

Metal Ion Catalysis of Phosphoryl Transfer from Phosphoenolpyruvate*

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ABSTRACT: The catalytic effect of mercury(II) and a number of other metal ions on the hydrolysis of phosphoenolpyruvate and ethyl phosphoenolpyruvate has been investigated. The mechanisms of catalysis and their effect on the phosphorylating properties of these substrate have been determined *via* kinetic and product studies.

The nonenzymic hydrolysis of phosphoenolpyruvate occurs only at elevated temperatures (Benkovic and Schray, 1968). The hydrolytic rate is enhanced by mercury(II) as first reported by Lohman and Meyerhof (1934) and is unusually large (Yasnikov, 1961), occurring at 0°. Concerted or preequilibrium protonation of the double bond of phosphoenolpyruvate by an acidic site in pyruvate kinase is suggested by the results of Rose (1960). In view of the fact that mercury(II) in its addition to double bonds may act as an analogy to protonation, we have investigated the mechanism and phosphorylating properties of this system.

In addition, in view of the biological role of magnesium (II) in the pyruvate kinase reaction (Boyer, 1962), we have sought to observe the effect of a number of other metal ions on phosphoenolpyruvate hydrolysis in aqueous solution.

Experimental Section

Materials. All reagents were as in the preceding paper (Benkovic and Schray, 1968). Metal nitrates (reagent grade, Baker) were utilized with the exception of $\text{Hg}(\text{ClO}_4)_2$ prepared by neutralization of HgO with perchloric acid (Baker reagent grade, 70%). Metal ions were standardized by the appropriate EDTA procedure of Flaschka (1959) or Schwarzenbach (1957). Dimethylformamide was Fisher reagent grade.

Apparatus. See preceding study.

Kinetics. Metal ion rates in runs maintained at $\mu = 0.2$ with KNO_3 were followed as stated previously with the pH range limited by metal ion hydrolysis. The initial second-order reactions of phosphoenolpyruvate and

The unusual properties of the mercury(II)-phosphoenolpyruvate reaction which proceeds *via* mercury(II) addition to the enol double bond and can lead to either phosphorylation of solvent or P_i release are discussed. The implications of these studies for the mechanism of the pyruvate kinase reaction are considered.

ethyl phosphoenolpyruvate with mercury(II) (HClO_4 - NaClO_4 , $\mu = 0.2$, buffer in addition to those previously described $\mu = 0.2$, KCl) were too rapid to follow but could be quenched by addition of aliquots to 12 M NaOH or to Fiske-Subbarow (1925) reagents. Catalytic mercury(II) runs (phosphoenolpyruvate, 20-fold excess) were monitored by orthophosphate release. On addition of mercury(II) to all solutions of substrate an initial precipitate is visible which dissolves on mixing.

Products. The mercuripyruvyl chloride and the ethyl ester of mercuripyruvyl chloride from the reaction of mercury(II) with phosphoenolpyruvate and ethyl phosphoenolpyruvate, respectively, in H_2O or $\text{C}_2\text{H}_5\text{OH}-\text{H}_2\text{O}$ could not be isolated by the procedures outlined by Chatt (1951) due to interference by phosphate. Magee (1965) has, however, isolated the mercuriacetaldehyde chloride from the similar reaction of mercury(II) with dimethoxyvinyl phosphate. Ethyl pyruvate (from ethyl phosphoenolpyruvate and from phosphoenolpyruvate by esterification of pyruvic acid) which resulted from dissociation of the alkylmercuric salts formed during product studies in H_2O or $\text{C}_2\text{H}_5\text{OH}-\text{H}_2\text{O}$ solvent was observed by gas chromatography (Perkin-Elmer R column, 140°, R_F 5 min). The procedure utilized for isolation was continuous extraction (5 hr) with CCl_4 or Et_2O of 0.1 M HCl solution of the alkylmercuric salts obtained by flash evaporation of the reaction mixture. Precipitation (presumably $\text{Hg}_3(\text{PO}_4)_2$) occurred toward the end of product studies in dimethylformamide- $\text{CH}_3\text{OH}-\text{H}_2\text{O}$ but not during H_2O or $\text{CH}_3\text{OH}-\text{H}_2\text{O}$ runs (mercury(II) and substrate $< 7 \times 10^{-3}$ M) except for carbonate (phosphoenolpyruvate) and succinate (ethyl phosphoenolpyruvate) buffers in the latter. Such precipitates were dissolved by acidification or dilution prior to orthophosphate assay. In order to obtain quantitative orthophosphate release from ethyl phosphoenolpyruvate in formate buffer (H_2O and $\text{CH}_3\text{OH}-\text{H}_2\text{O}$), additional mercury(II) was added during the experimental run because of concomitant reduction of mercury(II) to mercury. Mercury(II)-catalyzed product studies (from $\text{CH}_3\text{OH}-\text{H}_2\text{O}$ and $\text{CH}_3\text{OH}-\text{H}_2\text{O}$ -dimethylformamide) utilized a 1.1-fold excess of mercury(II) at 20°.

