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Electrophilic Catalysis. The Hydrolysis of Phosphoramidate[1]

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The hydrolysis of phosphoramidate is catalyzed by formaldehyde and hypochlorous acid. The formation of methyl phosphate and, in low yield, pyrophosphate is also induced by these compounds, in the presence of the appropriate phosphoryl acceptor. Evidence is presented that these reactions are examples of electrophilic catalysis, in which the catalyst forms a covalent bond with the substrate to give an intermediate with a better leaving group than the starting material. Formaldehyde does not accelerate the reaction of phosphoramidate with pyridine and neither formaldehyde nor hypochlorite catalysis results in a reaction with fluoride, in spite of the fact that pyridine and fluoride are effective nucleophilic reagents in uncatalyzed reactions with phosphoramidate. Although these results suggest that the catalyzed reactions involve an intermediate of high reactivity and low selectivity, similar to the monomeric metaphosphate monoanion, product analyses indicate that this hypothetical compound cannot exist as a free intermediate in these reactions. A nitrous acid induced hydrolysis of phosphoramidate is also described.

Nucleophilic catalysis, in which the catalyst forms an unstable intermediate by nucleophilic attack upon the substrate, is well known for reactions of acyl, carbonyl, and phosphoryl groups. Electrophilic catalysis, in which the catalyst is *subjected* to nucleophilic attack by the substrate and thereby forms a more reactive intermediate compound, is less well known. Examples of this type of catalysis include the facilitation of aromatic bromination by a second molecule of bromine,[2] the catalysis of hydrogen peroxide oxidations by carboxylic acids with the intermediate formation of peroxy acids,[3] certain pyridoxal-catalyzed reactions,[4] catalysis of the decomposition of hexamine by aldehydes,[5] and, possibly, catalysis by carbon dioxide of amide bond formation from dimethyl glutamate and N-carboxyamino acid anhydrides.[6] The pyridoxal phosphate-catalyzed decomposition of diethyl aminomalonate is a particularly interesting example,[7] which may have some mechanistic similarity to the formaldehyde-catalyzed reactions reported here. General and specific acid catalysis and catalysis by metal ions are special cases of electrophilic catalysis and, in fact, electrophilic catalysis may be viewed in its broadest sense as a form of catalysis by Lewis acids. Activation by oxidation also may be regarded as electrophilic activation and a number of systems in which phosphate is activated by oxidative processes are known.[8] A reversible oxidation would provide a powerful mechanism for enzymic electrophilic catalysis, but most examples of activation by oxidation are irreversible and, therefore, are not examples of catalysis. Electrophilic catalysis is of great potential utility for enzymes, since it provides a mechanism for increasing the susceptibility of a substrate to nucleophilic attack without requiring the enzyme to concentrate hydrogen ions and place them on the substrate. The experiments reported here describe evidence for electrophilic activation, and, in some cases, electrophilic catalysis of phosphoryl hydrolysis and transfer reactions of phosphoramidate (aminophosphate) by formaldehyde, hypochlorous acid, and nitrite.

(1) Supported by grants from the National Science Foundation and the National Cancer Institute of the National Institutes of Health (CA-03975).

(2) P. B. D. De La Mare and J. H. Ridd, "Aromatic Substitution," Academic Press, Inc., New York, N. Y., 1959, p. 116.

(3) W. D. Emmons, *J. Am. Chem. Soc.*, **76**, 3468, 3470 (1954); W. D. Emmons, A. S. Pagano, and J. P. Freeman, *ibid.*, **76**, 3472 (1954).

(4) E. E. Snell, in "The Mechanism of Action of Water-Soluble Vitamins," A. V. S. de Reuck and M. O'Connor, Ed., Little, Brown and Co., Boston, Mass., 1961, p. 18, and references therein.

(5) H. Tada, *J. Am. Chem. Soc.*, **82**, 255 (1960).

(6) D. G. H. Ballard and C. H. Bamford, *Nature*, **172**, 907 (1953); *Proc. Roy. Soc.* (London), **A223**, 495 (1954); A. Hubert, R. Buijle, and B. Hargitay, *Nature*, **182**, 259 (1958).

(7) J. W. Thanassi and J. S. Fruton, *Biochem.*, **1**, 975 (1962).

(8) W. P. Jencks, *Brookhaven Symp. Biol.*, **15**, 134 (1962).

Experimental

Materials.—Potassium phosphoramidate was prepared by the method of Stokes[9] and was stored at −15°. Solutions were made up just prior to use or were brought to a pH of 9 to 10 and were stored a few days at 3° before use. Reagent grade formaldehyde was standardized by titration with iodine and thiosulfate. Hypochlorous acid was prepared from calcium hypochlorite[10] and was stored at 3°. A concentrated solution was obtained by the addition of 4.4 *M* nitric acid to an equal volume of 2 *M* calcium hypochlorite at 0°, followed by distillation at 28° (20 mm.) into a chilled receiving flask; only the first fraction was collected and it was used immediately.

Methods.—The determination of inorganic phosphate release upon the hydrolysis of phosphoramidate is difficult because of the rapid hydrolysis of phosphoramidate under the conditions of most phosphate determinations.[11] In most experiments the measurement of inorganic phosphate appearance was followed by the following modification of the Martin and Doty[12] procedure: the aliquot to be analyzed (0.1 to 1.0 μmole) is made up to a volume of 5 ml. with water and 5.0 ml. of isobutyl alcohol is added. Ammonium molybdate, 1.0 ml. of a 2% solution in 2 *N* sulfuric acid, is added and the mixture is immediately shaken vigorously for exactly 5 sec. The layers are allowed to separate and 2.0 ml. of the isobutyl alcohol layer is added to 8.0 ml. of 2% sulfuric acid in absolute ethanol. To this solution is added 0.2 ml. of freshly diluted stannous chloride reagent, prepared by a 1:200 dilution of a stock solution of 10 g. of $SnCl_2 \cdot 2H_2O$ in 25 ml. of concentrated hydrochloric acid which is stored at 3°. The tubes are mixed thoroughly and the color is read at 660 mμ after 10 min. Even with this short period of shaking, about 5% of the phosphoramidate is hydrolyzed and appears as inorganic phosphate. However, for experiments which follow first-order kinetics this amount of hydrolysis is not a problem and the method was found to be adequate, although not ideal, for rate measurements. Phosphate was also determined by the Fiske and Subbarow method,[13] which measures the sum of inorganic phosphate and phosphoramidate, but not fluorophosphate, pyrophosphate, or methyl phosphate, and by precipitation of inorganic phosphate by magnesia mixture.[14] The reaction of phosphoramidate with fluoride may be followed conveniently by analysis by the Fiske and Subbarow method. In the presence of excess fluoride the disappearance of phosphoramidate follows pseudo-first-order kinetics with a rate constant equal to the sum of the rate constants for

(9) N. H. Stokes, *Am. Chem. J.*, **15**, 198 (1893); R. Klement and K. H. Becht, *Z. anorg. Chem.*, **254**, 217 (1947); R. W. Chambers and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 3749 (1958).

