Intramolecular and Divalent Metal Ion Catalysis. The Hydrolytic Mechanism of O-Phenyl N-(Glycyl)phosphoramidate

ERIC J. SAMPSON,[1a] JOHN FEDOR,
PATRICIA A. BENKOVIC, AND STEPHEN J. BENKOVIC*[1b]

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

Received August 24, 1972

The pH–rate profile for the hydrolysis of O-phenyl N-(glycyl)phosphoramidate (II) reveals intramolecular catalysis by the neighboring carboxylate function which serves to accelerate greatly the rate of P–O bond cleavage. In fact, P–O bond fission in the reference compound, O-(phenyl)phosphoramidate (I), is not detected. The catalysis of II is further enhanced (>10^2) by the addition of Zn^{2+} or Mg^{2+} ions, which do not affect the rate of hydrolysis of I. A mechanism is postulated featuring formation of a five-membered cyclic acyl phosphate (product studies in hydroxylamine buffer) which decomposes *via* water attack on phosphorus rather than carbon (^{18}O tracer experiments). These findings suggest that two types of biologically important catalysis may be incorporated into a model system in order to confer dramatic reactivity on a normally unreactive phosphate diester. These results contrast with the Cu(II)-catalyzed hydrolysis of salicyl phosphate, which apparently is of the general-acid type with carboxylate merely serving as a coordinating ligand.

Intramolecular models for biological phosphoryl transfer reactions at the diester level have been particularly useful in defining the probable existence of intermediate pentacovalent species on these pathways.[2,3] Previous quantitative investigations had focused mainly on the behavior of O-phosphate diesters and featured nucleophilic carboxyl or carboxylate catalysis.[3,4] A striking stereochemical aspect of carboxylate catalysis in the intramolecular diester systems is the preferential exocyclic group expulsion by an *o*-carboxylate, *e.g.*, loss of phenol during hydrolysis of phenyl (2-carboxyphenyl)phosphate, despite the relative leaving group pK_a values. This phenomenon has been attributed to a restricted pseudorotation of the dianionic pentacovalent intermediate.[4,5]

We posed several questions: (1) will the substitution of nitrogen for oxygen affect the stereochemical course; (2) will metal ions alter the mode of decomposition of the presumed intermediate; and (3) is a synergistic acceleration of the rate of hydrolysis by both intramolecular and metal ion catalysis feasible?[6] Answers to these questions compose the major thesis of this paper.

Experimental Section

Microanalyses for nitrogen and phosphorus were performed by Midwest Microlab. Twice distilled deionized water, D_2O (99.8% Diaprep) and $H_2{}^{18}O$ (8.1 atom %, Bio-Rad) were employed as solvents. Reagent-grade buffer materials, metal nitrates, and other solvents were used without further purification, except where noted. Descending paper chromatography was run on Schleicher and Schuell orange ribbon 589c paper in 0.1 *M* aqueous K_2CO_3–absolute ethanol (3.5:6.5) and developed with Hanes and Isherwood spray[7] (phosphate) and 1% ninhydrin spray (glycine). Nmr spectra in D_2O were measured on a Varian Associates A-60 spectrometer using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard. Uv spectra were obtained on a Cary 14 recording spectrophotometer. Mass spectra were measured on an MA 902-AEI spectrometer.

The monopotassium salt of O-(phenyl)phosphoramidate (I) was prepared from the diphenyl phosphoramidate precursor $[NH_2PO_3(C_6H_5)_2]$ by the method of Stokes,[8] uv $\lambda_{max}^{0.1\ M\ KOH}$ 262 mμ (ε 440).

Anal. Calcd for $C_6H_7N_1P_1O_3K\cdot H_2O$: N, 6.12; P, 13.52. Found: N, 6.40; P, 13.50.

The dipotassium salt of O-phenyl N-(glycyl)phosphoramidate (II) was prepared by an adaptation of the method of Zervas, *et al.*[9] Diphenyl phosphorochloridate (0.044 mol) was added dropwise to a rapidly stirring, ice-cold suspension of glycine ethyl ester hydrochloride (0.040 mol) in anhydrous pyridine (30 ml). The mixture was stirred for 2 hr and poured into ice water (100 ml). The diphenyl derivative separated as an oil that crystallized upon scratching. The white crystals were collected by filtration, washed with water, dried under vacuum, and recrystallized from ether, *m/e* 335 (calcd, 335), mp 76–77° (uncorrected).

A suspension of the diphenyl derivative (0.0057 mol) in 0.40 *N* potassium hydroxide (35 ml) was stirred for 6 hr at room temperature. The reaction mixture was filtered and the filtrate was titrated to pH 7 with glacial acetic acid. The solution was evaporated under vacuum to 3 ml and cold absolute ethanol (10 ml) was added. Precipitation of the desired salt was accomplished by the dropwise addition of cold acetone (10 ml). The product was isolated by filtration and further purified by dissolving in water (1 ml) and adding absolute ethanol (5 ml) followed by precipitation again with acetone. The compound was dried *in vacuo* and stored at −10°. The overall yield was approximately 50% and no attempts were made to maximize the yield. II showed nmr (D_2O) δ 3.48 (d, 2 H, $-NCH_2CO_2-$) and 7.33 (broad multiplet, 5 H, C_6H_5-), at pH 6; $\lambda_{max}^{0.1\ M\ KOH}$ 262 mμ (ε 480). Paper chromatography revealed one spot, R_f 0.53, which developed for phosphate, glycine, and phenol (visualized by uv irradiation).

