaction. In the presence of Tris and glycine the formyl phosphate disappearance is accompanied by formylation of the primary amines with a 100% yield in the case of glycine. Imidazole and pyridine also catalyze formyl phosphate breakdown. In the former case the reaction proceeds primarily by C–O cleavage, but no formylated product was observed. Differences in the hydrolyses of formyl phosphate and acetyl phosphate are discussed.

Recent studies on the mechanism of catalysis by N^{10}-formyltetrahydrofolate synthetase have strongly supported the suggestion that formyl phosphate is an enzyme-bound intermediate in the reaction (*1, 2*). The reaction is thought to occur with phosphorylation of formate by ATP, followed by formyl transfer to the N^{10}-position of tetrahydrofolate. Except for some early work (*3*) and some limited recent studies (*1*), little is known about the chemistry of formyl phosphate. To learn more about this unstable compound we have studied its hydrolysis. In this work we have compared the results with those for a well-studied acyl phosphate, acetyl phosphate (*4–8*), and have found some interesting differences.

EXPERIMENTAL

Materials. Formyl phosphate was synthesized from formyl fluoride and inorganic phosphate using the procedure of Jaenicke and Koch (*3*) with slight modifications described by Smithers *et al.* (*1*). Formyl fluoride was prepared by the method of Olah and Kuhn (*9*), except that the temperature used was maintained by a water bath at 90–95°C. The formyl phosphate preparations contained variable

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amounts of contaminating formate and phosphate. The concentration of formyl phosphate in solution was estimated from the area of the ^1H NMR signal, relative to that of the methyl groups of DSS,[2] added as an internal standard. The amounts of contaminating formate and phosphate were determined from areas of the ^1H and ^{31}P NMR signals, respectively, relative to those for formyl phosphate. The concentration of formyl phosphate was also determined colorimetrically and by ^1H NMR after conversion to the hydroxamate. Preparations were usually 65 to 75% formyl phosphate by weight. $H_2{}^{18}O$ and ^2H_2O were purchased from Cambridge Isotope Laboratories.

Rate studies. The hydrolysis of formyl phosphate was measured in two ways. In one type of assay ^{31}P NMR was used to measure the appearance of inorganic phosphate. In another assay the disappearance of formyl phosphate was followed by converting the remaining formyl phosphate to hydroxamate acid followed by formation of the colored Fe^{3+} complex (*10*). Aliquots (0.1 ml) of the reaction mixture were removed and added to 0.2 ml of 0.3 M neutralized $NH_2OH \cdot HCl$ in 0.05 M triethanolamine · HCl, pH 8.0. After at least 10 min, 0.1 ml of a 12% trichloroacetic acid solution and 0.2 ml of 6% $FeCl_3 \cdot 6H_2O$ in 0.5 M HCl were added. The absorbance at 505 nm was read within 30 min. By using a $NH_2OH \cdot HCl$ concentration lower than that recommended in the original procedure the formation of bubbles which causes difficulties in reading the absorbance (*10*) was avoided.

To test for the stability of the formyl hydroxamate formed and to determine if any *O*-formylhydroxylamine was produced, formyl phosphate was added to a solution of neutralized 0.3 M hydroxylamine and samples were removed over a period of 24 h and added to the $FeCl_3$ solution. There was no change in the resulting absorbance at 505 nm from 1 min to 24 h. If the *O*-acyl derivative had been formed, it should have converted slowly to the *N*-acyl derivative, with a corresponding increase in the absorbance with time (*11*). We also used ^1H NMR to test for the possiblity of *O*-acylhydroxylamine formation. After formyl phosphate and hydroxylamine were combined, the formyl phosphate signal had disappeared within 5 min, and a signal for the hydroxamate (7.880 ppm, with DSS as a standard) was present. No other signal was observed. The NMR spectrum did not change over a 24-h period.

In experiments in which 97% ^2H_2O was present the pD value was calculated by adding 0.4 to the pH meter reading (*12*). When hydrolyses were conducted at pH values above 7, the pH values were maintained during the course of the reaction by the addition of 2 M NaOH.