(10) N. G. Lordi and J. Epstein, *ibid.*, **80**, 509 (1958).

(11) T. Rathlev and T. Rosenberg, *Arch. Biochem. Biophys.*, **65**, 319 (1956).

(12) J. B. Martin and D. M. Doty, *Anal. Chem.*, **21**, 965 (1949).

(13) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

(14) L. F. Leloir and C. E. Cardini in "Methods in Enzymology," Vol. III, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, Inc., New York, N. Y., 1957, p. 840.

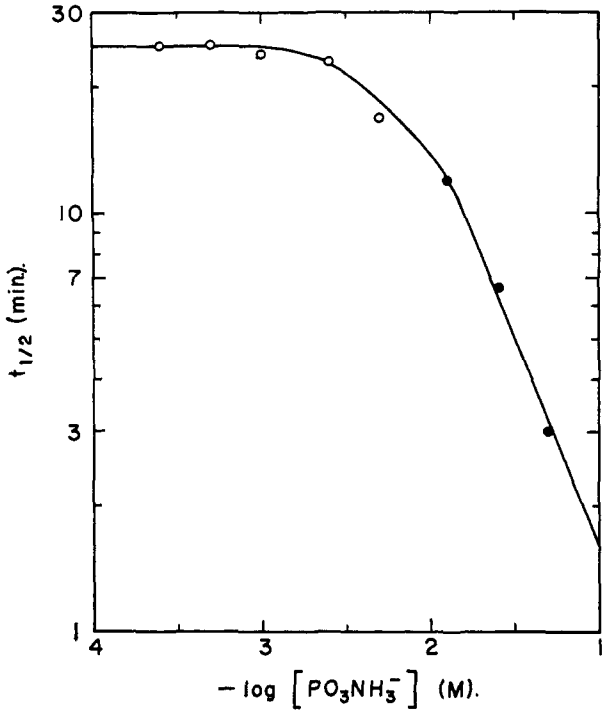


Fig. 1.—Logarithmic plot of the half-time for formaldehyde-catalyzed phosphoramidate hydrolysis against phosphoramidate concentration at 39°, ionic strength 1.0, pH 4.5: ●, rates determined by automatic titration; ○, rates determined by measurement of inorganic phosphate.

hydrolysis and reaction with fluoride; the ratio of these two rate constants is obtained from the ratio of inorganic phosphate to fluorophosphate at the end of the experiment. Inorganic pyrophosphate was determined by precipitation as the manganese salt, followed by acid hydrolysis and analysis by the Fiske and Subbarow method.[13] For the analysis of inorganic phosphate in the presence of chlorinated phosphoramidate an aliquot of the reaction mixture was added to 4 ml. of 0.025 *M* ammonia and phosphate was determined by the modified Martin and Doty procedure. The determination of inorganic phosphate in the presence of labile phosphate derivatives may be conveniently carried out by addition of an aliquot of the reaction mixture to a large excess of an alcohol and determination of inorganic phosphate after solvolysis of other phosphate derivatives. This method was used for the determination of the pseudo-first-order rate constant for the appearance of inorganic phosphate in the presence of nitrite. Aliquots of the reaction mixture (0.1 ml.) were added to ethylene glycol (0.9 ml.). The tubes were placed in a boiling water bath for 5 min. to decompose remaining phosphoramidate and the samples were analyzed for inorganic phosphate by the Fiske and Subbarow method in a final volume of 10 ml.

Inorganic pyrophosphate and triphosphate were identified by comparison with known compounds on paper electrophoresis in 0.04 *M* sodium acetate–0.01 *M* acetic acid buffers as described previously.[16]

Rate measurements were also carried out by automatic titration at constant pH with a Radiometer TTT1c titrator and titrigraph and by determination of ammonia liberation by the Conway diffusion technique into boric acid, followed by titration. Pseudo-first-order rate constants were obtained from plots of the extent of the reaction, $x_\infty - x_t$, against time on semilogarithmic graph paper and the relation $k = 0.693/t_{1/2}$.

Other pH measurements and titrations were carried out with a Radiometer PHM 4b pH meter equipped with a G200b glass electrode. Spectrophotometric measurements were carried out with a Zeiss PMQ II spectrophotometer equipped with a brass cell holder through which water from a thermostat was circulated.

Results

The Formaldehyde Reaction.—In the course of attempts to titrate phosphoramidate in the presence of

(15) A. Kornberg, *J. Biol. Chem.*, **182**, 779 (1950).
(16) G. Di Sabato and W. P. Jencks, *J. Am. Chem. Soc.*, **83**, 4393 (1961).

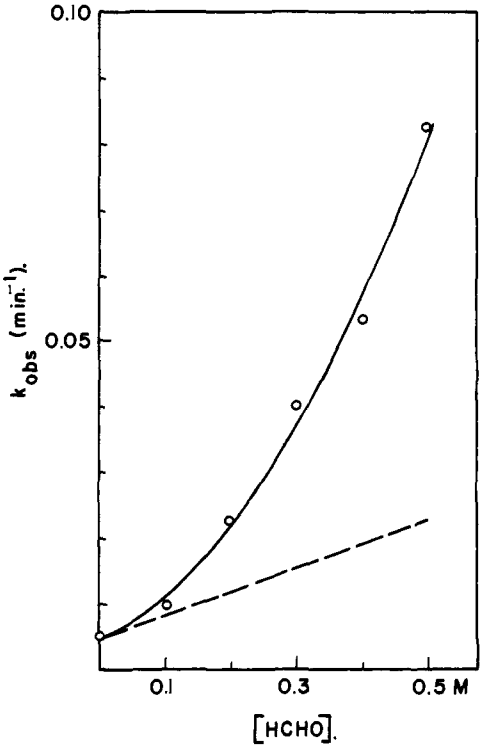


Fig. 2.—Dependence on formaldehyde concentration of the rate of phosphoramidate hydrolysis in 0.1 *M* acetate buffer, pH 4.62, ionic strength 1.0, at 39°: ———, calculated for the rate law of eq. 1; - - - -, calculated omitting the term second-order in respect to formaldehyde.

formaldehyde it was found that formaldehyde induces a rapid hydrolysis of phosphoramidate. For example, the half-time for the hydrolysis of phosphoramidate is decreased from 140 to 5 min. by the presence of 0.2 *M* formaldehyde at pH 7.3. The kinetics of the formaldehyde-catalyzed hydrolysis, which were studied under conditions in which formaldehyde was present in great excess over phosphoramidate, are complex: (1) At phosphoramidate concentrations of less than 10^{-3} *M* the disappearance of phosphoramidate follows pseudo-first-order kinetics at a given formaldehyde concentration. At higher concentrations the reaction is more than first order in respect to phosphoramidate. As shown in Fig. 1, a logarithmic plot of the half-time of the reaction against phosphoramidate concentration shows a half-time independent of phosphoramidate concentration, characteristic of a first-order reaction, at low phosphoramidate concentrations and a slope of 1.0, characteristic of a second-order reaction, at high phosphoramidate concentration.[17] Thus, the rate law contains terms both first and second order in respect to phosphoramidate.