Anal. Calcd for $C_8H_8N_1P_1O_5K_2\cdot H_2O$: N, 4.33; P, 9.58. Found: N, 4.42; P, 9.57.

Salicyl phosphate (III) was prepared according to the procedure of Chanley, *et al.*[10]

Dissociation Constants.—Values for dissociation constants for I and II were determined in a Metrohm cell (EA 662) at 25°, μ 0.2, KNO_3. Hydrogen ion corrections were applied as described by Albert and Serjeant[11] (Table I).

Apparatus.—Instrumentation used in this study has been described previously.[12] Kinetic runs were carried out in Kimax (No. 45066) screw-cap tubes whose threads were wrapped with Teflon tape to prevent evaporation. Tubes were maintained at reaction temperature (±0.1°) by immersion in a circulating water bath.

(1) (a) In partial fulfillment for the degree of Doctor of Philosophy, The Pennsylvania State University; (b) National Institutes of Health Career Development Awardee.

(2) D. A. Usher, D. I. Richardson, Jr., and D. G. Oakenfull, *J. Amer. Chem. Soc.*, **92**, 4699 (1970).

(3) K. J. Schray and S. J. Benkovic, *ibid.*, **93**, 2522 (1971).

(4) S. A. Khan, A. J. Kirby, M. Wakselman, D. P. Horning, and J. M. Lawlor, *J. Chem. Soc. B*, 1182 (1970).

(5) F. H. Westheimer, *Accounts Chem. Res.*, **1**, 70 (1968).

(6) S. J. Benkovic and L. K. Dunikoski, *J. Amer. Chem. Soc.*, **93**, 1526 (1971).

(7) C. S. Hanes and F. A. Isherwood, *Nature (London)*, **164**, 1107 (1949).

(8) N. H. Stokes, *Amer. Chem. J.*, **19**, 198 (1893).

(9) L. Zervas and P. G. Katsoyannis, *J. Amer. Chem. Soc.*, **77**, 5351 (1955).

(10) J. D. Chanley, E. M. Gindler, and H. Sobotka, *ibid.*, **74**, 4347 (1952).

(11) A. Albert and E. P. Serjeant, "Ionization Constants of Acids and Bases," Wiley, New York, N. Y., 1962.

(12) S. J. Benkovic and P. A. Benkovic, *J. Amer. Chem. Soc.*, **88**, 5504 (1966).

Kinetics.—Kinetic experiments were initiated by the addition of a 1-ml aliquot from a freshly prepared aqueous stock solution (0.01 *M*) of the phosphoramidate to 9 ml of the preequilibrated buffer solution or by the direct addition of the phosphoramidate (10^{-5} mol) to 10 ml of preequilibrated buffer solution.

Hydrolysis of I was monitored by analysis for ammonia employing the following modification of the Weatherburn method.[13] The aliquot (0.2 ml) to be analyzed (0–6 μmol in ammonia) was added to 5.0 ml of reagent A, which consists of 5.0 g of phenol and 25 mg of sodium nitroprusside made up to 500 ml with water. The tube was covered with Parafilm and shaken vigorously to mix. To this solution, 5 ml of reagent B, which consists of 2.5 g of sodium hydroxide and 4.2 ml of commercially available Clorox made up to 500 ml with water, was added. The tube was thoroughly mixed and the intensity was read at 625 mμ after 20 min of incubation at 35°. Compound I gave no initial reading so that apparently no hydrolysis occurred under the assay conditions. Duplicate runs agreed within ±5%.

The spontaneous and metal ion catalyzed hydrolysis of II, at pH >6, was monitored by measuring phenoxide ion at 285 mμ by withdrawing 1-ml aliquots and adding 1 ml of 1 *N* potassium hydroxide. An alternative analysis measured the production of orthophosphate by the method of Martin and Doty,[14] as modified by Jencks.[15] Duplicate runs agreed within ±4%. In the presence of magnesium ion, the addition of KOH precipitated $Mg(OH)_2$, which was removed by centrifugation prior to measurement of the absorption.

The disappearance of II at pH <6 was monitored by measuring the liberation of phenoxide ion at 285 mμ by the following procedure. A 1-ml aliquot was added to 1 ml of 1 *N* potassium hydroxide and the absorbance was measured. This solution was sealed into a breakseal ampoule and incubated in a 75° water bath for 24 hr, and the absorbance at 285 mμ was again measured. Under the assay conditions the dianion of phenyl phosphate—a competing product at pH <6—is stable, so that any increase in absorbance at 285 mμ arises from phenolate due to the total hydrolysis of the remainder of II. The concentration of II at a given time therefore is proportional to ΔOD_t, the difference between the two absorbance readings at 285 mμ. Hydrolysis of phenyl phosphate monoanion (10^{-3} min^{-1}, 75°),[16] a competing reaction at pH <6, therefore does not interfere. The observed first-order rate constants for the hydrolysis of II, at pH <6, were calculated from slopes of log ΔOD_t against time. All plots were linear to at least three half-lives and duplicate runs agreed within ±4%.

The hydrolysis of III at 25° was monitored by measuring orthophosphate release by the method of Martin and Doty. Initial ester concentrations were *ca.* 5×10^{-3} *M*.

Acyl trapping reactions with II were carried out in 0.67 *M* hydroxylamine hydrochloride, recrystallized prior to use, at pH 7.2, 75°. The rate was determined by measuring the production of phenoxide ion at 285 mμ as described above. With hydroxylamine in excess, pseudo-first-order kinetics were observed. Similar trapping experiments were attempted with III employing 0.4 *M* hydroxylamine (as free base) at pH 5.6, 25°, in the absence and presence of metal ion.