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TABLE I: Metal Ion Catalyzed Hydrolysis of Phosphoenolpyruvate^a at 75.35° ($\mu = 0.2$).

Metal Ion	pH	$k \times 10^8$ min ⁻¹	M	Metal Ion	pH	$k \times 10^8$ min ⁻¹	M
None	2.5	8.50		Nickel(II)	2.50	9.77	0.015
	3.6	7.60			3.62	7.70	0.015
	4.0	6.65			4.67	8.62	0.015
	4.68	6.30			5.30	9.76	0.015
	5.30	5.65		Cobalt(II)	2.47	9.76	0.015
Copper(II)	2.45	10.4	0.015		3.63	7.67	0.015
	3.53	69 ^b	0.015		4.68	7.95	0.015
	4.02	99 ^b	0.015		5.30	6.15	0.015
Aluminum(III)	2.61-2.42	14.7	0.019	Calcium(II)	2.50	9.95	0.015
	3.38-3.31	16.9	0.019		3.63	7.65	0.015
	4.03-3.75	2.8	0.019		4.68	6.12	0.015
Iron(III)	1.07	7	0.019		5.32	4.43	0.015
Magnesium(II)	2.51	10.24	0.015	Zinc(II)	2.49	7.62	0.015
	3.63	7.34	0.015		3.62	7.25	0.015
	4.66	4.97	0.015		4.16	6.87	0.015
	5.35	4.69	0.015		4.67	4.47	0.015
Manganese(II)	2.37	7.83	0.015				
	3.58	8.77	0.015				
	4.67	6.74	0.015				
	5.26	2.48	0.015				

^a About 1×10^{-3} M. ^b Estimated from per cent hydrolysis at selected time intervals.

Results

Results. The data for the metal ion studies, excluding mercury(II), are found in Table I. Copper(II) at pH 4.02 (70% dianion, 30% monoanion of phosphoenolpyruvate) catalyzes the hydrolysis *ca.* tenfold. Aluminum(III) catalyzes reaction of the monoanion *ca.* twofold but retards the dianion by *ca.* twofold. Magnesium(II), cobalt(II), and zinc(II), metal ion activators of pyruvate kinase (Boyer, 1962), as well as nickel(II), copper(II), and iron(III), have only slight acceleratory and inhibitory effects.

Mercury(II) was found to yield enzyme-like catalysis of phosphoenolpyruvate and ethyl phosphoenolpyruvate

as shown in Figure 1. A rapid initial reaction followed by steady-state kinetics to near completion is evident. The rate of the initial reaction is decreased and the steady-state reaction is increased, as a function of increasing $[\text{Cl}^-]$ and $[\text{OAc}^-]$, primarily due to the complexation of mercury(II). Quenching of the catalytic reactions (15-sec to 30-min reaction times at 4°, conditions under which the succeeding steady-state rate is greatly decreased) indicates initial release of 1 mole of phosphate/mole of mercury(II). Extrapolation of the HClO_4 buffer catalysis run at 20° to zero time yields a higher value (3:1 phosphate to mercury(II)) but the validity of such an extrapolation is somewhat tenuous due to phosphate complexation equilibria.

The solvolytic product distributions in $\text{MeOH-H}_2\text{O}$ (1:1) in the mercury(II)-catalyzed phosphoenolpyruvate and ethyl phosphoenolpyruvate reactions (shown in Figure 2) exhibit a striking contrast between the catalyzed and noncatalyzed reactions (see preceding study). P_i is the exclusive product at pH 2 and remains the major product (on a mole to mole basis) over the pH range investigated whereas the noncatalyzed reactions feature nonselective phosphorylation (69.9 mole % P_i).