(2) The reaction also contains terms both first and second order in respect to formaldehyde (Fig. 2). The rate constant (examined at low phosphoramidate concentration so that the reaction is pseudo first order in respect to phosphoramidate disappearance) increases more rapidly than the first power of the formaldehyde concentration and follows the rate law

$$k_{obsd} = 0.005 + 0.037[HCHO] + 0.23[HCHO]^2 \quad (1)$$

The calculated rate according to this rate law is shown as the solid line in the figure, while the part of the re-

(17) A. A. Frost and R. G. Pearson, "Kinetics and Mechanism," John Wiley and Sons, Inc., New York, N. Y., 1953, p. 41.

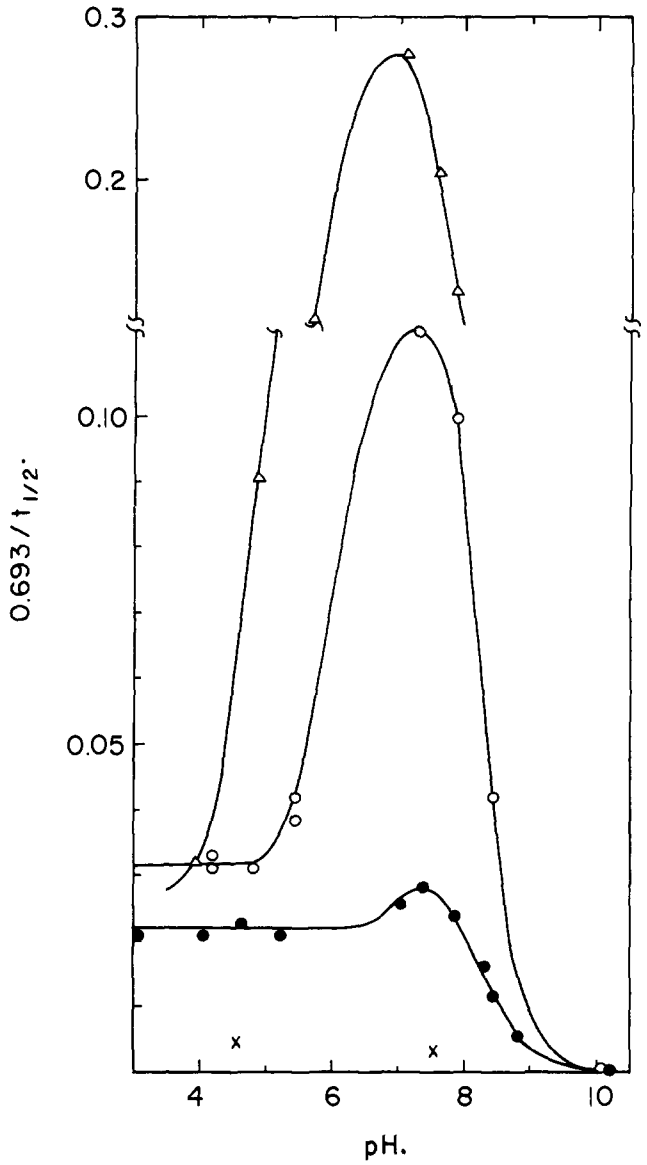


Fig. 3.—Dependence on pH of the hydrolysis of phosphoramidate catalyzed by 0.2 *M* formaldehyde at 39°, ionic strength 1.0: ●, 10^{-3} *M* phosphoramidate; ○, 5×10^{-3} *M* phosphoramidate; Δ, 10^{-3} *M* phosphoramidate plus 0.05 *M* ammonium chloride; ×, same, but no formaldehyde; acetate buffers, 0.1 *M*, pH 4–6; N-methylmorpholine buffers, 0.1 *M*, pH 7–9; triethylamine buffer, 0.1 *M*, pH 10.

action which is first order in respect to formaldehyde is shown by the dashed line.

(3) At low phosphoramidate concentration the reaction is independent of pH below pH 7, in the region in which phosphoramidate exists as the monoanion, and drops off above pH 8 as the phosphoramidate is converted to the dianion (Fig. 3, closed circles). There is a small hump in the pH–rate profile at pH 7. At higher phosphoramidate concentration this hump is greatly magnified and the rate of the pH-independent reaction at lower pH values is also slightly increased (Fig. 3, open circles). Thus the reaction which is second order in respect to phosphoramidate shows a sharp pH–rate maximum, while the reaction which is first order in respect to phosphoramidate occurs at a rate proportional to the fraction of phosphoramidate present as the monoanion.

(4) In the presence of formaldehyde, the reaction is markedly accelerated by ammonium ion and shows a

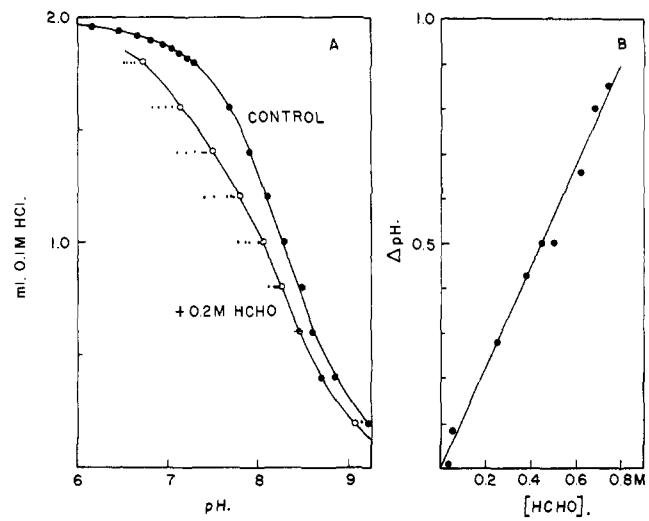


Fig. 4.—Effect of formaldehyde on the titration curve of phosphoramidate at 25°, extrapolated to zero time: A, effect of 0.2 *M* formaldehyde. The small circles are the experimental points used for extrapolation to zero time (open circles); B, The effect of formaldehyde concentration on the pH of a half-neutralized solution of 0.02 *M* phosphoramidate.

pH–rate maximum similar to that observed at high concentrations of phosphoramidate (Fig. 3, triangles). In the absence of formaldehyde, ammonium ion has no effect on the hydrolysis rate (Fig. 3, crosses). This behavior strongly suggests that ammonium ion can take the place of the second molecule of phosphoramidate to accelerate the hydrolysis rate in the reaction which occurs with a pH–rate maximum.

