The Kinetics and Mechanisms of Phosphoenolpyruvate Hydrolysis*

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ABSTRACT: The hydrolytic mechanisms of phosphoenolpyruvate and its reference compound ethyl phosphoenolpyruvate have been investigated. Rate constants for the protomeric species, activation energies, D_2O and nonaqueous solvent effects, and methyl phosphate–inorganic phosphate product distribution in methanol–H_2O solvent have led to the postulation of: (1) abnormally rapid phosphoenolpyruvate mono- and dianion and ethyl phosphoenolpyruvate monoanion hydrolysis *via* metaphosphate expulsion possibly involving a cyclic transition state, (2) unusual neutral species hydrolysis, (3) only small (3–40-fold) rate enhancement which was attributable to enol–ketone conversion energy, and (4) no demonstrable kinetically important role for the neighboring group of phosphoenolpyruvate.

Phosphoenolpyruvate is a key biological metabolite in the phosphorylation of glucose (Kundig *et al.*, 1964) and ADP[1] (Boyer, 1962). Accordingly, phosphoenolpyruvate is classified as a high-energy compound characterized by a free energy of hydrolysis, $\Delta G^{\circ\prime} = -13$ kcal mole^{-1} at pH 7, as compared with monoalkyl phosphates, $\Delta G^{\circ\prime} = -3$ kcal mole^{-1} (Mahler and Cordes, 1966). Yet the ratio of hydrolytic rate constants of phosphoenolpyruvate to methyl phosphate (Bunton *et al.*, 1958) is only 10^2 (75°, pH 4), whereas an order of magnitude calculation, assuming the difference in free energy of hydrolysis should be reflected in the relative activation energy, yields a ratio of 10^6. Moreover, the possibility arises that the nonenzymic and enzymic transfer of phosphate from phosphoenolpyruvate could be influenced by the neighboring carboxyl group, as evidenced in other ester hydrolyses (Bruice and Benkovic, 1966). Previous investigations (Weil-Malherbe and Green, 1951; Yasnikov, 1961) have been preliminary and contradictory.

In this study we have examined the mechanism of nonenzymic phosphate transfer from phosphoenolpyruvate and in the following paper we report on the catalysis of phosphoenolpyruvate phosphorylation by metal ions.

Methods

Phosphoenolpyruvate was prepared as the monocyclohexylamine salt by the method of Clark and Kirby (1963), mp 141–146 dec, uncor. *Anal.* Calcd for $C_9H_{18}NO_6P$: C, 40.44; H, 6.74; P, 11.61. Found: C, 40.76; H, 7.05; P, 1170. Ethyl phosphoenolpyruvate was prepared as the didyclohexylamine salt, mp 170.6–171.5 uncor by an adaptation of the method of Cramer and Vogel (1959) for preparation of phosphoenolpyruvate. *Anal.* Calcd for $C_{17}H_{35}N_2O_6P$: C, 51.77; H, 8.88; P, 7.87. Found: C, 50.77; H, 8.80; P, 7.83. The infrared spectrum (nujol) showed peaks at 1720 (sharp), 1640 (shoulder), 1620 (sharp), 1323 (sharp), 1145–1190 (broad multiplet), and 1108 cm^{-3} (strong). Complete hydrolysis yielded P_i quantitatively and the presence of ethyl pyruvate was shown by gas chromatography (no other organic component being detected).

Imidazole was recrystallized (chloroform) before use and chloropyridine (Aldrich) was used as obtained. Methanol (Baker reagent grade), D_2O (99.8% Diaprep), dioxane (purified by distillation over sodium), dimethylformamide (Fisher reagent grade), and twice-distilled deionized water were employed as solvents. All other buffer materials were reagent grade (Baker, Eastman).

Apparatus. Instrumentation used in this study has previously been described (Benkovic and Benkovic, 1966). All kinetic runs were carried out in Kimax screw-cap tubes (No. 45066-A) with Teflon-lined caps (No. 9447-B3) maintained at constant temperature (±0.1) by a circulating water bath.