Determination of C–O and P–O bond cleavage. To determine the site of bond cleavage during hydrolysis, reactions were carried out in $H_2{}^{18}O$ at different pH values, and the ^{13}C and ^1H NMR spectra were examined. Substitution of a single ^{18}O oxygen in each of these compounds produces an approximate upfield shift of 0.02 ppm (*13, 14*). Formyl phosphate (15 mg for pH 1, 4, and 7; 10 mg for pH 9; and 5 mg for pH 13) was dissolved in 200 μl of 97% $H_2{}^{18}O$ and the pH was adjusted with 10 M NaOH for 12 M HCl. Samples at pH 4, 7, and 9 were left at room

[2] Abbreviations used: DMSO, dimethyl sulfoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt.

temperature for 12–16 h. The pH 9 sample had to be adjusted with small additions of 2 M NaOH during the first 2 h. The samples at pH 1 and 13 were hydrolyzed for 1 h. At the end of the reaction all samples were adjusted to pH 7 and diluted to 700 μl with ^2H_2O before the NMR spectra were recorded. For ^{31}P spectra the samples were made 2 mM in EDTA. To examine for solvent exchange into the products, solutions of formate and phosphate, adjusted to the appropriate pH value, were incubated in $H_2{}^{18}O$ for the same length of time as the sample. Exchange occurred only into formate and only at pH 1.

NMR spectra. NMR spectra of products of reactions were recorded on a Varian XL-300 at 300 MHz for ^1H, 75 MHz for ^{13}C, and 121.4 MHz for ^{31}P.

RESULTS

Formyl phosphate is an unstable compound, and a certain amount of hydrolysis occurs during its synthesis and isolation, resulting in contamination by formate, phosphate, and a very small amount of pyrophosphate. Thus, it is not possible to study the hydrolysis reaction in the complete absence of buffer, but since hydrolysis rates were determined at low formyl phosphate concentrations, the amount of buffer resulting from the formyl phosphate preparation is insignificant. At pH 7 and 25°C the observed first-order rate constant of hydrolysis was 8.84×10^{-3} min^{-1} (Table 1). The corresponding reported value for acetyl phosphate is 0.56×10^{-3} min^{-1}.

The effect of pH on the rate of hydrolysis is presented in Fig. 1. From pH 5 to about pH 8.5 the rate constant is essentially independent of pH. A small increase between pH 5 and 3 is probably due to the protonation of the dianion species. Protonation of the dianion was observed from the changes in chemical shift of the

TABLE 1

Observed First-Order Rate Constants for the Hydrolysis of Formyl and Acetyl Phosphate at pH 7

	$k_{obs} \times 10^3$ (min^{-1})	
Conditions	Formyl phosphate	Acetyl phosphate
H_2O	8.8 (25°)	0.56(25°)[a]
H_2O	39.6 (40°)	15.0 (50°)[b]
		4.3 (39°)[a]
10% CH_3CN	6.9 (25°)	
25% CH_3CN	5.2 (25°)	
50% CH_3CN	4.1 (25°)	4.4 (39°)[a]
		13.1 (50°)[b]
50% DMSO	4.3 (25°)	
With NaCl	9.0 (0.5 M, 25°)	4.3 (0.6 M, 39°)[a]

[a] From Ref. (7).
[b] This work.

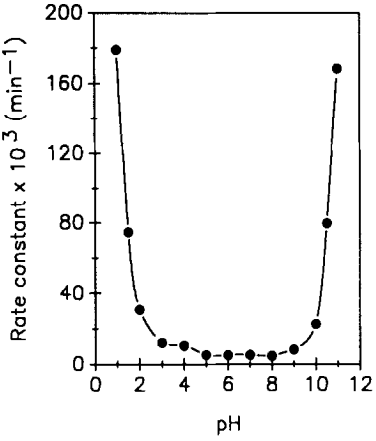


FIG. 1. Effect of pH on the observed rate constant for hydrolysis. Formyl phosphate (8 mM) was incubated at 20°C. The initial pH values of the solutions were adjusted with either 2 M HCl or 2 M NaOH. The disappearance of formyl phosphate was followed by the hydroxamate assay as described under Experimental.