On the basis of this information, the following rate law is proposed for the formaldehyde-catalyzed hydrolysis of phosphoramidate

$$\text{rate} = k_{\text{hyd}}[\text{PA}^-] + k_1[\text{PA}^-][\text{HCHO}] + k_2[\text{PA}^-][\text{HCHO}]^2 + k_3[\text{PA}^-][\text{HCHO}]^a[\text{RNH}_3^+] \quad (2)$$

In this equation, RNH_3^+ may be either a second mole of phosphoramidate or a mole of added ammonium ion. The order of the k_3 term in respect to formaldehyde is not known, as indicated by the exponent a, and it is probable that the k_3 term is really two terms, first and second order in respect to formaldehyde, as was shown for the reactions at low phosphoramidate concentration. Only the k_{hyd}, k_1, and k_2 terms have been evaluated quantitatively (eq. 1). Equation 2 accounts for the increase of the k_3 term with decreasing pH, as the phosphoramidate is converted from the dianion to the monoanion, but it does not account for the decrease in rate on the acid side of the pH–rate maximum. However, a reasonable explanation for this rate decrease emerges from a consideration of the probable mechanism of the reaction, as discussed below.

Evidence for a rapid combination of formaldehyde with phosphoramidate was obtained by rapid titration experiments (Fig. 4). Although a conventional formol titration curve could not be obtained because of the rapid formaldehyde-induced hydrolysis of phosphoramidate, the effect of formaldehyde on the titration curve of phosphoramidate was evaluated by measurements of the pH as a function of time after the addition of formaldehyde to a partially neutralized solution of phosphoramidate and extrapolation of the readings

to zero time. It is apparent that the titration curve is shifted to lower pH values by 0.2 *M* formaldehyde, with a larger shift at lower pH values (Fig. 4A). The decrease in the pH of a solution of half-neutralized phosphoramidate becomes larger as the formaldehyde concentration is increased and there is no tendency of the curve to level off as the formaldehyde concentration is increased to 0.8 *M* (Fig. 4B). Plots of the data according to Levy and Silberman[18] suggest that the association constants for the combination of formaldehyde with phosphoramidate are on the order of 2 and 6 for the reactions with 1 and 2 moles of formaldehyde, respectively. Although these should not be regarded as more than very approximate values, they do provide further support for the view that phosphoramidate exists in aqueous solution as the zwitterion and also indicate that under the experimental conditions of the rate measurements, only a small fraction of the phosphoramidate is combined with formaldehyde.

The formaldehyde-induced reaction is a true catalysis. A solution of 0.5 *M* phosphoramidate in the presence of 0.2 *M* formaldehyde (0.25 *M* N-methylmorpholine buffer, final pH 7.45, 38°) was one-third hydrolyzed after 1 min. and underwent further hydrolysis with a half-time of approximately 17 min.

Phosphoramidate reacts readily with pyridine[11,19] and with fluoride ion.[8,20] In the former case the product is hydrolyzed rapidly, so that hydrolysis is observed, while in the latter case fluorophosphate is the product.[21] However, formaldehyde does not significantly accelerate the reaction of phosphoramidate with either pyridine or fluoride. As shown in Fig. 5, formaldehyde induces almost exactly the same rate increase in the absence as in the presence of pyridine. Similarly, no fluorophosphate was formed during the formaldehyde-catalyzed hydrolysis of phosphoramidate in the presence of 0.2 *M* fluoride and 0.4 *M* formaldehyde at pH 5.5, although a 48% yield of fluorophosphate was obtained from the uncatalyzed reaction of phosphoramidate under the same conditions. It was shown that fluorophosphate does not react with formaldehyde under these conditions.

In the presence of methanol, methyl phosphate is a product of the formaldehyde-induced solvolysis of phosphoramidate. In 30 and 50% aqueous methanol at 39° the yields of methyl phosphate were found to be 50 and 70%, respectively. These are only slightly less than the yields of 54 and 73%, respectively, which were observed for the uncatalyzed solvolysis of phosphoramidate in the same solvent, in good agreement with the results of Chanley and Feageson.[19] In concentrated aqueous solution, pyrophosphate is a product of the formaldehyde-induced reaction of phosphoramidate. In the presence of 1.1 *M* formaldehyde, 5% of the initial (1.7 *M*) potassium phosphoramidate appeared as pyrophosphate after 30 min. at 39°; a somewhat larger yield of 15%, as well as smaller

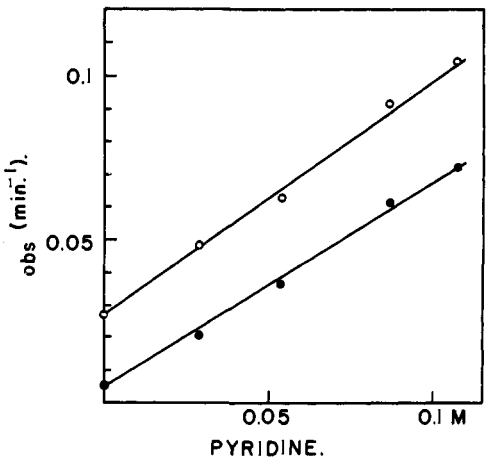


Fig. 5.—Effect of formaldehyde on the rate of pyridine-catalyzed hydrolysis of 2.5×10^{-3} *M* phosphoramidate in 0.1 *M* acetate buffer, pH 5.6, ionic strength 1.0, at 39°: O, 0.2 *M* formaldehyde; ●, no formaldehyde.

amounts of a material which migrates on paper electrophoresis with the same mobility as tripolyphosphate, was obtained after the slower, uncatalyzed hydrolysis of phosphoramidate. The formation of pyrophosphate during the solvolysis of phosphoramidate has been reported previously.[22]

Pyridoxal also catalyzes the hydrolysis of phosphoramidate, although less efficiently than formaldehyde. In the presence of 0.2 *M* pyridoxal at pH 3.7 and 39° the pseudo-first-order rate constant for the hydrolysis of phosphoramidate was found to be 0.010 min.$^{-1}$, compared to the value of 0.005 min.$^{-1}$ for the uncatalyzed reaction.

The Hypochlorite Reaction.—Hypochlorite causes an almost instantaneous hydrolysis of phosphoramidate in neutral or slightly acid solution, but not in alkaline solution. The following experimental observations are pertinent to a consideration of the mechanism of the reaction: (1) At alkaline pH equimolar amounts of hypochlorite and phosphoramidate react to give a product with an absorption maximum at 252 mμ (ε 200). This is presumably the monochlorophosphoramidate, O_3PNHCl^{-2}. Chloramine has a λ_{max} of 245 mμ (ε 416) and several monosubstituted chloramines have maxima at 253–254 mμ (ε 350–380).[23] Several N-chlorophosphoramidate esters with an aromatic substituent on the nitrogen atom have been prepared from the reaction of the corresponding phosphoramidate with *t*-butyl hypochlorite and are moderately stable in the absence of acid.[24] Upon acidification, the product of the reaction of phosphoramidate with hypochlorite undergoes hydrolysis to inorganic phosphate with a half-time of less than 1 min. It may be differentiated from inorganic phosphate under the conditions used for phosphate determinations if an aliquot is added to a solution of 0.025 *M* ammonia before the addition of molybdate and rapid extraction with isobutyl alcohol. In alkaline solution the product decomposes slowly over several days.