The product composition of the phosphoenolpyruvate reaction was studied as a function of dimethylformamide concentration in $\text{MeOH-H}_2\text{O}$ -dimethylformamide solutions with acetate, KCl or KNO_3 present, aqueous pH 4.69, and HClO_4 , aqueous pH 2.1. The significant finding (see Figure 3) is the decrease in per cent P_i at pH 4.69 with increasing dimethylformamide concentration to a product composition which is nearly identical with the noncatalyzed solvolysis. Dimethylform-

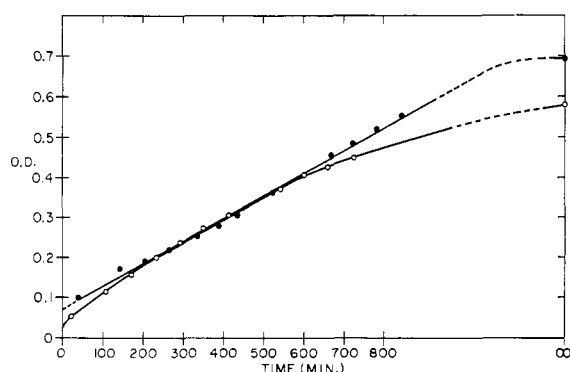


FIGURE 1: Mercury(II)- (1×10^{-3} M) catalyzed hydrolysis of phosphoenolpyruvate in HClO_4 (pH 2), in phosphoenolpyruvate (3.4×10^{-2} M) (●), and in acetate (pH 4.7) phosphoenolpyruvate (2.9×10^{-2} M) (○) at 20.0°.

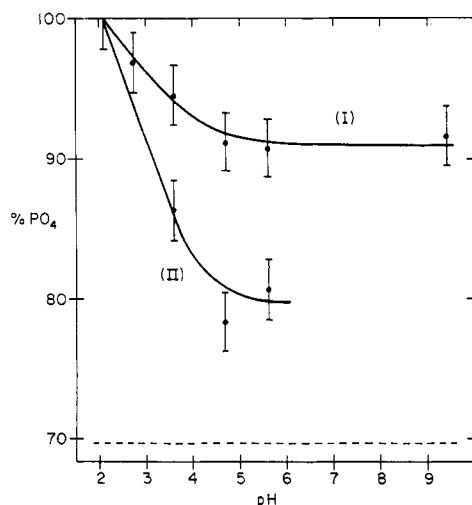
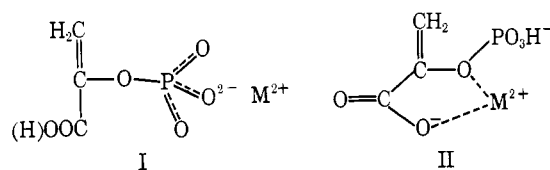


FIGURE 2: Per cent PO_4 product in $\text{CH}_3\text{OH}-\text{H}_2\text{O}$ solvolysis of phosphoenolpyruvate (curve I) and ethyl phosphoenolpyruvate (curve II) catalyzed by mercury(II) at 20.0° . Expected product distribution from metaphosphate (-----) is shown for comparison; (I) represents error.

amide has no effect on the pH 2.1 solvolysis product. Higher dimethylformamide concentrations were not attempted due to substrate insolubility. Runs in dimethylformamide- H_2O showed 100% P_i formation thus indicating the absence of pyrophosphate generation.

Discussion

Transition states analogous to I and II have been proposed for hydrolysis of acetyl phosphate catalyzed by magnesium(II), calcium(II), and zinc(II) (Oestreich and



Jones, 1966), salicyl phosphate by $\text{ZrO(IV)} \cong \text{iron(III)} > \text{VO(IV)} > \text{UO}_2(\text{VI}) > \text{copper(II)}$ (Hofstetter *et al.*, 1962), and methoxyethyl phosphate by La(OH)_2 gel (Butcher and Westheimer, 1955). The above transition states ascribe catalysis to either chelate formation, *i.e.*, the formation of a cyclic intermediate involving the pyruvate moiety (II), or charge neutralization, *i.e.*, reduction of the net negative charge of the phosphate group through electrostatic interaction with the positively charged metal ion (I).