Buffers employed in the spontaneous hydrolysis of I and II were nitric acid (pH <1.5), oxalate (0.2 *M*, pH 1.5–2.0), glycine (0.1 *M*, pH 2.0–3.0), citrate (0.033 *M*, pH 3.0–4.0), acetate (0.2 *M*, pH 4.0–5.5), phosphate (0.033 *M*), pH 6.0–7.0), and Tham (0.2 *M*, pH 7.2–9.0) with μ 0.2, KNO_3. Buffers used in the metal ion catalyzed hydrolysis of II were acetate (0.002 *M*, pH 4.0–5.5) and Tham (0.002 *M*, pH 7.2–9.2) with μ 0.2, KNO_3. Acetate buffer (0.4 *M*, μ 1.0, KCl) was employed for the spontaneous and metal ion catalyzed hydrolysis of III.

Buffer effects in both the spontaneous and metal ion catalyzed hydrolysis were negligible over a 0.1 *M* change in buffer concentration. The pH was measured at 25° (glass electrode) upon initiation and after completion of the kinetic runs; those exhibiting pH drift greater than ±0.05 unit were discarded. Buffer corrections were applied to those runs at 75° employing the apparent heats of ionization from data in ref 17. Deuterium oxide buffers were *ca.* 98% D_2O after correction for addition of hydrogen acids and bases. The kinetic deuterium solvent isotope effect (pH 6.6) was calculated utilizing rates measured in identical H_2O and D_2O buffers in the pH-independent region.

^{18}O Tracer Experiments.—The dipotassium salt of II (15 mg) was hydrolyzed to completion (*i.e.*, to glycine, inorganic phosphate, and phenol) in 2 ml of 8.1% ^{18}O-enriched acetate buffer (pH 5.8, μ 0.1, 75°) or 0.4 *M* chloroacetate buffer (pH 2.9, μ 0.2, 75°). The solution was added to an Amberlite IR-120 column (1 × 5 cm) in the ammonium form, and eluted first with water (40 ml) and then 1 *N* ammonium hydroxide (40 ml). The fraction eluted with water contained inorganic phosphate (Martin and Doty method) and phenol (spectrophotometric assay at 285 mμ). Isolation of inorganic phosphate and conversion of the oxygens to carbon dioxide has been described previously.[18,19] The fraction eluted with ammonium hydroxide was evaporated *in vacuo* to dryness.

A fraction of the residue was dissolved in a minimal amount of water and chromatographed according to the procedure of Fieser for the identification of glycine.[20] The remainder was redissolved in 0.1 ml of 0.01 *M* ammonium hydroxide and the silver glycinate precipitated upon the addition of solid silver nitrate (10% excess). Pyrolytic decarboxylation of silver glycine in a vacuum train yielded carbon dioxide, isolated by the method in ref 19.

The dipotassium salt of II (15 mg) was hydrolyzed to completion in 2 ml of 8.1% ^{18}O-enriched acetate buffer (pH 6.0, μ 0.1, 35°) in the presence of 0.5 *M* $Zn(NO_3)_2$. The resulting white precipitate, $Zn_3(PO_4)_2$, which formed, was isolated by centrifugation, washed with 95% ethanol and absolute ether, and dried *in vacuo*. The zinc phosphate was converted to potassium dihydrogen phosphate by the method of Haake and Westheimer.[21] The oxygen of potassium dihydrogen phosphate was converted to carbon dioxide according to the method of Boyer, *et al.*[19]

The relative isotopic abundances occurring in the carbon dioxide were determined on an MS 902 AEI mass spectrometer by measuring peak heights directly from the ion signal collector. Tank carbon dioxide was run as a standard prior to determinations.

Products.—The products of hydrolysis of I and II at t_∞ were identified by paper chromatography utilizing glycine, R_f 0.50, inorganic phosphate, R_f 0.12, and phenyl phosphate (uv visualization and Hanes and Isherwood spray) as standards. The observed product of hydrolysis of I at pH 2.0 was phenyl phosphate. The observed products of hydrolysis of II at pH 2.0 were glycine and phenyl phosphate, and a trace amount of inorganic phosphate, and at pH >6 in the absence and presence of metal ions were glycine, phenol, and inorganic phosphate at all temperatures.

Spectrophotometric scanning (340–210 mμ) at t_∞ of the reaction solutions of II at pH >6 disclosed ultraviolet spectra identical with quantitative liberation of phenol. Below pH 6, the products of hydrolysis are pH dependent and the mole fraction of phenol produced *via* P–O bond cleavage was calculated from the ratio of the phenol concentrations measured at t_∞ to the initial concentration of II obtained by total hydrolysis to phenol. In practice this was accomplished by starting with a known concentration of II and measuring the OD of phenolate ion (see above) against time until successive readings at *ca.* 1–3-hr intervals agreed within experimental error. Since the hydrolysis of phenyl phosphate to phenol and inorganic phosphate was a competing reaction, the mole per cent of phenol was calculated for only those runs at pH values in which II hydrolyzed at least 50-fold faster than phenyl phosphate.

The products of the hydrolysis of III in the absence and pres-

(13) W. W. Weatherburn, *Anal. Chem.*, **39**, 971 (1967).

(14) J. B. Martin and D. M. Doty, *ibid.*, **21**, 965 (1949).

(15) W. P. Jencks and M. Gilchrist, *J. Amer. Chem. Soc.*, **86**, 1410 (1964).

(16) Calculated from data of A. J. Kirby and A. G. Varvoglis, *ibid.*, **89**, 415 (1967).