Kinetics. The hydrolytic rates of phosphoenolpyruvate and ethyl phosphoenolpyruvate were determined by monitoring the release of orthophosphate (Fiske and Subbarow, 1925); controls indicate no hydrolysis during the development procedure. Aliquots of the reaction mixture, 0.75–3.0 × 10^{-3} M in substrate, were withdrawn and quenched at 0° at appropriate time intervals. Duplicate runs agreed within ±4%. Pseudo-first-order kinetics were observed to at least 3 half-lives. Buffers employed were HCl (pH < 2.5), formate (0.2 M, pH 2.8–3.8), acetate (0.2 M, pH 4.0–5.4), succinate (0.067 M, pH 5.7–6.1), Tris (0.2 M, pH 6.8–7.8), and carbonate (0.2 M, pH 9.0–10.0), all at $\mu = 0.2$ (KCl). Observed rates were invariant with changing buffer concentration (acetate, 0.04–0.2 M; Tris. 0.05–

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TABLE I: Dissociation Constants of Phosphoenolpyruvate and Ethyl Phosphoenolpyruvate.

Compound	Solvent	Temp (°C)	pK_{a_1}	pK_{a_2}	pK_{a_3}
Phosphoenolpyruvate	H_2O	35	1.41 ± 0.18[a]	3.56 ± 0.03	6.22 ± 0.02
		75	1.59 ± 0.18[b]	3.67 ± 0.05[a]	6.40 ± 0.05[a]
	D_2O	35	1.85 ± 0.18[a]	4.01 ± 0.02	6.67 ± 0.03
		75	2.03 ± 0.18	4.12 ± 0.05[a]	6.85 ± 0.05[a]
Ethyl phosphoenolpyruvate	H_2O	75		6.5	

[a] Values utilized in rate calculation. [b] Estimated, see Harned and Embree (1934).

TABLE II: Rate Constants for the Hydrolysis of Phosphoenolpyruvate and Ethyl Phosphoenolpyruvate.

Compounds	Solvent	$k_{H^+} \times 10^3$ (M^{-1} min^{-1})	$k_1 \times 10^3$ (min^{-1})	$k_2 \times 10^3$ (min^{-1})	$k_3 \times 10^3$ (min^{-1})
Phosphoenolpyruvate	H_2O	4.3	6.80[a]	8.80	6.20
	D_2O		4.85	8.02	4.56
k^{H_2O}/k^{D_2O}			1.40 ± 0.07	1.10 ± 0.05	1.36 ± 0.07
Ethyl phosphoenolpyruvate	H_2O			1.97	
	D_2O			2.05	
k^{H_2O}/k^{D_2O}				0.97 ± 0.05	

[a] Profiles calculated assuming $k_1 = 0$ and $pK_{a_1} = 1.0$ did not give curves that approximated the experimental results.

0.2 M) or the presence of air or light. As a further precaution all phosphoenolpyruvate runs were carried out in the presence of EDTA (1.7×10^{-3} M). The pH of all buffers was determined at 75° employing an EA 121 H Metrohm electrode. Kinetic runs with a pH variation greater than ±0.02 during the course of the run were not utilized. Deuterium oxide buffers were 98% D_2O after correction for hydrogen acids and bases.

Dissociation Constants. The dissociation constants for phosphoenolpyruvate ($\mu = 0.2$) were determined titrimetrically in a Metrohm cell (EA 662) at 35° by the procedure outlined by Albert and Serjeant (1962). The method of Noyes (Britton, 1955) for separation of overlapping dissociation constants was used for pK_{a_2} and pK_{a_3} of phosphoenolpyruvate and the pK_{a_1} of phosphoenolpyruvate is corrected for the hydrogen ion concentration as per Britton (1955). Samples of phosphoenolpyruvate were titrated to pK_{a_2} and pK_{a_3} at 35° and the change in pH in going from 35 to 75° was determined using the high-temperature electrode. Determination of the pK_{a_2} of ethyl phosphoenolpyruvate was by estimation from the pH–rate profile assuming the dianion to be unreactive. Determination of the dissociation constants of phosphoenolpyruvate in D_2O was the same as for aqueous determinations at 35° of pK_{a_2} and pK_{a_3}. The glass electrode pH values were corrected to pD values by the method of Fife and Bruice (1961) and temperature corrections were assumed to be the same as in the aqueous case within experimental error (Gary *et al.*, 1965). The value of pK_{a_1} was calculated by the method of Bell (1959) for change of solvent from water to deuterium oxide. All dissociation constants are compiled in Table I.