The rate of formation of this compound, followed at 245 mμ in the presence of excess phosphoramidate, follows pseudo-first-order kinetics. The pseudo-first-

(18) M. Levy and D. E. Silberman, *J. Biol. Chem.*, **118**, 723 (1937); D. French and J. T. Edsall, *Advan. Protein Chem.*, **2**, 277 (1945).

(19) J. D. Chanley and E. Feageson, *J. Am. Chem. Soc.*, **85**, 1181 (1963).

(20) M. Halmann, A. Lapidot, and D. Samuel, *J. Chem. Soc.*, 1299 (1963).

(21) Halmann, *et al.*,[20] describe the reaction of fluoride with phosphoramidate as an acceleration of hydrolysis, but also refer to a nucleophilic attack of fluoride on phosphoramidate. The reaction must be predominantly a nucleophilic reaction of fluoride with phosphoramidate because fluorophosphate is the principal product of the reaction and fluoride causes an increase in the rate of phosphoramidate disappearance.[8]

(22) O. T. Quimby, A. Narath, and F. H. Lohman, *J. Am. Chem. Soc.*, **82**, 1099 (1960).

(23) W. S. Metcalf, *J. Chem. Soc.*, 148 (1942).

(24) J. I. G. Cadogan and W. R. Foster, *ibid.*, 3076 (1961).

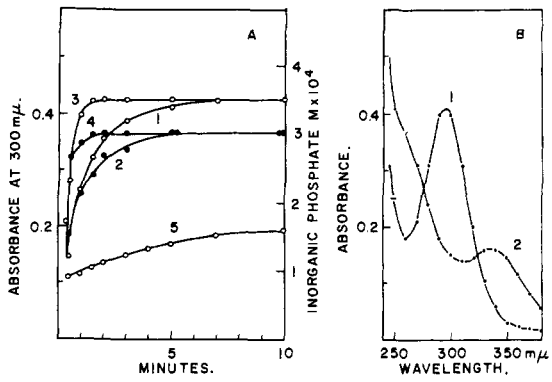


Fig. 6.—The hydrolysis of 3×10^{-4} M phosphoramidate induced by 6.6×10^{-4} M hypochlorous acid at pH 3.0, 25°. A, time course of the reaction: O, absorbance at 300 mμ (left scale); ●, appearance of inorganic phosphate (right scale). Curves 1 and 2, phosphoramidate alone; curves 3 and 4, same, plus 10^{-3} M potassium chloride; curve 5, ammonia instead of phosphoramidate. Solutions adjusted to final pH of 3.0 with perchloric acid. B, spectra of the products of the reaction of phosphoramidate (curve 1) and ammonia (curve 2).

order rate constants are proportional to the concentration of phosphoramidate and inversely proportional to the concentration of hydroxide ion (Table I). The results may be satisfactorily accounted for by the rate law of eq. 3 with $k_2 = 2.1 \times 10^8$ M^{-1} min.$^{-1}$ (based

$$\text{rate} = k_2[O_3PNH_2^{-2}][HOCl] \qquad (3)$$

on a value of 4.1×10^{-8} for the dissociation constant of hypochlorous acid[25]). The formation of chloramine from ammonia follows the rate law of eq. 3 with a similar value of $k_2 = 3.7 \times 10^8$ M^{-1} min.$^{-1}$; chlorination of more basic alkylamines proceeds somewhat more rapidly: for methylamine,[26] $k_2 = 3.0 \times 10^{10}$; dimethylamine,[26,27] $k_2 = 3.0 \times 10^{10}$ or 3.8×10^9; and diethylamine,[27] $k_2 = 1.25 \times 10^9$ M^{-1} min.$^{-1}$.

TABLE I

REACTION OF PHOSPHORAMIDATE WITH HYPOCHLORITE AT 25°

$[OCl^-]$, $M \times 10^4$	$[PO_3NH_2^{-2}]$, $M \times 10^3$	[KOH], M	k_{obsd},[a] min.$^{-1}$	$k_2 \times 10^{-8}$,[b] M^{-1} min.$^{-1}$
4.0	2.0	0.15	0.70	2.2
4.0	2.0	.10	1.04	2.1
4.0	2.0	.05	1.80	1.8
4.0	4.0	.05	4.20[c]	2.2
2.0	2.0	.05	2.10	2.2
4.0	2.0	.05	2.00[d]	2.0

[a] Pseudo-first-order rate constant obtained from readings at 245 mμ in a 5-cm. cuvette over the first half-time, during which pseudo-first-order kinetics were followed accurately. [b] $k_{obsd} = k_2(O_3PNH_2^{-2})(H^+)/K_{HOCl}$. [c] Approximate value. [d] In 1.0 M KCl.

(2) At acidic pH the increase in absorbance at 245 mμ due to the formation of the monochloro derivative of phosphoramidate occurs at a rate which is too rapid to measure accurately. In the pH range 2 to 7 the reaction occurs with a half-time of less than 20 sec. This is followed by a slower decrease in absorbance at 245 mμ and an increase in absorbance at 300 mμ. The increase in absorption at 300 mμ is accompanied by the liberation of inorganic phosphate (Fig. 6A, curves 1 and 2). The product of this reaction has an absorption maximum at 295 mμ (ε 255) (Fig. 6B), which is characteristic of dichloramine (λ_{max} 297 mμ (ε 265)). Inorganic phosphate was shown to be the other product of the reaction by precipitation as the magnesium–ammonia complex and by placing an aliquot in 90% methanol, allowing the solution to stand for 1 to 3 hr., and analyzing for inorganic phosphate. Under these conditions a chlorinated phosphoramidate would be expected to give methyl phosphate as the predominant solvolysis product, but only inorganic phosphate was found.

The rate of dichloramine formation, measured at 300 mμ, and the rate of inorganic phosphate liberation are increased markedly by 1.4×10^{-3} M chloride ion (Fig. 6A, curves 3 and 4). This suggests that chlorine, formed from the reaction of chloride with hypochlorous acid, is a more active chlorinating species for the addition of a second mole of chlorine than is hypochlorous acid. The addition of both chlorine atoms must occur before the hydrolysis of the substrate because (a) inorganic phosphate liberation does not occur until 2 moles have reacted and (b) the observed dichloramine formation does not involve a rapid hydrolysis followed by chlorination of ammonia because the chlorination of ammonia is sluggish under these conditions and gives a mixture of products which includes trichloramine (λ_{max} 340 mμ) rather than the dichloramine which is a principal product of the reaction with phosphoramidate (Fig. 6A, curve 5; Fig. 6B, curve 2).[23,28] The rates of dichloramine and inorganic phosphate formation from phosphoramidate were found to be the same at pH 2.6, 2.8, and 3.0 ($t_{1/2}$ = 0.7 ± 0.1 min. under experimental conditions similar to those of Fig. 6), which indicates that the rate-determining step of the reaction is independent of pH over this range.