^{31}P resonance allowing a pK_a of about 4.5 to be calculated. The hydrolysis rate increased dramatically below pH 3 and above pH 9. The effect of temperature on hydrolysis was determined at pH values of 1, 7, and 11. The Eyring plots were linear over the temperature range used and the thermodynamic activation parameters at 20°C calculated from the data are presented in Table 2. An interesting point is that the $\Delta S^{\ddagger}$ value is dependent upon the pH of the hydrolysis reaction.

Organic solvents are known to affect the rate of reactions which involve H_2O in the rate limiting step. We found such an effect with CH_3CN and DMSO (Table 1) with about a 50% reduction in the rate constant in 50% CH_3CN or 50% DMSO.

The site of bond cleavage (C–O or P–O) at different pH values was examined by conducting the hydrolysis in the presence of $H_2{}^{18}O$ and recording the ^{31}P and ^{31}C NMR spectra of the products. The results, presented in Table 3, showed the

TABLE 2

Kinetic Constants, Activation Parameters, and Solvent Isotope Effects at Different pH Values[a]

pH	k_{obs} (min^{-1})	$\Delta G^{\ddagger}$ (kcal/mol)[b]	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (e·u)	k_H/k_D
1.0	0.14	21.6 ± 1.1	14.5 ± 0.8	−21.3 ± 2.0	0.9
7.0	0.004	22.7 ± 0.3	19.2 ± 0.2	−11.8 ± 0.8	1.6
11.0	0.24	20.4 ± 0.7	12.9 ± 0.5	−25.5 ± 1.6	3.3[c]

[a] Values are given with standard errors.

[b] At 20°C.

[c] Determined in 0.2 M triethylamine as a buffer to prevent a change in the pH during hydrolysis.

TABLE 3

Percentage of C–O and P–O Bond Cleavage at Different pH Values

	From ^{13}C spectra[a]		From ^{31}P spectra[b]	
pH	C–O	P–O	C–O	P–O
1[c]	—	—	100	0
4	71	29	69	31
7	44	56	44	56
9	81	19	82	18
13	100	0	100	0

[a] Corrections were made for a 15% contamination of formyl phosphate with formate. Values were determined from relative areas of the peaks and are ±10%.

[b] Corrections were made for a 23% contamination of formyl phosphate with phosphate. Values were determined from relative areas of the peaks and are ±10%.

[c] At pH 1 solvent exchange into formate was too rapid to permit calculations of C–O cleavage from the ^{13}C spectrum.

presence of [^{18}O]formate and [^{18}O]phosphate at the pH values of 4, 7, and 9. At pH 13 ^{18}O was found only in formate. At pH 1 no ^{18}O was found in the phosphate, but a rapid exchange with solvent precluded determining the incorporation of ^{18}O into the formate. NMR spectra of samples after hydrolysis at pH 7 are presented in Fig. 2. Although there is some error in the values in Table 3 at pH 4, 7, and 9 because of the lack of complete resolution of the bands of the ^{16}O- and ^{18}O,^{16}O-compounds and the correction for contaminating formate and phosphate, it is clear that the lowest amount of C–O cleavage occurred at 7 and that this increased as the pH was lowered or raised. In a previous report (*1*) we estimated the amount of P–O cleavage at pH 7 to be around 90%, higher than the 56% reported here. In the earlier work, however, complete resolution of the ^{31}P spectra was not achieved, and the ^{13}C spectrum was not examined. It has been reported that acetyl phosphate hydrolysis near neutrality occurs by way of P–O bond cleavage (*15*). However, the extent of P–O and C–O cleavage is influenced by the presence of divalent ions (*8*). By conducting hydrolysis of acetyl phosphate in $H_2{}^{18}O$ at pH 7. and 40°C and examining the ^{31}P and ^{13}C NMR spectra, we found that 90% of the reaction proceeded by P–O cleavage and 10% by C–O cleavage.