Products. Phosphoenolpyruvate hydrolysis quantitatively yielded only orthophosphate over the pH range investigated. Pyruvic acid was quantitatively determined by the salicylaldehyde method (Berntsson, 1955) yielding 97 ± 2% pyruvic acid at pH 4.6 and 104 ± 3% at pH 2.7. Ethyl phosphoenolpyruvate kinetics are first order to >3 half-lives indicating no carbethoxy group hydrolysis.

Product determinations in alcohol–water were carried out as previously described (Benkovic and Benkovic, 1966).

Results

Kinetics. The pH–rate profiles for the hydrolysis of phosphoenolpyruvate and ethyl phosphoenolpyruvate are shown in Figure 1 and the former is similar to that found by Weil-Malherbe and Green (1951). The smooth curve for phosphoenolpyruvate was calculated from

$$k_{obsd} = \frac{a_H^3(k_1 + k_{H^+}a_H) + k_2K_1a_H^2 + k_3K_1K_2a_H)}{K_1K_2(a_H + K_3) + a_H^2(K_1 + a_H)} \quad (1)$$

assuming all species to be hydrolytically reactive, excluding the trianion, where k_{H^+} is the second-order rate

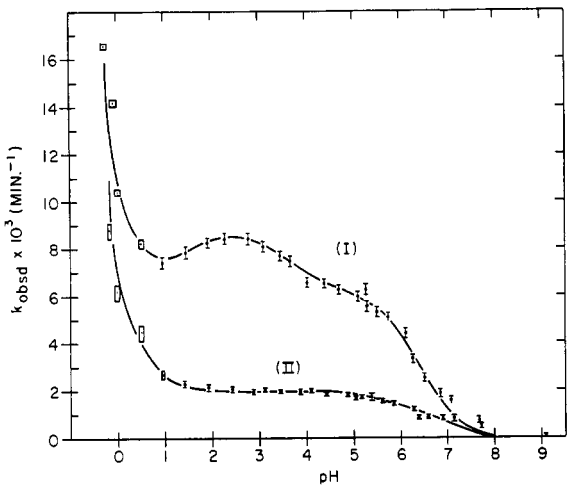
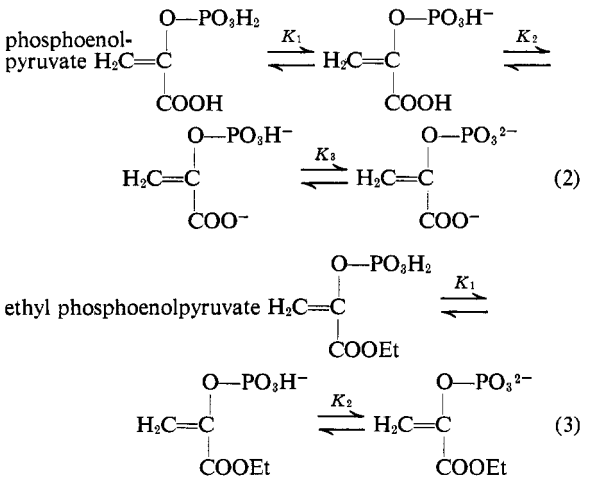


FIGURE 1: The pH–rate profiles of phosphoenolpyruvate (I) and ethyl phosphoenolpyruvate (II). Vertical lines represent experimental error. The solid curves are theoretical (eq 1).