Our results are similar to salicyl phosphate where copper(II) is an effective catalyst but magnesium(II), manganese(II), etc., are not. Possible factors in this catalysis include ionic radius (Genge and Salmon, 1959), electron affinity as measured by oxidation potential, and charge density which have been discussed elsewhere (Bruice and Benkovic, 1966). Winstein and Lucas (1938) have shown that no measurable complexes between olefins and copper(II), iron(II), cadmium(II), cobalt(II), or

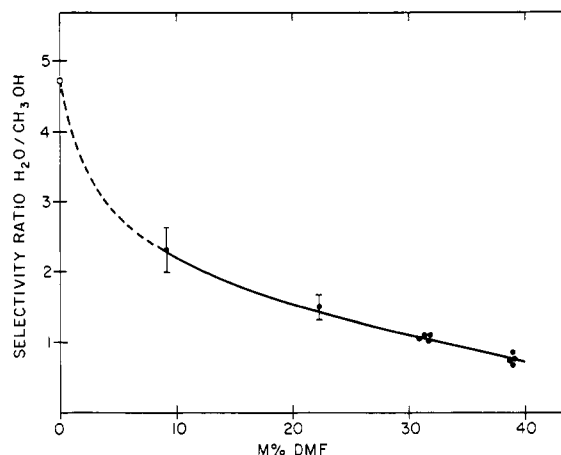
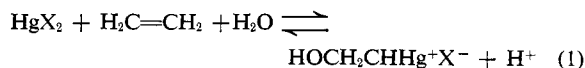


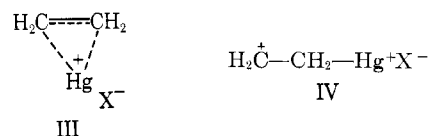
FIGURE 3: Selectivity for water in mercury(II)-catalyzed solvolysis of phosphoenolpyruvate in dimethylformamide- $\text{H}_2\text{O}-\text{CH}_3\text{OH}$ (pH 4.69 acetate buffer, 20°). Nonselectivity yields a value of 1.

zinc(II) are formed. Thus these metal ions are distinctly different from mercury(II).

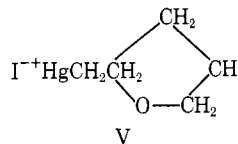
The reaction of mercury(II) with olefins is well known (Sand and Hofmann, 1900) and in aqueous solution results in an oxymercuration reaction (eq 1). In the case of unsymmetrical olefins mercury(II) adds to the least substituted position (Markovnikov addition) (Lucas *et al.*, 1939). There is apparently still some doubt as to the



mechanism of this reaction, particularly in regards the existence of a mercurinium ion (III) *vs.* a classical carbonium ion (IV) (Halpern and Tinker, 1967), but the salient point is that a covalent bond between mercury(II) and carbon is formed. Furthermore this oxymercuration

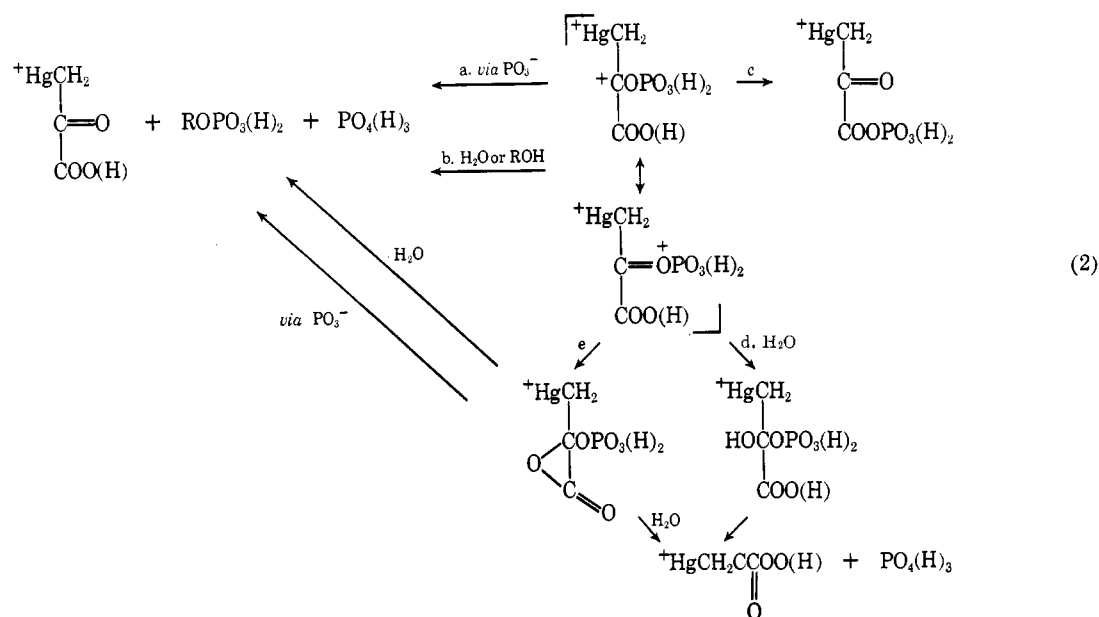


reaction is reversible in acidic solution (Kreevoy and Turner, 1964; Chatt, 1951). Internal quenching is also possible with suitably placed nucleophiles, *e.g.*, the formation of V from 1-penten-5-ol (Halpern and Tinker, 1967).