(17) G. Kortum, W. Vogel, and K. Andrussow, "Dissociation Constants of Organic Acids in Aqueous Solution," Butterworths, London, 1961.

(18) (a) S. J. Benkovic and E. J. Sampson, *J. Amer. Chem. Soc.*, **93**, 4009 (1971). (b) The decomposition of IV is given by the expression $k_p = k_{obsd}K_a k_{-b}k_h/k_b k_{-h}$, where $K_a = 10^{-4}$ *M*, $k_h/k_{-b} \cong 10^{-6}$ (approximation for the steady-state concentration of IV), and $k_{-h}/k_h = 10^{-13}$ *M*. The latter is estimated from the equation of Branch and Calvin: G. E. Branch and M. Calvin, "The Theory of Organic Chemistry," Prentice-Hall, Englewood Cliffs, N. J., 1941. The calculated value of $k_p \cong 10^9$ sec^{-1} should be compared to that deduced for k_{-h} (*ca.* 10^{-2} sec^{-1}), since k_h is diffusion controlled. Implicit in the above preequilibrium derivation is the assumption that $k_h[H^+] \gg k_p$, which is untenable at pH ≧ 7 in view of the calculated value of k_p.

(19) P. D. Boyer, D. J. Graves, C. H. Suelter, and M. E. Demsey, *Anal. Chem.*, **33**, 1906 (1961).

(20) L. F. Fieser, "Experiments in Organic Chemistry," D. C. Heath, Boston, Mass., 1957, p 130.

(21) P. Haake and F. Westheimer, *J. Amer. Chem. Soc.*, **83**, 1102 (1961).

TABLE I
RATE AND DISSOCIATION CONSTANTS OF I AND II

Compd	k_H, M^{-1} min^{-1}	k_1, min^{-1}	$k_2 \times 10^3$, min^{-1}	$k_3 \times 10^3$, min^{-1}	pK_{a_1}[a]	pK_{a_2}[a]
I[b]	25	0.15			2.23 ± 0.1	
II[b]		1.25	8.1	8.77	1.9 ± 0.2	4.12 ± 0.03
II[c]	1.78	0.023	0.71	0.12	1.9 ± 0.2	4.12 ± 0.03

[a] Dissociation constants were determined at 25° (μ 0.2). [b] Rate constants were determined at 75°. [c] Rate constants were determined at 35°.

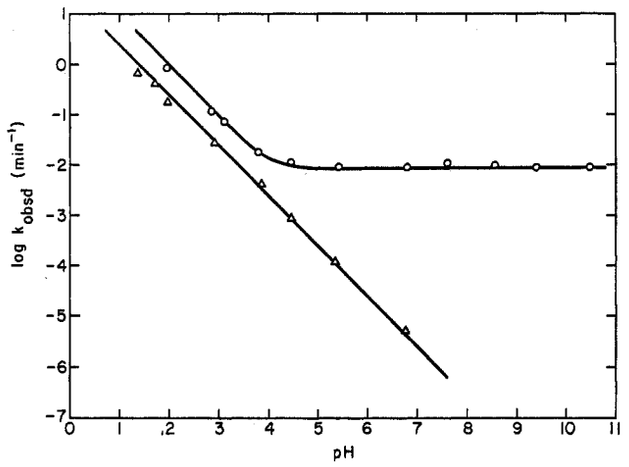


Figure 1.—The log k_{obsd}–pH rate profile for the hydrolysis of I, △, and II, O, at 75°, μ 0.2. Solid lines are theoretical curves calculated from values listed in Table I.

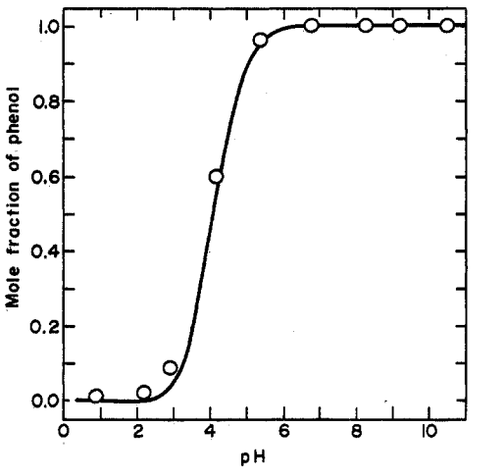


Figure 2.—Plot of the mole fraction of phenol liberated at t_∞ *vs.* pH, for the hydrolysis of II at 35°, μ 0.2. The solid line is the theoretical curve calculated from eq 3 utilizing $pK_{a_2} = 4.12$.

ence of metal ion previously have been shown to be salicylic acid and inorganic phosphate.[22,23]

The mole fraction of glycylhydroxamic acid produced in the acyl trapping experiment described above was calculated from the ratio of hydroxamic acid concentrations measured at t_∞ to the value observed for the total solvolysis of glycine ethyl ester hydrochloride under identical conditions (0.015 *M* substrate, 0.67 *M* hydroxylamine, pH 7.2, 75°). Hydroxamic acid was developed by the procedure of Lippman and Tuttle.[24] Glycine controls revealed no significant hydroxamic acid formation at identical reagent concentrations (0.015 *M*). Duplicate runs agreed within ±15%.