constant associated with hydronium ion catalyzed hydrolysis of the neutral species and k_1, k_2, and k_3 are defined as first-order rate constants for the hydrolysis of the neutral, mono-, and dianion species, respectively. The macroscopic dissociation constants, K_1, K_2, and K_3, may be identified with those measured experimentally and refer to the ionizations of the phosphate (pK_{a_1} and pK_{a_3}) and carboxyl (pK_{a_2}) groups as illustrated in eq 2 and 3. The values of k_1, k_2, and k_3 utilized appear in Table II. Equation 1 was also used to determine



k_1, k_2, and k_3 in D_2O (Table II) utilizing the pK_a (D_2O) values listed in Table I. The value of k_2 in H_2O and D_2O for the monoanion of ethyl phosphoenolpyruvate was graphically determined. Calculation of k_{H^+} and k_1 of ethyl phosphoenolpyruvate was not attempted due to concomitant hydrolysis of the carbethoxy group below pH 2. Hydroxamic acid assay (Lippmann and Tuttle, 1945) revealed no greater than 30% hydrolysis of the carbethoxy group during the period of the kinetic run at pH < 2. Therefore it is evident from Figure 1 that both the neutral and acid-catalyzed species hydrolyze at an appreciable rate.

TABLE III: Activation Parameters[a] for Hydrolysis of Phosphoenolpyruvate and Ethyl Phosphoenolpyruvate.

Compound	Species	$\Delta H^{\ddagger}$ (kcal)	$\Delta S^{\ddagger}$ (eu)
Phosphoenol-pyruvate	Neutral	23.8 ± 0.9	−7.5 ± 3.1
	Mono-anion	25.5 ± 1.0	−1.1 ± 3.6
	Dianion	25.7 ± 0.9	−3.6 ± 3.2
Ethyl Phospho-enolpyruvate	Mono-anion	27.6 ± 1.0	+2.7 ± 3.4

[a] Calculated from $E_{ac} = -2.303R\ (\log k_2 - \log k_1)/((1/T_2) - (1/T_1))$. $\Delta H^{\ddagger} = E_{ac} - RT$ and $\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta F^{\ddagger})/T$, where $T = 75°$.

Inspection of Table II reveals that for phosphoenolpyruvate the order of rate constants is $k_2 > k_1 \approx k_3 > k_{H^+}$. However, k_2 is only *ca.* 1.3-fold greater than k_1 or k_3. Thus the state of ionization of the carboxyl group has little influence on the hydrolysis of phosphoenolpyruvate. This is also evident in that k_2 and k_3 of phosphoenolpyruvate are only *ca.* fourfold greater than k_2 of ethyl phosphoenolpyruvate.

The activation parameters for phosphoenolpyruvate and ethyl phosphoenolpyruvate are found in Table III.

The pH–rate profile in dioxane–water (50:50, v/v) for both phosphoenolpyruvate and ethyl phosphoenolpyruvate are exhibited in Figure 2. The observed rates of the dioxane–water runs are plotted against the aqueous buffer pH for purpose of comparison. The assumption that substrate and buffer pK_a change is similar in changing from water to dioxane–water is reasonable (Chanley and Feaguson, 1963). Examination of Figure 2 reveals that the effect of mixed solvents is small (neutral phosphoenolpyruvate, 1.1-fold increase; phosphoenolpyruvate monoanion, 1.1-fold increase; phosphoenolpyruvate dianion, 1.05-fold decrease; ethyl phosphoenolpyruvate monoanion, 1.15-fold decrease).

The solvolysis of phosphoenolpyruvate and ethyl phosphoenolpyruvate in MeOH–H_2O (50:50, v/v) also describes a profile similar to that in aqueous solution with 1.1- to 1.7-fold decreases for all species.

Attempts to detect catalysis or phosphate transfer to amines such as imidazole and chloropyridine were unsuccessful.

Products. The products of the solvolysis of phosphoenolpyruvate and ethyl phosphoenolpyruvate carried out in MeOH–H_2O (50:50, v/v) at 55° are listed in Table IV. Examination of data reveals that (1) the mole fractions of P_i and methyl phosphate closely approximate the mole fractions in the solvent, and (2) the product composition is independent of pH.

Discussion

The rate of hydrolysis of alkyl and aryl phosphate monoester monoanions may generally be predicted

TABLE IV: Water–Methanol Solvolytic Products of Phosphoenolpyruvate and Ethyl Phosphoenolpyruvate.[a]

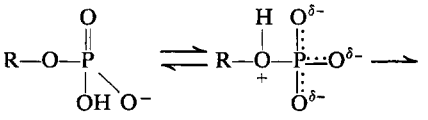
Compound	pH	% PO_4
Phosphoenolpyruvate	1.31	67.6
	3.69	68.0
	5.48	74.0
Ethylphosphoenolpyruvate	4.44	69.7

[a] Mole % P_i in 69.1 mole % H_2O mixture.