Hydrolysis at pH 7 was conducted in the presence of varying mole fractions of ^2H_2O (Fig. 3). The reaction proceeds with a solvent isotope effect of 1.6 and the linearity of the data in Fig. 3 indicates that a single proton is transferred during the

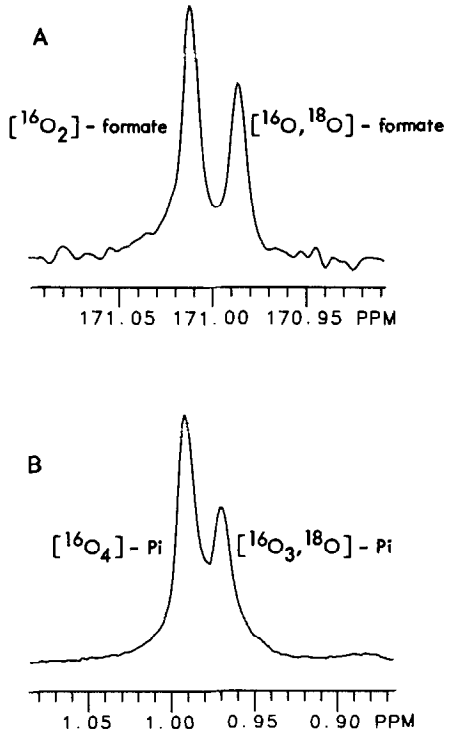


FIG. 2. ^{13}C and ^{31}P NMR spectra of formate and P_i formed by hydrolysis in $H_2{}^{18}O$. (A) Proton decoupled ^{13}C spectrum. Spectrometer conditions included the following: sweep width, 2400 Hz; pulse width 3.5 μs; tip angle, 18.5°; number of transients, 28,000; expansion, 80×. Chemical shifts are reported with reference to the methyl resonance of TMS. T = 20°C. (B) Proton-decoupled ^{31}P spectrum. Spectrometer conditions included the following: sweep width, 6000 Hz; pulse width, 17.3 μs; tip angle, 26°; number of transients, 96; expansion, 135×. Chemical shifts are reported with reference to 85% H_3PO_4. T = 20°C.

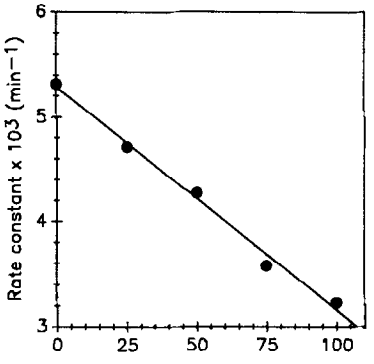


FIG. 3. Effect of ^2H_2O on the observed rate constant for hydrolysis. Formyl phosphate (8 mM) was incubated at 20°C in various mole fractions of ^2H_2O; pH (pD) = 7.

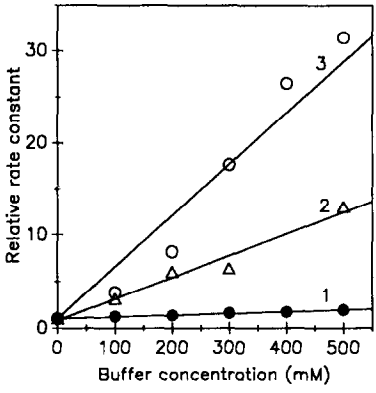


FIG. 4. Effect of three buffers on the observed rate constant for the disappearance of formyl phosphate. Formyl phosphate (8 mM) was incubated with the appropriate buffer at 20°C. Formyl phosphate disappearance was measured by the hydroxamate assay. 1, Hepes/NaOH, pH 7.6. 2, Tris/HCl, pH 8.0. 3, imidazole/HCl, pH 6.9.

reaction. At pH 1 and 11 the solvent isotope effect was 0.9 and about 3.3, respectively (Table 2). The presence of ^2H_2O also influenced the ratio of C–O and P–O cleavage at pH 7. In the presence of 57% [^{2}H], 97% $H_2{}^{18}O$, ^{18}O incorporation into formate decreased by 20% of that obtained in 97% $H_2{}^{18}O$. This result is consistent with a reaction which proceeds by C–O cleavage and which has a solvent isotope effect of 2 to 3.