The reaction is slowed, but not stopped, by the addition of ammonium ion, which competes with phosphoramidate for reaction with hypochlorous acid. The reaction of 2.5×10^{-3} M phosphoramidate with 0.01 M hypochlorous acid in acetate buffer at pH 4.4 is complete in less than 2 min. at 39°, but proceeds with half-times of 20 and 6 min. in 0.2 and 0.005 M ammonium chloride, respectively. The hydrolysis is catalytic; the reaction of 0.012 M phosphoramidate with 0.006 M hypochlorous acid results in an immediate hydrolysis of 25% of the phosphoramidate, followed by a slower hydrolysis of the remaining phosphoramidate with a half-time of 14 min. These results indicate that the hydrolysis can occur by a cyclic process in which the various species of chlorinated ammonia can supply the chlorine which is required for phosphoramidate hydrolysis.

As in the case of the formaldehyde reaction, the hypochlorous acid-induced hydrolysis in 0.2 M potassium fluoride does not give rise to detectable amounts of fluorophosphate. In aqueous methanol, methyl phosphate is a product of the hypochlorite-induced reaction, but the yields are smaller than in the formaldehyde-induced reaction. In 30 and 50% methanol the yields of methyl phosphate in the former reaction were found to be 35 and 56%, respectively. The hypochlorous acid-induced solvolysis of concentrated solutions of phosphoramidate gives a low yield of inorganic pyrophosphate; the solvolysis of a 1.6 M solution in

(25) R. P. Mauger and F. G. Soper, *J. Chem. Soc.*, 71 (1946).
(26) I. Weil and J. C. Morris, *J. Am. Chem. Soc.*, **71**, 1664 (1949).
(27) C. R. Edmond and F. G. Soper, *J. Chem. Soc.*, 2942 (1949).
(28) R. E. Corbett, W. S. Metcalf, and F. G. Soper, *ibid.*, 1927 (1953).

the presence of 1.45 M hypochlorous acid resulted in the conversion of about 2% of the phosphoramidate into pyrophosphate after 8 min. Hypochlorite does not show a detectable reaction with inorganic pyrophosphate or fluorophosphate.

The Nitrous Acid Reaction.—Phosphoramidate undergoes rapid hydrolysis in the presence of sodium nitrite at a rate which increases with increasing acidity. Nitrogen was identified as a product of the reaction by gas chromatography.[29] In the presence of 0.1 M nitrite at pH 3.9 the half-time for phosphoramidate disappearance is less than 1 min. Quantitative rate measurements may be carried out conveniently in the pH range 4.5 to 5.5. The disappearance of phosphoramidate was found to follow (pseudo) first-order rather than zero-order kinetics. As shown in Table II the rate increases with increasing nitrite concentration at a constant pH value in 0.1 M acetate buffer. The results show that the reaction is predominantly first order in respect to nitrite concentration and that any second-order term must be small. The rates also increase with increasing acidity under conditions in which very little nitrite is in the form of nitrous acid (Table II). There is a small increase in rate with increasing acetate buffer concentration. If the rates are expressed according to the rate law of eq. 4, the values of k_3 show a moderate increase with both increasing

TABLE II

NITRITE-INDUCED HYDROLYSIS OF PHOSPHORAMIDATE AT 25°[a]

Nitrite, M	pH	k_{obsd}, min.$^{-1}$	$k_3 \times 10^{-4}$,[b] M^{-2} min.$^{-1}$
0.04	5.18	0.0108	2.2
.08	5.17	.0165	2.1
.12	5.18	.025	2.5
.16	5.18	.032	2.6
.20	5.17	.039	2.5
.10	5.26	.015	1.8
.10	5.05	.032	3.0
.10	4.87	.061	4.1
.10	4.71	.079	3.8
.10	4.52	.133	4.3
.10	4.76	.087[c]	4.7[c]
.10	4.72	.098[d]	4.9[d]
.10	4.72	.116[e]	5.8[e]

[a] In 0.1 M potassium acetate buffer; 0.005 M phosphoramidate; analyses by ethylene glycol method. [b] $k_3 = (k_{obsd} - k_{hyd})/[NO_2^-][H^+]$. [c] Acetate buffer, 0.2 M. [d] Acetate buffer, 0.4 M. [e] Acetate buffer, 0.6 M.

$$\text{rate} = k_3[PA^-][NO_2^-][H^+] \quad (4)$$

acidity and acetate buffer concentration. This indicates that there are further terms in the rate law for acid catalysis, the exact nature of which cannot be specified from the data.

Discussion

The following mechanism is proposed for the formaldehyde-catalyzed hydrolysis of phosphoramidate

$$^{-2}O_3PNH_3^+ + HCHO \underset{}{\overset{\text{fast}}{\rightleftharpoons}} {}^{-2}O_3PN^+H_2CH_2OH \quad (5)$$

$$^{-2}O_3PN^+H_2CH_2OH + HCHO \overset{\text{fast}}{\rightleftharpoons} {}^{-2}O_3PN^+H(CH_2OH)_2 \quad (6)$$

$$^{-2}O_3PN^+H_2CH_2OH \xrightarrow{k_3} H_2PO_4^- + H_2NCH_2OH \quad (7)$$

$$^{-2}O_3PN^+H(CH_2OH)_2 \xrightarrow{k_4} H_2PO_4^- + HN(CH_2OH)_2 \quad (8)$$

$$^{-2}O_3PN^+H_2CH_2OH + H_3N^+R \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} {}^{-2}O_3PN^+H_2CH_2N^+H_2R \quad (9)$$

$$^{-2}O_3PN^+H_2CH_2N^+H_2R \xrightarrow{k_6} H_2PO_4^- + H_2NCH_2N^+H_2R \quad (10)$$

In these equations, the ionic forms shown are not necessarily the predominant forms in solution, but they would be expected to be present to some extent. Phosphoramidate exists as the zwitterion in the crystalline state[30,31] and a comparison of the pK_a values of phosphoramidate (3.0 and 8.2) with the pK_a value of 7.2 for the related compound, $Et_2NP(OEt)OH$, strongly suggests that it must exist in the zwitterionic form in solution as well.[32] This conclusion receives further support from the observation that the rapid combination of formaldehyde with the amino group of phosphoramidate results in a decrease in the pH of the solution (Fig. 4) similar to that observed with other partially protonated amines. The rates of the condensation steps with amines are undoubtedly proportional to the concentrations of the free base form of the amines rather than the protonated species. Steps 5 to 8 of this scheme are based on (a) the observed rapid condensation of formaldehyde with phosphoramidate, (b) the terms in the rate law which are first and second order in respect to formaldehyde, (c) the evidence that formaldehyde addition is not complete even in 0.8 M formaldehyde, and (d) the fact that the rate of the reaction is proportional to the concentration of phosphoramidate monoanion in dilute solution. The pathway of steps 9 and 10 is based on the marked potentiation of the hydrolysis by an amine, which can be either ammonia or a second molecule of phosphoramidate. We suggest that step 10 is rate determining at alkaline pH, while step 9 becomes rate determining at lower pH as the amines become protonated and the rate of formation of the methylenediamine adduct is consequently retarded. Such a change in rate-determining step would account for the observed pH–rate maximum for the amine-potentiated reaction if it is assumed that the amine condensation step requires the free base form of the amine. Experiments in which the effect of the addition of formaldehyde to ammonium chloride at pH 4.5 was compared to the effect of mixing the reactants at pH 9, followed by rapid titration to pH 4.5, showed that the reaction of formaldehyde with ammonia is very slow at pH 4.5. The evidence for the exact nature of steps 9 and 10 is less complete than that for steps 5 through 8; in particular, it is possible that the amine-potentiated reaction involves species containing more than 1 mole of formaldehyde. The fact that the reaction is catalytic shows that the formaldehyde-amine which is formed as a hydrolysis product, is able to react further to induce the hydrolysis of phosphoramidates.