(Bunton *et al.*, 1967; Kirby and Varvoglis, 1967) from the pK_a of the departing alcohol moiety according to the expression

$$\log k_{\text{hydrolysis}} = 0.91 - 0.27\ \text{p}K_a \qquad (4)$$

The small dependence of $k_{\text{hydrolysis}}$ upon pK_a has been interpreted as indicating that alcohol and not alcoholate ion is the leaving group. This conclusion in conjunction with much additional experimental evidence (Bruice and Benkovic, 1966; Cox and Ramsay, 1964) has led to the following generalized mechanism of hydrolysis for phosphate monoester monoanions. It should be



$$\text{R—OH} + [PO_3]^- \xrightarrow{H_2O} H_2PO_4^- \qquad (5)$$

noted that, as ROH becomes increasingly acidic, *i.e.*, 2,4-dinitrophenol (Kirby and Varvoglis, 1967), a negative deviation from the above expression is found and the hydrolysis is characterized by a kinetic deuterium solvent isotope effect (see Table V). Such an observation is consistent with proton transfer becoming partially rate determining.

We will first consider mechanistic criteria for the hydrolysis of ethyl phosphoenolpyruvate and phosphoenolpyruvate monoanions. The similarity between ethyl phosphoenolpyruvate, phosphoenolpyruvate, and other phosphate monoester monoanions (see Table V) is revealed by (1) the unreactivity of the dianion or trianion, (2) the absence of an appreciable deuterium solvent isotope effect, (3) the small entropy of activation, (4) the nonselective formation of methyl and inorganic phosphate upon solvolysis in CH_3OH–H_2O solutions, and (5) insensitivity of the hydrolytic rate to nonaqueous solvents (DiSabato and Jencks, 1961). These findings are in accord with a unimolecular mechanism and the expulsion of the nonselective, highly reactive monomeric metaphosphate.

Attempts to measure the hydrolysis of the neutral species of ethyl phosphoenolpyruvate and its acid-catalyzed reaction are subject to experimental difficulties. Nevertheless it is obvious that the former does undergo hydrolysis at a rate comparable with the rate of

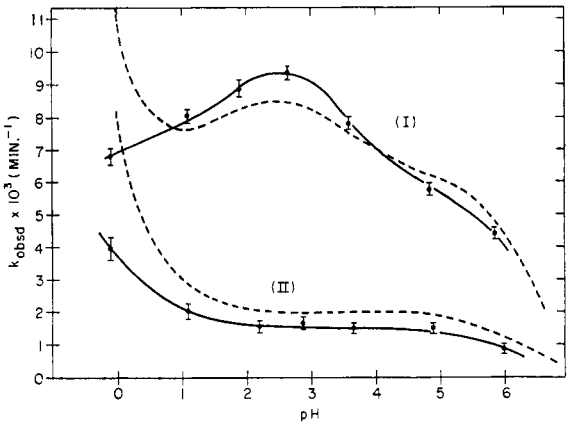


FIGURE 2: The pH–rate profile in dioxane–water (50:50, v/v) (solid line) *vs.* aqueous hydrolysis for phosphoenolpyruvate (I) and ethyl phosphoenolpyruvate (II).

the monoanion, presumably *via* P–O bond cleavage. Similar behavior has been observed with aromatic phosphate monoesters (Bunton *et al.*, 1967) and has been ascribed to a bimolecular attack of water on the neutral species. Further discussion of this point will be deferred until phosphoenolpyruvate neutral species hydrolysis is considered.