The influence of buffers was examined to determine whether the reaction near neutral pH was buffer-catalyzed and whether different buffers would alter the course of the reaction. The results with Hepes, Tris, and imidazole are shown in Fig. 4. There is a small buffer effect by Hepes with the rate at a 0.5 M concentration being 50% greater than that in the absence of buffer. On the other hand, imidazole and Tris at the same concentration increased the rate about 30- and 12-fold, respectively. Other buffers tested were pyridine and glycine. A summary of the effects of these compounds at their respective pK_a values is presented in Table 4. Formylated products were detected by the appearance of an absorption band in ^{1}H NMR spectra at about 8 ppm and were found in the presence of Tris and glycine, but not with imidazole and pyridine. ^{31}P NMR did not reveal the presence of a stable phosphoramidate.

DISCUSSION

This study was undertaken to learn more about the stability of formyl phosphate, which is a putative intermediate in the reaction catalyzed by N^{10}-formyltetrahydrofolate synthetase. We have found that certain differences exist in the mechanism of hydrolysis of formyl phosphate and a well-studied homolog, acetyl phosphate. Formyl phosphate hydrolysis at pH 7 occurs at a higher rate, is enhanced in buffer, and decreases in ^2H_2O and organic solvents. Acetyl phosphate

TABLE 4

Influence of Buffers on the Rate of Formyl Phosphate Disappearance, Position of Bond Cleavage, and Products of the Reaction

Buffer[a]	pH	Rate enhancement	% C–O bond cleavage[b]	Percentage of formylated product[c]
Imidazole	6.9	8.2	86	0
Pyridine	5.2	8.5	35	0
Glycine	9.6	56	100	100
Tris	8.0	6.0	67	51
Hepes	7.6	1.2	62	0

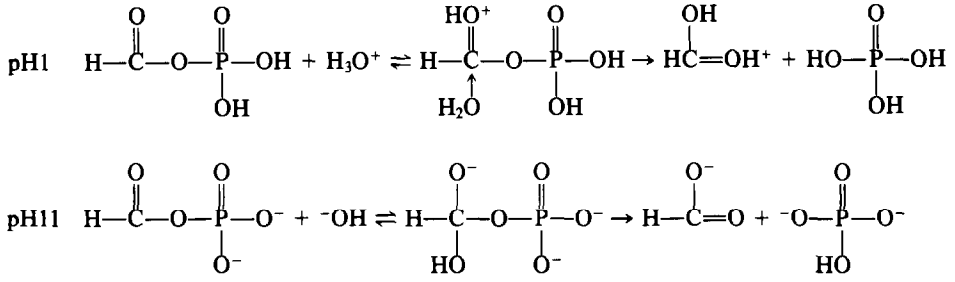
[a] All at 0.2 M and 20°C.

[b] Determined from ^{13}C and ^{31}P NMR spectra.

[c] Percentage of formyl phosphate converted to the formylated product as determined from the ^{1}H NMR spectra with DSS as an internal standard. The values presented refer to the final products. As discussed in the text it is likely that unstable intermediates are formed in the cases of imidazole- and pyridine-catalyzed hydrolysis.

hydrolysis is not affected by buffer, organic solvent, or $^{2}H_2O$ (*7*). These results indicate that the rate-limiting step in the hydrolysis of formyl phosphate at pH 7 does involve a bimolecular reaction with H_2O. However, the solvent isotope effect is not the value of 2 to 3 expected for such a reaction (*7, 16*). In addition, the $\Delta S^{\ddagger}$ value of -11.8 e.u. is approximately 33 to 50% of that expected for a bimolecular reaction (*7, 17*). This information, together with the fact that ^{18}O from solvent appears in the formate and phosphate, indicates that, at pH 7, formyl phosphate hydrolysis occurs by two pathways. One pathway, involving P–O bond cleavage, would be similar to the pathway for acetyl phosphate hydrolysis, and would occur by a dissociative mechanism or one with an extended associative transition state (*18*). This pathway would account for about 55% of the reaction. Because formyl phosphate hydrolysis proceeds with approximately 45% C–O bond cleavage at pH 7, a second pathway, involving H_2O attack on the carbonyl carbon, must also take place. A combination of these pathways would result in an isotope effect, solvent effects, and a $\Delta S^{\ddagger}$ value which would be an average of those found for acetyl phosphate hydrolysis and those expected for a bimolecular associative mechanism. One might expect a nonlinear Eyring plot for a reaction involving two different pathways. Perhaps the difference in $\Delta H^{\ddagger}$ between the mechanisms and the limited temperature range which could be used would preclude detecting such nonlinearity.