Consistent with these data the following mechanisms should be considered for the mercuric ion activation of phosphoenolpyruvate solvolysis. Mercury(II) + phosphoenolpyruvate yields reaction 2.

Examination of products in mixed solvents is mech-



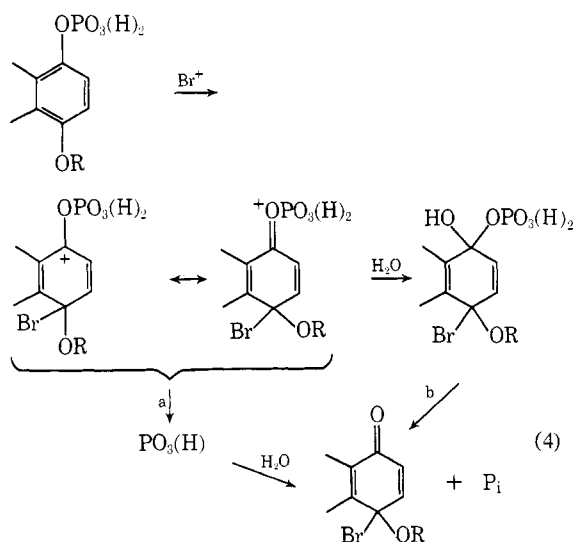
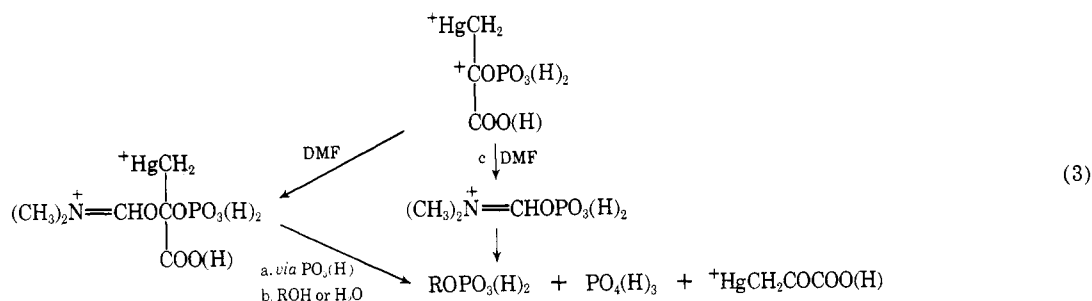
anistically informative. The complete generation of inorganic phosphate at pH 2 from both ethyl phosphoenolpyruvate and phosphoenolpyruvate (Figure 2) demands C–O bond fission as both metaphosphate generation and bimolecular attack on phosphorus would be expected to yield inorganic and methyl phosphate. The formation of acyl phosphate (pathway 2c) and α -lactone (pathway 2e) is excluded on the grounds that esterification of the carboxyl function does not alter the product composition. Thus the mechanism at low pH is that of pathway 2d. Capture of the cation by alcohol would give a ketal-like species which under the reaction conditions would presumably be in equilibrium with the active species of 2d.

As pH increases, P–O bond cleavage becomes partially competitive most probably *via* a unimolecular pathway since bimolecular attack should have been more rapid at low pH due to lack of ion–dipole repulsion between the ionized phosphate and the nucleophiles. The P–O bond cleavage now accounts for 30% of the phosphoenolpyruvate reaction pathway and 60% for ethyl phosphoenolpyruvate. P–O fission is most readily envisioned *via* eq 2a. Explanation of the quantitative pH dependence of the per cent methyl phosphate is hampered by (1) the change in mercury(II) solvation with pH, (2) uncertainty of substrate pK_a 's in mixed solvent, (3) buffer effects, and (4) phosphate–mercury(II) complexation, but is qualitatively in agreement with increasing P–O cleavage *via* metaphosphate from ionized substrate species. Utilizing this reaction scheme and assuming that the rate of aqueous quench of the carbonium ion is diffusion controlled, $10^{10} \text{ M}^{-1} \text{ sec}^{-1}$ (Eigen, 1964) the rate of P–O bond cleavage at pH 4–8 from the carbonium ion (or its resonance form, the protonated ketone) is *ca.* $5 \times 10^9 \text{ sec}^{-1}$. It is surprising that unimolecular elimination of protonated ketone is not more rapid.