It is probable that the pyridoxal-induced hydrolysis occurs by a similar mechanism, although dehydration

(29) We are indebted to Dr. Colin Steele for this analysis, which was carried out with a 6-ft. column of Linde Molecular Sieve 13X, 30–60 mesh, at room temperature, with helium as carrier.

(30) E. Hobbs, D. E. C. Corbridge, and B. Raistrick, *Acta Cryst.*, **6**, 621 (1953).

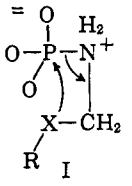
(31) D. E. C. Corbridge and E. J. Lowe, *J. Chem. Soc.*, 493 (1954).

(32) E. W. Crunden and R. F. Hudson, *ibid.*, 3591 (1962).

to a Schiff base intermediate might be considered as a possible additional step in this reaction.

The significance of these reactions derives in part from the fact that they involve at least three, and possibly four, molecules in the catalytic mechanism. This type of catalysis should be greatly favored if the catalyzing molecules were brought together in a single molecule, as might be expected to be the case on the surface of an enzyme, in order to overcome the entropy barrier to the formation of the reactive adducts.

A number of different mechanisms may be considered for the hydrolytic steps (eq. 7, 8, and 10). The evidence for a rapid addition of formaldehyde to the amino group of phosphoramidate, the rapid rate of the reaction, and the formation of pyrophosphate and methyl phosphate in the formaldehyde-catalyzed reaction show that the reaction involves an adduct of formaldehyde and phosphoramidate rather than a bimolecular attack of hydrated formaldehyde on phosphoramidate. The reaction does not appear to be due to an intramolecular displacement of ammonia by the hydroxymethyl (I, X = O) or aminomethyl (I, X = NH) group of the adduct because (a) such a reaction of the phosphoramidate with formaldehyde



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and ammonia would lead only to the regeneration of starting materials and therefore would not explain the rapid hydrolysis; (b) such a mechanism does not explain the formation of pyrophosphate in the formaldehyde-catalyzed reaction; (c) the amino group does not participate readily in intramolecular displacements on phosphorus[33]; and (d) it would be difficult for a weakly basic uncharged oxygen atom to displace a strongly basic amine, even in an intramolecular reaction. An intramolecular displacement involving an oxygen anion might give a rapid hydrolytic reaction, but would be difficult to reconcile with the observed pH dependence of the reaction and would not account for the formation of methyl phosphate in the presence of methanol. The rate acceleration by formaldehyde is most reasonably explained by an increase in the leaving ability of the amino group of phosphoramidate upon the addition of either one or two molecules of formaldehyde (eq. 7 and 8) or formaldehyde and an amine (eq. 10). The detailed mechanism of the solvolytic step will be considered further below.

The following mechanism is proposed for the hypochlorite-catalyzed hydrolysis of phosphoramidate

$$^{-2}O_3PNH_2 + HOCl \longrightarrow {}^{-2}O_3PNHCl + H_2O \quad (11)$$

$$^{-2}O_3PNHCl + HOCl \longrightarrow {}^{-2}O_3PNCl_2 + H_2O \quad (12)$$

$$^{-2}O_3PNCl_2 \xrightarrow{\text{fast}} HPO_4^{-2} + HNCl_2 \quad (13)$$

$$^{-2}O_3PNH_2 + HNCl_2 \longrightarrow {}^{-2}O_3PNCl_2 + NH_3 \quad (14)$$

Steps 11 to 13 of this scheme are based on (a) the observed rapid formation of $^{-2}O_3PNHCl$ in alkaline solution without hydrolysis of the product; (b) the rapid increase in absorption at 245 mμ in acidic solution, which is characteristic of the formation of $^{-2}O_3PNHCl$ and occurs more rapidly than the hydrolytic step; and (c) the parallel release of inorganic phosphate and formation of dichloramine, which occurs more rapidly and with fewer side reactions than in the reaction of hypochlorous acid with ammonia under the same conditions. Step 12 is strongly catalyzed by chloride ions, which indicates that chlorine is a better chlorinating agent toward monochlorophosphoramidate than is hypochlorous acid (this process is nucleophilic catalysis of electrophilic catalysis). Step 14 is based on the observation that the reaction is truly catalytic; *i.e.*, chlorine must be transferred from the products to unreacted phosphoramidate. A similar process is presumably involved in the slower hydrolysis observed in the presence of a large excess of ammonia, in which all of the hypochlorite must react with ammonia to form the various chloramines before it can react with phosphoramidate. It is probable that monochloramine and perhaps trichloramine can also act as chlorine donors in a process analogous to that of eq. 14.

Similar considerations apply to the mechanism of the hydrolytic step as in the case of the formaldehyde-catalyzed reaction. Chlorination will make the amino group of phosphoramidate a much better leaving group. A direct nucleophilic attack of hypochlorite on phosphoramidate, followed by a secondary reaction to give products, is ruled out by the spectrophotometric evidence that phosphoramidate is chlorinated before hydrolysis occurs; by the fact that the formation of dichloramine parallels the release of inorganic phosphate and occurs in much higher yield than in the reaction of hypochlorite with the hydrolysis products; and by the fact that the rate of the reaction is increased further by chloride ion, which suggests that a chlorination step must occur before hydrolysis.

Hypochlorite induces the hydrolysis of several other phosphate compounds by somewhat different mechanisms. The hydrolysis of isopropyl methylphosphonofluoridate is accelerated by hypochlorite by means of nucleophilic catalysis.[34] The hypochlorite-catalyzed hydrolysis of triethyl phosphorothiolate probably proceeds by a nucleophilic reaction, but the hydrolysis of this compound is also accelerated by oxidation by hypochlorite and by molecular chlorine.[10] The hydrolysis of tetramethylphosphorodiamidic anhydride (schradan) is markedly accelerated by oxidation of one or more of the methyl groups.[35] Westheimer has suggested an attractive mechanism for this hydrolysis which involves a concerted decomposition to give formaldehyde and a monomeric metaphosphate derivative.[36] However, this reaction is of a quite different nature from the formaldehyde-catalyzed hydrolysis of phosphoramidate.