Results and Discussion

The pH–rate profiles for the hydrolysis of *O*-(phenyl)-phosphoramidate (I) and *O*-phenyl *N*-(glycyl)phosphoramidate (II) are shown in Figure 1. The products of hydrolysis of I are ammonia and phenyl phosphate over the pH range investigated. The solid line for I was calculated from eq 1, where k_H is the second-order

$$k_{obsd} = (k_H a_H + k_1)\left(\frac{a_H}{K_{a_1} + a_H}\right) \quad (1)$$

rate constant associated with hydronium ion catalyzed hydrolysis of the neutral species, k_1 is the first-order rate constant for the spontaneous hydrolysis of the neutral species, K_{a_1} is the dissociation constant for the neutral species, and a_H is the activity of hydrogen as measured by the glass electrode. The solid lines for the pH–rate profiles (Figure 1, 75°, Figure 3, 35°) and pH product distribution profile (Figure 2) for II were calculated from eq 2 and 3, respectively, where k_H, k_1, and K_{a_1} are defined as above, K_{a_2} is assigned to the dissociation

$$k_{obsd} = \frac{a_H^2(k_H a_H + k_1) + K_{a_1}(k_2 a_H + K_{a_2}k_3)}{a_H(a_H + K_{a_1}) + K_{a_1}K_{a_2}} \quad (2)$$

$$\text{Mole fraction of phenol} = \left(\frac{K_{a_2}}{K_{a_2} + a_H}\right) \quad (3)$$

constant of the carboxyl function, and K_2 and k_3 are first-order rate constants associated with the hydrolysis of the mono- and dianion, respectively. Values of the rate and dissociation constants utilized in eq 1, 2, and 3 appear in Table I.

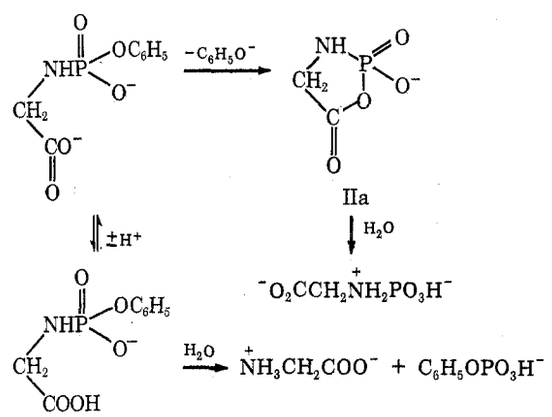
At pH <2, hydrolysis of II proceeds *via* P–N bond cleavage with concomitant formation of glycine and phenyl phosphate. The mechanisms of hydronium ion and spontaneous hydrolysis of the neutral species of mono- and unsubstituted phosphoramidates have been investigated previously;[25,26] therefore the subsequent study has been restricted to the pH-independent region where the observed catalysis is maximum. At pH 6–11, phenol, inorganic phosphate (quantitative formation), and glycine (qualitative) are observed as the products of hydrolysis. Presumably the P–O bond is preferentially cleaved, forming phenol and *N*-phosphorylglycine. The subsequent hydrolysis of *N*-phosphorylglycine to glycine and inorganic phosphate is anticipated to be 30-fold faster at 75° than the hydrolysis of II based on the structure–reactivity correlation for the hydrolysis of phosphoramidate monoester monoanions and the estimated pK_a of glycine (9.6, 75°).[18] Alternatively, the formation of phenol by sub-

(22) M. L. Bender and J. H. Lawlor, *J. Amer. Chem. Soc.*, **85**, 3010 (1963).
(23) R. Hofstetter, Y. Murakami, G. Mont, and A. E. Martell, *ibid.*, **84**, 3041 (1962).
(24) F. Lippman and C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).
(25) I. Oney and M. Caplow, *J. Amer. Chem. Soc.*, **89**, 6972 (1967).
(26) S. J. Benkovic and P. A. Benkovic, *ibid.*, **90**, 2646 (1968), and references cited therein.

sequent hydrolysis of phenyl phosphate as a result of P–N bond cleavage appears unlikely, since phenyl phosphate dianion hydrolyzes 10^6 fold less rapidly than II.[16]

The involvement of the neighboring carboxylate anion in the hydrolysis of II is supported by (1) the broad pH-independent region at pH >6, (2) the large rate enhancement for P–O fission observed at pH >7, *ca.* 10^4, even though amine expulsion in I is at least a factor of 10^2 faster than phenol,[18] and (3) the change in products resulting from the titration of a group (pK_a 4.1) as shown in Figure 2. A small deuterium solvent kinetic isotope effect observed at pH 6.6 for II, $k_H/k_D = 1.2$, suggests that a proton transfer is not involved in the rate-determining step. Furthermore, the entropy of activation in the pH-independent region is −15 eu. This $\Delta S^{\ddagger}$ value is identical with those reported for benzyl phosphoenolpyruvate[3] and phenyl-(2-carboxyphenyl)phosphate,[4] in which nucleophilic catalysis by a neighboring carboxyl and/or carboxylate group has been implicated. These collective data appear to be in accord with the mechanism shown in Scheme I.

SCHEME I



In an attempt to trap the cyclic acyl intermediate IIa, the solvolysis of II was conducted in 0.67 *M* hydroxylamine.[3] A small but detectable concentration of hydroxamic acid (8 ± 2% of the theoretical) was produced. However, the observed rate of phenol release was *ca.* tenfold greater (0.086 min^{-1}, 75°) in the presence of hydroxylamine than for the spontaneous hydrolysis. This finding may be rationalized in terms of preferential nucleophilic attack on phosphorus by hydroxylamine prior to formation of IIa. Competing intermolecular catalysis by hydroxylamine is further supported by the observation of similar processes at rates that would be competitive for both *O*-phosphate diesters and phosphoramidate monoesters in the presence of added nucleophiles.[27,28]

The results of the total hydrolysis of II in 8.1% ^{18}O-enriched acetate buffer (pH 5.8) are shown in Table II. These data are interpreted as indicating the incorporation of two oxygen atoms of solvent per molecule of inorganic phosphate and no incorporation of excess ^{18}O into the carboxyl moiety of glycine during the hydrolysis of II. A control experiment with glycine and inorganic phosphate revealed that no exchange with the solvent occurred with either product during the time of the hydrolysis.