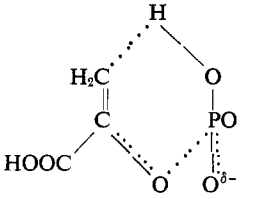
It should be noted, however, that the rates of hydrolysis of the ethyl phosphoenolpyruvate and phosphoenolpyruvate monoanions are not predicted by eq 4. The observed rates are *ca.* 3- to 22-fold more rapid than calculated, employing a pK_a range of 10.8–12.4 and 10–12.4 for the ionization of the enol of the ester and acid pyruvates, respectively. The latter were determined as follows: (1) by assuming a $K_a = 10^{-18}$ for the pyruvate carbanion (in analogy to acetone, $K_a = 10^{-20}$; acetophenone, $K_a = 10^{-19.2}$; acetonylacetone, $K_a = 10^{-18.7}$; chloroacetone, $K_a = 10^{-16.5}$ (Bell, 1959) and the relative σ^* constants of the substituent groups (Taft, 1956)) and a mole fraction enol content of 2.5×10^{-6} (acetone; Schwarzenbach and Wittwer, 1947) which yields a calculated enol pK_a of 12.4, and (2) from the pK_a's of the hydroxyl function of lactic acid and ethyl lactate estimated from the data of Ballinger and Long (1960) and corrected for the presence of the double bond by interpolation from the ΔpK_a (2.0) obtained for a series of amine–enamine comparisons (Stamhaus *et al.*, 1965). The second calculation gives pK_a's of 10 and 10.8 for the enols of phosphoenolpyruvate and ethyl phosphoenolpyruvate monoanions, respectively. These calculations yield pK_a's independent of hydrogen-bonding effects. Although one may question the assumptions inherent in the above calculations, it is reasonable that the pK_a values and correspondingly the computed rates are minimal since hydrolysis of the completely ionized species would have been detected experimentally if the pK_a for enolic ionization were <9.

The postulated mechanism for phosphoenolpyruvate and ethyl phosphoenolpyruvate monoanion hydrolysis is illustrated in eq 6. The cyclic mechanism rationalizes the rate enhancement observed with these species by invoking a transition state with a degree of ketone character rather than expulsion of the respective enol.

TABLE V: Comparison of Some Typical Monoester Monoanion Hydrolysis Parameters.

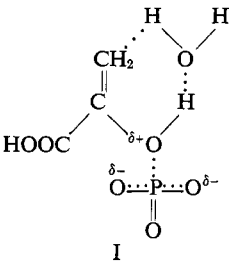
Compound	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	k^{H_2O}/k^{D_2O}	Selectivity H_2O/MeOH	References
Methyl phosphate	30.6	−2.2	0.87		Bunton *et al.* (1958)
Acetyl phosphate	22.5	−3.6	1.1		DiSabato and Jencks (1961)
Salicyl phosphate (dianion)	23.5	−1.2	0.96		Chanley *et al.* (1952) Bender and Lawlor (1963)
Glucose 6-phosphate	31.2	0			Degani and Halmann (1966)
	32.1	+2.3			Bunton and Chaimovitch (1966)
Isopropyl phosphate	32.1	+2.4			Kugel and Halmann (1967)
p-Carboxyphenyl phosphate (dianion)	26.8	−3.1			Chanley and Feaguson (1955)
2,4-Dinitrophenyl phosphate	24.2	−6.0	1.45	1.10[a]	Kirby and Varvoglis (1967)
Phenyl phosphate	28.4	+0.9		0.96[a]	Chanley and Feaguson (1963)
Ethyl phosphoenolpyruvate monoanion	27.6	+2.7	0.97	1.03[b]	
Phosphoenolpyruvate dianion	25.7	−3.6	1.36	1.27[b]	
Phosphoenolpyruvate monoanion	25.5	−1.1	1.1	0.95[b]	
Phosphoenolpyruvate neutral	23.8	−7.5	1.40	0.93[b]	

[a] At 100°. [b] At 55°.