The nitrite-induced hydrolysis of phosphoramidate differs from the reactions of formaldehyde and hypochlorite in that it is not catalytic, but is almost certainly similar in that it involves an electrophilic induction of hydrolysis. A detailed kinetic study of this reaction has not been undertaken, but the limited re-

(33) D. M. Brown and G. O. Osborne, *J. Chem. Soc.*, 2590 (1957).

(34) J. Epstein, V. E. Bauer, M. Saxe, and M. M. Demek, *J. Am. Chem. Soc.*, **78**, 4068 (1956).

(35) E. Y. Spencer, Special Publication No. 8, The Chemical Society, London, 1957, p. 171.

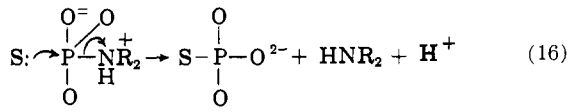
(36) F. H. Westheimer, ref. 35, p. 180.

sults which have been obtained demonstrate that the reaction is acid catalyzed in the pH range in which nitrite is present as the anion, is first order in respect to phosphoramidate, and is predominantly first order in respect to nitrite at a given pH value. These results and the fact that the observed rates are slower than the rates of formation of the nitrosating species, dinitrogen trioxide,[37,38] show that the formation of a reactive nitrosating agent is not the rate-determining step of the reaction. In view of the predominantly first-order dependence of the rate in respect to both nitrite and phosphoramidate, it appears probable that an adduct of these compounds is formed in a preliminary step, followed by a decomposition to give products (eq. 15). An adduct of phosphoramidate and nitrous acid could be formed either as an anhydride

$$PA^- + NO_2^- + H^+ \longrightarrow [PA{\cdot}NO] \longrightarrow \longrightarrow \text{products} \qquad (15)$$

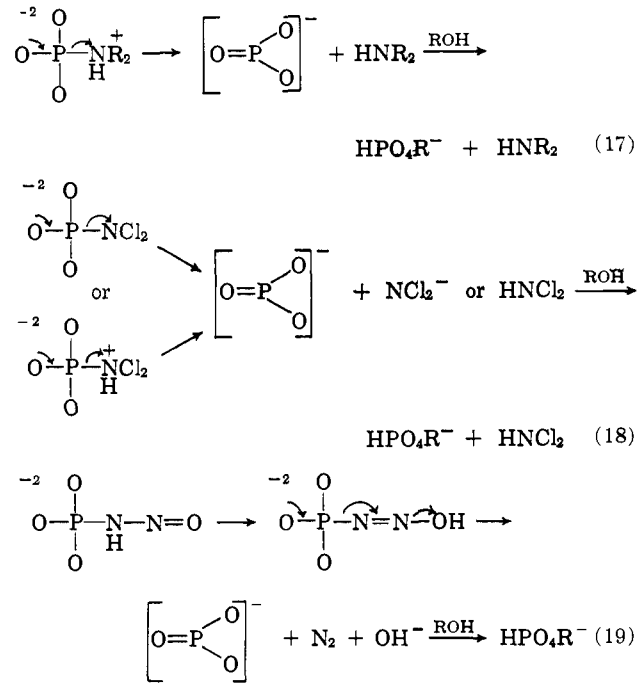
with a phosphate oxygen atom[37] or by addition to the amino group. Decomposition of the adduct could occur directly or after an intramolecular rearrangement to a less stable species in which the P–N bond has been replaced by a P–O bond.[39]

In all three of these reactions the increase in solvolysis rate results from a combination of phosphoramidate with an electrophilic reagent; the hypochlorite reaction is a special case in which the electrophilic reagent is an oxidizing agent. For the formaldehyde and hypochlorite reactions and probably also for the nitrite reaction the function of the electrophilic reagent is to convert the amino group into a better leaving group. The solvolytic step could then take place either by a nucleophilic attack of solvent or phosphate (eq. 16) or by the intermediate formation of the monomeric metaphosphate monoanion[40] followed by a



(16)

rapid reaction with solvent or phosphate (eq. 17–19). Pyridine and fluoride ion are effective nucleophilic reagents toward phosphoramidate.[8,11,19,20] The observations that the rate of the reaction with pyridine is not increased by formaldehyde and that fluorophosphate is not formed in either the formaldehyde- or the hypochlorite-catalyzed reactions suggest that the catalytic mechanism does not involve simply an increase in the susceptibility of phosphoramidate toward nucleophilic attack. These findings would be consistent with an elimination mechanism to form metaphosphate, in which the nucleophilic reagent attacks the highly reactive intermediate after the rate-determining step. Chanley and Feageson have presented evidence that the uncatalyzed solvolysis of phosphoramidate involves nucleophilic attack by solvent, with a high degree of selectivity toward the nucleophilic reagent, while the solvolysis of other



phosphate compounds which may proceed through metaphosphate ion shows much less selectivity toward the nucleophilic reagent in methanol–water mixtures.[19] However, the metaphosphate ion cannot be a *free* intermediate in the formaldehyde- and hypochlorite-catalyzed reactions, because different yields of methyl phosphate are obtained when these two reactions are carried out in the same methanol–water mixture and the yields of methyl phosphate in both reactions are higher than those obtained with *p*-nitrophenyl phosphate and phenyl phosphate in the same solvent mixtures (Table III). Similarly, the yield of pyrophos-

TABLE III

THE SOLVOLYSIS OF PHOSPHORAMIDATE IN METHANOL–WATER MIXTURES

	Temp., °C.	Yield of methyl phosphate, % 30% methanol	50% methanol
Phosphoramidate	39	54	73
Phosphoramidate[a]	37	54	73
Phosphoramidate + HCHO	39	50	70
Phosphoramidate + HOCl	39	35	56
Phenyl phosphate[a]	55	27	42
p-Nitrophenyl phosphate[a]	37	22	38

[a] Chanley and Feageson.[19]

phate is somewhat lower in the hypochlorite- than in the formaldehyde-catalyzed reaction, and the yields in both cases are lower than in the uncatalyzed solvolysis of phosphoramidate. These apparently contradictory results suggest that the catalyzed reactions involve little or no participation of the nucleophilic reagent, but that the metaphosphate-like intermediate is not completely separated from the attacking or leaving groups. In this respect the situation appears to be analogous to that in carbon chemistry, in which there is a somewhat hazy borderline region between nucleophilic displacement and carbonium ion mechanisms.

(37) J. H. Ridd, *Quart. Rev.* (London), **15**, 418 (1961).

(38) C. A. Bunton, D. R. Llewellyn, and G. Stedman, *J. Chem. Soc.*, 568 (1959).

(39) P. J. Bunyan and J. I. G. Cadogan, *ibid.*, 1304 (1962).

(40) W. W. Butcher and F. H. Westheimer, *J. Am. Chem. Soc.*, **77**, 2420 (1955); C. A. Bunton, D. R. Llewellyn, K. G. Oldham, and C. A. Vernon, *J. Chem. Soc.*, 3574 (1958).