(27) A. J. Kirby and M. Younas, *J. Chem. Soc. B*, 1165 (1970).
(28) G. W. Jameson and J. M. Lawlor, *ibid.*, 53 (1970).

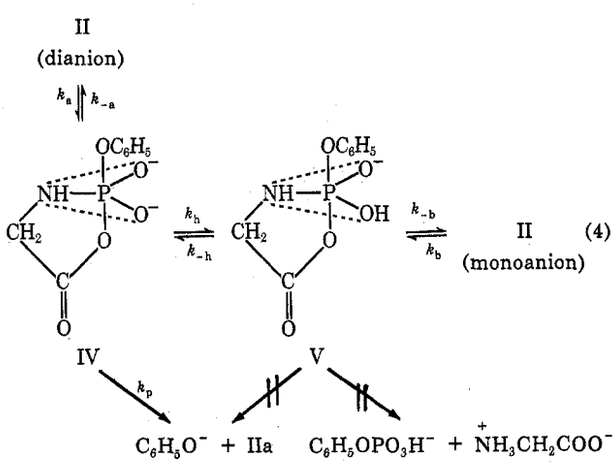
TABLE II
^{18}O TRACER STUDIES ON THE HYDROLYSIS OF II[a]

Compd	^{18}O excess	Atoms of solvent incorporated per molecule of product[b]
II (pH 5.8)	3.8 (in KH_2PO_4)	1.9[c]
II (pH 5.8)	0.0 (in glycine)	0
II + Zn^{2+} (0.5 *M*, pH 5.8)	3.6 (in KH_2PO_4)	1.8[c]
KH_2PO_4 (pH 5.8)	0.0	0
Glycine (pH 5.8)	0.0	0
Glycine (pH 2.9)	0.0	0

[a] 8.1% ^{18}O-enriched buffer. [b] Error bounds ±7%. These values were corrected for the natural abundance of ^{18}O. [c] These results suggest that 2.0 atoms of solvent are incorporated per molecule of inorganic phosphate. Similar small discrepancies in ^{18}O tracer studies have been observed in other cases, *e.g.*, P. C. Haake and F. H. Westheimer, *J. Amer. Chem. Soc.*, **83**, 162 (1961)

The incorporation of two atoms of solvent per molecule of inorganic phosphate may be viewed as occurring in two consecutive hydrolysis steps. Ring closure followed by preferential attack by water on the phosphoryl center of IIa would introduce the first atom of solvent. Incorporation of the second atom of solvent presumably occurs during the subsequent hydrolysis of *N*-phosphorylglycine. We have previously shown that P–N bond cleavage in the hydrolysis of *N*-(*n*-butyl)phosphoramidate results in the incorporation of only one oxygen atom of solvent per molecule of inorganic phosphate.[18a] These results are in accord with the mechanism in Scheme I.

Although Scheme I is written without explicitly invoking pentacoordinate intermediates, previous studies have implicated their presence.[3,4] Furthermore, their decomposition in this case is anticipated to be rate limiting, since expulsion of carboxylate or carboxyl should be orders of magnitude greater than amine or phenolate. The two probable species, in accord with the preference rules, are IV and V.[5]



The hydrolysis of the monoanion through preequilibrium formation of species V and IV followed by the latter's decomposition is kinetically indistinguishable from hydrolysis of the dianion. However, the calculated rate coefficient, k_p, for collapse of IV to IIa and phenoxide greatly exceeds that for either deprotonation or protonation of V, *i.e.*, k_{-h} and $k_h[H^+]$, invali-

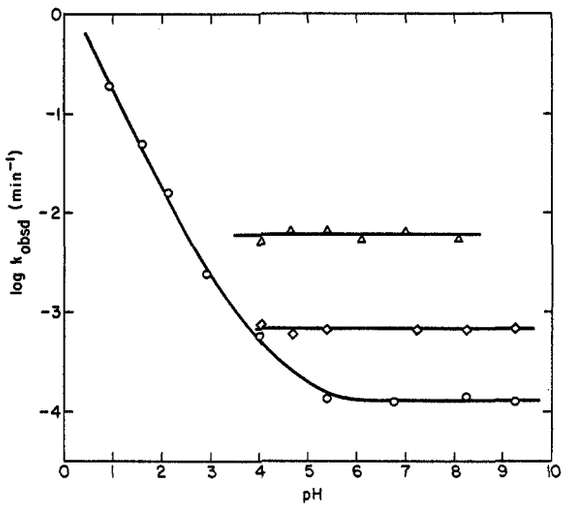


Figure 3.—The log k_{obsd}–pH rate profile for the spontaneous (O), Mg^{2+} (10^{-2} M) catalyzed (◇), and Zn^{2+} (10^{-2} M) catalyzed (△) hydrolysis of II (10^{-3} M) at 35°, μ 0.2.

dating the required preequilibrium assumption.[18b] It is also unlikely that the proton transfer step to solvent is in itself rate determining in view of the high sensitivity of the k_3 term to changes in the para substituent of the phenol.[4]