(6)

The free energy of such a transition state, therefore, partially reflects the *ca.* 6 kcal mole^{-1} greater stability of the ketone of pyruvate relative to its enol (Hill and Morales, 1951; Oesper, 1950). One may also view the development of ketone character as increasing the acidity of the leaving group (Arnett, 1963) thus accelerating the rate of hydrolysis. The absence of appreciable D_2O solvent isotope effects indicates a small degree of proton transfer in the cyclic transition state. Though the six-membered ring appears planar, it actually places the hydrogen above the double bond, the favorable geometry for proton transfer (Corey and Sneen, 1956). The proposed mechanism leads to metaphosphate formation and thus is consistent with the remaining experimental observations. A similar cyclic mechanism has been formulated for the hydrolysis of acetyl phosphate monoanion (DiSabato and Jencks, 1961) whose rate likewise exhibits a marked positive deviation from that predicted by eq 4. Except for the enhanced rate the hydrolysis of acetyl phosphate, phosphoenolpyruvate, and ethyl phosphoenolpyruvate monoanions is experimentally indistinguishable from that of other phosphate monoester monoanions. It appears that the possibility of proton transfer *via* a six-membered cyclic transition state may circumvent the formation of the unstable zwitterion species (I) although this advantage is somewhat balanced by a reduction in the presumed "driving force" of the reaction, the electronic density in the phosphoryl moiety. However, in the present case one cannot completely eliminate this possibility of proton transfer from the zwitterion species (I) *via* an intervening water



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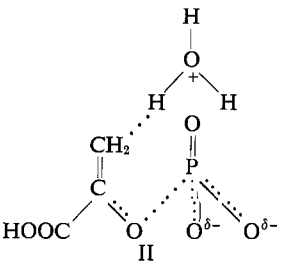
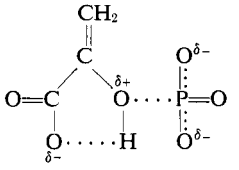


II

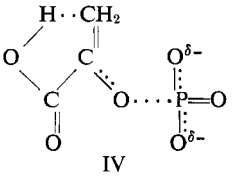
molecule or the kinetically indistinguishable hydronium ion catalyzed hydrolysis of the dianion species (II). Proton transfer without solvent assistance is unlikely on chemical grounds. Although reactions involving rate-determining hydronium ion protonation of olefins generally exhibit rates much slower in D_2O than H_2O (k^{D_2O}/k^{H_2O} = 0.5) the small extent of proton transfer may cancel the anticipated effect (Bunton and Shiner, 1961). It should be noted that no catalytic function has been ascribed to the carboxyl moiety since phosphoenolpyruvate monoanion is only *ca.* fourfold more reactive than ethyl phosphoenolpyruvate. The present re-

sults qualitatively parallel the anticipated inductive effects for the COOH and COOEt moieties based on σ^* constants (Taft, 1956) with the proviso that the reaction rate is increased by electron withdrawal.

The analysis of the rate data for the hydrolysis of phosphoenolpyruvate anion is complicated by a kinetic ambiguity. If the assumed active species possesses a protonated carboxyl (pK_a of enol 10–12.4) then the calculated rate enhancement is 5- to 17-fold neglecting the fact that it is not the thermodynamically favored species. On the other hand, if the active species is presumed to possess an ionized carboxyl function (pK_a of enol 12.4–13.5) then the calculated rate enhancement is 17–43-fold. Assuming the hydrolysis proceeds *via* the protonated carboxyl species it is necessary to assign a catalytic function to the moiety as illustrated in III and IV. These mechanisms feature general acid catal-