At higher pH there is a quantitative difference in metaphosphate generation from the two substrates. This difference reveals that a maximum of 30% of the reaction

might be the result of carboxyl participation. However, solvolysis of phosphoenolpyruvate at pH 4.7 in $\text{C}_2\text{H}_5\text{OH-H}_2\text{O}$ mixtures does not yield ethyl pyruvate above that detected for esterification of pyruvic acid. Calculations that assume nonselectivity of the α -lactone for ethanol or water reveal pathway 2e cannot exceed 10% of the reaction, otherwise ethyl phosphoenolpyruvate would have been detected. Moreover, acyl phosphate was not detected as an intermediate in the catalytic run although its steady-state formation cannot be completely eliminated. Interaction between the electropositive mercury and the carboxyl moiety may also be responsible for the above observation.

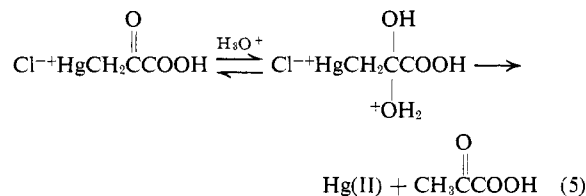
The higher percentage of methyl phosphate production upon addition of dimethylformamide (Figure 3) can be interpreted as (1) a solvent effect or (2) as a direct involvement of dimethylformamide. Several possible mechanisms for the latter are shown in reaction 3. It has been shown (Cohen and Lapidot, 1967) that intermediates analogous to that of eq 3a–c exist in the bromine oxidation of 2-methylnaphthalene-1,4-diol diphosphate, ruling out a simple solvent effect. This general system, investigated by several workers (Clark *et al.*, 1961; Lapidot and Samuel, 1962; Durckheimer and Cohen, 1964; Tomasi and Dallam, 1964), proceeds *via* eq 4a,b and is similar to the Hg(II)–phosphoenolpyruvate system. In aqueous solution C–O cleavage (eq 4b) is the primary reaction pathway (Lapidot and Samuel, 1962) but in dimethylformamide, P–O cleavage leads to phosphate esters or condensed phosphates *via* mechanisms analogous to eq 3. Thus the question is largely one of molecularity. Equation 3a envisions a bimolecular quenching of the carbonium ion which then decomposes unimolecularly to metaphosphate which is nonselectively solvated. Not depicted is solvation of metaphosphate by dimethylformamide, followed by product formation through bimolecular displacements on this dimethylformamide– $\text{PO}_3(\text{H})_2$ substrate. This appears to violate microscopic reversibility because of the similarity of this species to the initial mercury(II)–pyruvate adduct. Bi-



molecular attack by dimethylformamide on phosphorus as in eq 3c and by solvent as in eq 3b is unlikely because (1) as indicated by the mercury(II) results in $\text{CH}_3\text{OH-H}_2\text{O}$ at low pH (exclusive P_i formation) such displacements do not readily occur and (2) only P_i is generated in dimethylformamide- $\text{CH}_3\text{OH-H}_2\text{O}$ solvents at pH 2, *i.e.*, where bimolecular displacements on phosphorus are favored (Bruice and Benkovic, 1966).

Thus we favor unimolecular decomposition *via* eq 3a. Increasing dimethylformamide concentration leads to greater trapping of the cation and metaphosphate formation since expulsion of dimethylformamide and regeneration of the amide linkage is energetically much more facile than loss of P_i . The mole % dimethylformamide at which metaphosphate product distribution is achieved indicates *ca.* threefold selectivity of dimethylformamide over H_2O in quenching the cation. The slight selectivity for methanol is readily explained in that reactions known to proceed *via* metaphosphate-like transition states yield as great as fourfold methanol selectivity at low temperatures (Kirby and Varvoglis, 1967).