Our ^{18}O incorporation results show that C–O bond cleavage also contributes a small amount to acetyl phosphate hydrolysis. The greater contribution of attack on the carbonyl carbon to formyl phosphate hydrolysis when compared to acetyl phosphate hydrolysis at neutral pH is probably due to two facts. Water attack on the formyl carbon would be more favorable than that on the acetyl carbonyl due to the greater steric hindrance of the methyl group compared to the hydrogen and



SCHEME 1. Proposed mechanisms of hydrolysis at pH 1 and 11.

to the greater inductive effect of the methyl group which would make the carbonyl carbon in the acetyl group less electrophilic than the formyl carbon. Steric hindrance would predominate as the major factor since the bimolecular aminolysis and hydroxide ion hydrolysis of formate ester is 10^3 times faster than that of acetate ester (*19*). A difference in the pK_a's of formic and acetic acid (1 unit) suggests that the inductive effect also contributes to the reaction at the carbonyl group in formyl phosphate.

The pH profile for the hydrolysis of formyl phosphate is similar to that for acetyl phosphate hydrolysis. In the neutral pH region the rate constant is unchanged. There is a small increase in the rate constant as the pH is decreased from 5 to 4, which apparently is explained by the conversion of the dianion to the monoanion species. At low and high pH values the rate constants increase dramatically. Accompanying these increases is a change to C–O bond cleavage as the sole pathway, changes in the $\Delta S^{\ddagger}$ giving values which are more consistent with bimolecular reactions, and changes in the solvent isotope effects to 0.9 at pH 1 and to about 3.3 at pH 11. Suggested mechanisms of hydrolysis at these pH values are presented in Scheme 1.

Since ^2H_2O is three times as strong an acid as H_2O the ratio of the equilibrium constants, K_H/K_D, for the first half of the reaction at pH 1 would be approximately 0.33 and, if the second half had a solvent isotope effect of 2 to 3, an overall solvent isotope effect of 1 or less might be expected. $^-O^2H$ is also a stronger base than ^-OH so that at pH 11, K_H/K_D for the first half of the reaction would be about 0.5. If the proton transfer step shown in the second half of the reaction had a k_H/k_D value of about 6, an overall isotope effect of about 3 would result. An alternative mechanism at pH 11 is one in which hydroxide ion acts as a base in assisting H_2O attack.

The primary amines Tris and glycine reacted with formyl phosphate to give the respective formylated products; 100% of the formyl phosphate was converted to the *N*-formamide in the presence of glycine and, in the case of Tris, 50% of the formyl phosphate was converted to the amide. The difference may be due to the greater basicity of the glycine amino group and to the bulkiness of the trihydroxymethyl group in Tris. When imidazole was used as the nucleophile formyl imidazole was not detected. The fact that ^{18}O was found primarily in the formate when

imidazole was present is probably best explained by the formation and subsequent hydrolysis of formyl imidazole. A formylated product also was not observed in the presence of pyridine and the fact that the amount of C–O bond cleavage was reduced compared to that obtained for simple hydrolysis suggests that pyridine may act partially by attacking the phosphorus atom followed by hydrolysis of the phosphoramidate (*20, 21*).

The results presented in this work demonstrate that formyl phosphate is much less stable than acetyl phosphate at pH values around neutrality. Moreover, approximately 45% of formyl phosphate is hydrolyzed by cleavage of the C–O bond at pH 7, compared to about 10% of acetyl phosphate. It is clear that formyl phosphate is a very effective formylating agent and, from a chemical view, is a reasonable intermediate in the formylation of tetrahydrofolate in the reaction catalyzed by N^{10}-formyltetrahydrofolate synthetase.

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