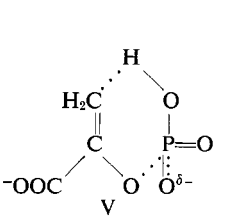


III

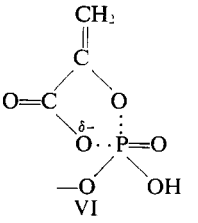


IV

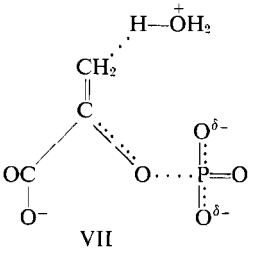
ysis of metaphosphate expulsion and are in accord with the experimental formulation of a nonselective phosphorylating agent and the detection of a small but significant deuterium solvent isotope effect. Mechanism III resembles that postulated for the hydrolysis of salicyl phosphate dianion whose rate is 220 times greater than the *para* isomer (Bender and Lawlor, 1963; Chanley and Feaguson, 1955). The validity of employing eq 4 to examine phosphate monoester hydrolysis for evidence of abnormal mechanistic behavior is verified by noting that salicyl phosphate dianion hydrolysis is 190-fold faster than predicted. Presumably the COOH group facilitates hydrolysis by stabilization of the zwitterionic species. Mechanism IV postulates the development of ketone character *via* proton transfer to the vinyl bond but through an unfavorable five-membered ring which has the carboxyl group in an energetically disfavored lactone conformation. The absence of dramatic carboxyl group catalysis as observed for salicyl phosphate would then arise from the requirement of proton transfer *via* a five- rather than six-membered transition state. The activation energy for proton transfer increases as the position of the proton deviates from an axis joining the donor and acceptor atom (Adam *et al.*, 1968). If one assumes that the carboxyl moiety of the active species is actually ionized then mechanisms V and VI may occur. The enhanced rate is not attributed to substituent effects because COO^- is not electron withdrawing but may be rationalized in terms of (1) electrostatic repulsion between the COO^- and the departing metaphosphate anion, (2) intramolecular nucleophilic catalysis (the intermediate acyl phosphate would hydrolyze rapidly under the reaction conditions to yield metaphosphate), or (3) hydronium ion catalysis involving proton donation to the trianion species. The small deuterium isotope effect could be generated from solvent



V



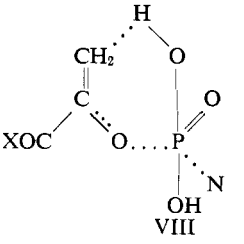
VI



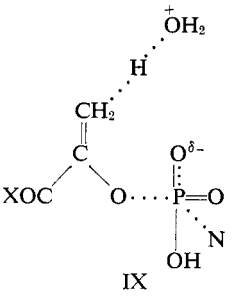
VII

reorganization in any of the above mechanisms or for reasons discussed above. It should be emphasized that if one corrects for the intrinsic higher reactivity of the enol phosphate substrate (ethyl phosphoenolpyruvate as the model), catalysis by COO^- increases the rate only 2- to 14-fold. Thus, regardless of the state ionization of the carboxyl moiety, it does not function as a efficient catalyst.

The relatively large neutral hydrolysis rate for phosphoenolpyruvate and ethyl phosphoenolpyruvate is atypical of phosphate monoesters. It is generally observed only with systems that hydrolyze *via* carbonium ion intermediates, *i.e.*, glucose 1-phosphate (Bunton *et al.*, 1958), or possess rather acidic leaving groups such as *p*-nitrophenyl phosphate (Bunton *et al.*, 1967). The proposed mechanisms for the present system must accommodate (1) a $\Delta S^{\pm}$ of -7 eu, (2) a significant deuterium solvent isotope effect, $k^{H_2O}/k^{D_2O} = 1.4$, (3) the equimolar product ratios observed in mixed CH_3OH–H_2O solvent, and (4) the unusual reactivity. Two possible mechanisms, VIII and IX, involve the generation of ketone char-



VIII



IX

acter through proton transfer from a thermodynamically more acidic phosphoryl oxygen or a hydronium ion concurrent with partial phosphoryl transfer to a solvent molecule (N). Thus one avoids generation of the presumed high-energy protonated metaphosphate. We have not assigned a unique function to the carboxyl moiety due to the observation of neutral species hydrolysis with ethyl phosphoenolpyruvate, although arguments similar to those discussed above could be advanced. The phosphoryl moiety in the above mechanisms would be

highly activated and subject to indiscriminate solvation by methanol or water.

In conclusion the nonenzymic hydrolysis of enol phosphates partially reveals their phosphorylating potential through the detection of significant rate enhancements which surprisingly occur with all the ionic species. However, not all the available free energy arising from enol to ketone conversion, actually less than 0.5%, is kinetically realized. Moreover, in the pH region 1–7, the hydrolysis generates a nonselective phosphorylating entity. It is quite possible that a cyclic mechanism may be operative in enol phosphate hydrolysis but in the particular case of phosphoenolpyruvate the carboxyl group is of minor importance kinetically.

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