At pH 2.9 the observed kinetic terms k_1 and k_2 assigned to the neutral and monoanionic species of II comprise 90% of k_{obsd}. However, the appearance of glycine and phenyl phosphate as products upon protonation of II is not accompanied by incorporation of ^{18}O into glycine at pH 2.9, which would be diagnostic of an intermediate acyclic acyl phosphate arising from carboxyl attack which then decomposes *via* C–O bond cleavage. Hence V is either not in prototropic equilibrium with IV as suggested above or the former reacts mainly through k_{-b}. The formation of glycine and phenyl phosphate as products therefore may be assigned to the operation of a competing intermolecular pathway owing to the increased reactivity of the P–N relative to the P–O bond toward acid-catalyzed hydrolysis. Species similar to V, however, are competent in neighboring carboxyl catalysis in *O*-phosphate diester hydrolyses.[3,4]

The pH–rate profiles for the metal ion catalyzed hydrolysis of II in the presence of Zn^{2+} and Mg^{2+} at a [metal]/[substrate] = 10 are shown in Figure 3. Catalysis by such metal ions has not been observed with phosphate diesters previously and was not observed with I. Throughout the pH region of interest the reactive forms of the metal ions presumably are $[Zn(H_2O)^6]^{2+}$ and $[Mg(H_2O)^6]^{2+}$. As before, phenol was released quantitatively.

Plots of log k_{obsd} *vs.* $[M_T]$ for both Zn^{2+} and Mg^{2+} ions are shown in Figure 4. The theoretical curves are calculated from eq 6 based on a scheme assuming preequilibrium formation of a reactive 1:1 ester–metal ion complex ME, where $[ME] \ll [M^{2+}]$. Given eq 5 it

$$M^{2+} + E \underset{}{\overset{K}{\rightleftharpoons}} ME \xrightarrow{k_M} \text{products} + M^{2+} \qquad (5)$$
$$\underset{k_0}{\longleftarrow}$$

may be shown that k_{obsd} is described by eq 6, where $[M_T]$ is the initial stoichiometric concentration of

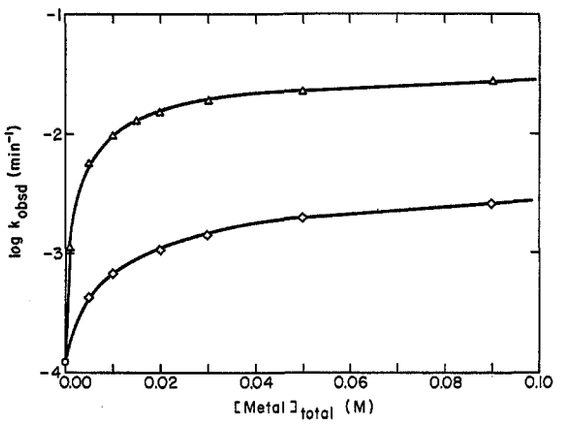


Figure 4.—Plot of log k_{obsd} *vs.* $[M_T]$ for the metal ion catalyzed hydrolysis of II (10^{-3} M), at 35°, μ 0.2, pH 5.5, Mg^{2+} (◇), and Zn^{2+} (△). $[M_T]$ is the initial stoichiometric concentration of the metal ion.

TABLE III
RATE AND ASSOCIATION CONSTANTS FOR THE METAL ION CATALYZED HYDROLYSIS OF II (35°, μ 0.2)

Metal ion	k_M[a] × 10^3, min^{-1}	k_0[b] × 10^4, min^{-1}	K,[c] M^{-1}
Zn	36	1.2	35
Mg	4.8	1.2	13.5

[a] k_M is the first-order rate constant associated with the hydrolysis of the ester–metal ion complex. [b] k_0 is the first-order rate constant for the spontaneous hydrolysis of II. [c] K is the association constant for formation of the ester–metal ion complex.

metal ion; k_0, k_M, and K are defined in Table III. The values of k_{obsd} calculated from eq 6 utilizing the

$$k_{obsd} = \frac{k_0 + K[M_T]k_M}{1 + K[M_T]} \qquad (6)$$

rate constants and dissociation constants in Table III are in satisfactory agreement with the experimentally determined points (see Figure 4). The values of K suggest metal–oxygen ion complexes, implying that ME may represent an initial metal ion–carboxylate complex.[29]

The ^{18}O data (Table II) confirm that two atoms of solvent are incorporated per molecule of inorganic phosphate product during the Zn^{2+}-catalyzed hydrolysis of II. We interpret this finding as indicating that the integrity of the mechanism of bond breaking and bond formation at the phosphorus center of II is unaffected by the presence of metal ions (see above).

The spontaneous and Cu(II) ion catalyzed hydrolysis of salicyl phosphate has been partially reexamined particularly in view of an earlier postulation which featured nucleophilic attack by carboxylate on phosphorus in the presence of the metal ion.[23] This mechanism contrasts with the generally accepted view of general acid catalysis by carboxyl in the spontaneous reaction.[22] Such a scheme requires the intermediacy of a cyclic acyl phosphate (salicyloyl phosphate) and/or an acyclic acyl phosphate (*O*-hydroxybenzoyl phosphate). Either species should lead to a salicyl hydroxamate in the presence of hydroxylamine. The observed rate constants, 2.8×10^{-7} and 3.5×10^{-6} min^{-1} [5×10^{-3} M Cu(II)], are in satisfactory agree-

(29) L. Sillen and A. E. Martell, "Stability Constants of Metal-Ion Complexes," Special Publication 25, Chemical Society, London, 1964.

ment with earlier measurements.[22,23] However, no hydroxamate could be detected during the course of the metal ion catalyzed hydrolysis. The observation of hydroxamate products in other related di- and triester phosphate hydrolyses catalyzed by neighboring carboxyl or carboxylate groups warrants its presence.[30] From this result one may infer that nucleophilic catalysis by carboxylate is limited to di- and triester systems, as for II, and that the occurrence of metal ion catalysis in salicyl phosphate hydrolysis may be attributed to an amplification of the general acid catalysis observed in the absence of metal ion.