The observed kinetics (Figure 1) are consistent with the initial rapid formation of the mercury(II)-phosphoenolpyruvate addend resulting in a phosphate "burst" in HClO_4 buffer. It is known that the mercury(II) and olefin reaction to form an oxymercurial is very rapid, $\sim 5 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ (Halpern and Tinker, 1967). Moreover, the mercuripyruvate salt formed may then undergo dissociation as discussed above *via* eq 5. The regenerated mercury(II) then catalyzes hydrolysis of another mole of phosphoenolpyruvate. This slow dissociation and



subsequent more rapid reaction generates the steady-state kinetics seen in Figure 1. The rapidity of reaction, change in mercury(II) solvation over pH, and buffer and counter ion effects precluded a kinetic analysis.

In conclusion copper(II) is seen to catalyze phosphoenolpyruvate hydrolysis by a factor of 10 *via* a chelation or complexation mechanism. Mercury(II) catalyzes hydrolysis by a factor of $>10^6$ *via* addition to the enol double bond. This may be viewed as complete protonation in analogy to the partial protonation seen in the noncatalyzed hydrolysis with the resultant larger rate enhancement. The intermediate carbonium ion formed may then undergo (1) quenching by water yielding a ketal-like intermediate which generates inorganic phosphate or (2) immediate generation of metaphosphate. Addition of dimethylformamide to the solution results in quenching of the intermediate ion by dimethylformamide and subsequent metaphosphate expulsion yielding complete metaphosphate generation at 40 mole % dimethylformamide. Mercury(II) is regenerated from the mercuripyruvyl chloride under reaction conditions and is thus a true catalyst.

We have shown that phosphoenolpyruvate hydrolyzes quite slowly (in light of a large hydrolytic ΔG°) *via* monomeric metaphosphate with only small acceleration due to ketonization energy release (see preceding paper). This hydrolysis may be activated, however, by mercuration of the double bond of phosphoenolpyruvate realizing the energy of ketonization. Protonation of this double bond by the enzyme pyruvate kinase would be expected to yield a similar activation and would generate a similar carbonium ion. Although we have not observed bimolecular general acid catalysis it is possible that a carboxyl function may be utilized as demonstrated for vinyl ether hydrolysis (Fife, 1965). The mercuration intermediate, protected from aqueous quenching, also yields metaphosphate as opposed to bimolecular attack. This model then appears to give evidence for enzymic preprotonation of the double bond followed by metaphosphate generation. The metal ion role (magnesium(II), manganese(II), or cobalt(II)) would be one of orientation to prevent phosphorylation of the enzyme and possibly activation *via* charge neutralization of the phos-

phoryl moieties. The neighboring carboxyl group appears to have no demonstrable function.

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L-Lysine- α -Ketoglutarate Aminotransferase. I. Identification of a Product, Δ^1 -Piperidine-6-carboxylic Acid*

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ABSTRACT: The product derived from L-lysine was isolated from the L-lysine- α -ketoglutarate aminotransferase system of *Flavobacterium fuscum* and its properties were studied. Radioactivity from DL-[1- 14 C]lysine was incorporated exclusively into a product which reacted with *o*-aminobenzaldehyde. Chromatographic and electrophoretic studies, characterization of the bisulfite and *o*-aminobenzaldehyde adducts and the con-

densation product, and comparison with authentic Δ^1 -piperidine-2-carboxylic acid offer evidence that the product is Δ^1 -piperidine-6-carboxylic acid. In this aminotransferase reaction, ϵ -amino group of L-lysine is transferred to α -ketoglutarate to yield glutamate and α -aminoadipate- δ -semialdehyde which is immediately converted into the intramolecularly dehydrated form, Δ^1 -piperidine-6-carboxylic acid.

Although the metabolism of lysine has been extensively studied as reviewed by Meister (1965) and Broquist and Trupin (1966), the mechanism of the enzymatic deamination of lysine has, until recently, remained unsolved. Both D and L isomers of lysine are

known to be scarcely deaminated by the respective amino acid oxidases (Bender and Krebs, 1950; Greenstein *et al.*, 1953; Scannone *et al.*, 1964). In 1960, the occurrence of a bacterial enzyme which catalyzes the conversion of L-lysine into δ -aminonorvalerate, ammonia, and carbon dioxide was reported (Hagihira *et al.*, 1960). This enzyme was later demonstrated to be an oxygenase (Itada *et al.*, 1961), which has been crystallized (Takeda and Hayaishi, 1966). This oxygenase reaction involves initially the oxygenative de-

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