One may postulate several alternative mechanisms for the role of the metal ion in the catalysis of II. The metal ion may serve to neutralize the negatively charged phosphoryl oxygen, thereby reducing the electrostatic repulsion encountered by the carboxylate anion, and facilitating displacement. The importance of this effect, however, is apparently a factor of tenfold. This estimate is based on the ratio of the rate constants for phenoxide expulsion by carboxylate from the triester, diphenyl(2-carboxyphenyl) phosphate, and the diester, phenyl(2-carboxyphenyl) phosphate, the latter as the dianion, after correction for differences in the sensitivity of phosphorus to nucleophilic attack in the two systems.[31] Alternatively, the metal ion may act as an effective acid catalyst, lowering the pK_a of the departing phenol.[6] The structure–reactivity correlation for the hydrolysis of substituted aryl(2-carboxyphenyl) phosphates reveals a very high dependency (β −1.26) on the basicity of the leaving phenol.[4] Therefore, a change of 2 pK_a units in the pK_a of the leaving phenol, owing to chelation of the metal ion with the ether oxygen, would rationalize the rate acceleration. However, the pK_a of the stronger La^{3+}–phenolate complex is only 2 units below that for phenol, implying that this rationale is not entirely satisfactory.[29] A third and final argument invokes stabilization of the possible intermediate pentacovalent species by the metal ion and the associated transition states leading to and from this species. The plausibility of this latter suggestion will be the subject of a future communication.

Model systems which feature intramolecular catalysis or catalysis by biologically important Zn^{2+} or Mg^{2+} ions are of particular interest, since the interactions involved may closely resemble those in an enzyme–substrate complex.[32] The results of this study indicate that both of these types of catalysis may be integrated into one model system to confer dramatic reactivity to a normally unreactive phosphate diester.

Registry No.—I, 38401-04-6; II, 28401-05-7; diphenylphosphorochloridate, 2524-64-3; glycine ethyl ester hydrochloride, 623-33-6; diphenyl *N*-(glycyl)-phosphoramidate, 38401-06-8.

Acknowledgment.—This work was supported by a grant from the National Institutes of Health, GM 13306.

(30) S. J. Benkovic in "Comprehensive Chemical Kinetics," C. H. Banford and C. F. Tipper, Ed., American Elsevier, New York, N. Y., 1972.

(31) R. H. Bromilow, S. A. Khan, and A. J. Kirby, *J. Chem. Soc., Perkin Trans. 2*, 911 (1972).

(32) G. J. Lloyd and B. S. Cooperman, *J. Amer. Chem. Soc.*, **93**, 4883 (1971). These authors recently have described a model system which features phosphoryl transfer from phosphoryl imidazole to the Zn^{2+}–pyridine-2-carbaldoxime anion *via* a ternary complex.

Phosphorus Derivatives of Nitrogen Heterocycles. 3. Carbon–Phosphorus Bonding in Pyridyl-2- and -4-phosphonates[1]

DEREK REDMORE

Tretolite Division, Petrolite Corporation, St. Louis, Missouri 63119

Received September 1, 1972

A postulate that the extent of d_π–p_π conjugation for a phosphorus substituent on a pyridyl ring is greater for attachment at the 4 position than at the 2 position has been examined in a series of pyridyl-2- and -4-phosphonates by measurement of several physical properties. Although the ^{31}P nmr spectra of the pyridylphosphonate esters suggest the presence of d_π–p_π conjugation for attachment at the 4 position, ultraviolet and mass spectra of these esters and pK_a determinations on the corresponding acids argue strongly against such conjugation. The general conclusion that all the pyridylphosphonates show an absence of d_π–p_π conjugation is based on a comparison of physical properties with those of phenylphosphonates, a system in which d_π–p_π conjugation has been shown to be absent by other workers.

There exists considerable current interest concerning the extent of d_π–p_π bonding in the C–P bond of phosphorus substituents attached to aryl and heteroaryl rings.[2] From the ultraviolet and proton magnetic resonance spectra it has been concluded that d_π–p_π bonding exists in the C–P bonds of furan, thiophene, and pyrrole derivatives but that it is probably absent in pyridine derivatives. Although the spectra for pyridyl-2-phosphonates support this view, the corresponding pyridyl-4-phosphonates give indications of some d_π–p_π interaction.[2,3] To examine this possibility a more detailed examination has been made of the ^{31}P nmr spectra of the esters and acids, the mass spectra of esters, and pK_a and uv measurements for pyridyl phosphonic acids.

P^{31} Nmr Spectra.—The magnitude of the ^{31}P chemical shift of the phosphonate group can be correlated with the electron-donating ability of the attached organic radical.[4] It should be possible, therefore, to

(1) This work was presented, in part, at the 7th Midwest Regional Meeting of the American Chemical Society, St. Louis, Mo., Oct 1971. The present interpretation of the data differs considerably from this earlier presentation.

(2) D. Redmore, *Chem. Rev.*, **71**, 315 (1971).

(3) D. Redmore, *J. Org. Chem.*, **35**, 4114 (1970).

(4) J. G. Riess, J. R. Van Wazer, and J. Letcher, *J. Phys. Chem.*, **71**, 1925 (1967); C. C. Mitsch, L. D. Freedman, and C. G. Moreland, *J. Magn. Resonance*, **3**, 446 (1970).