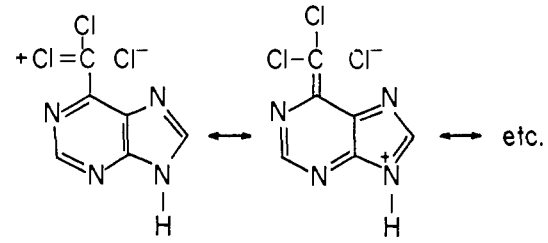
reactivity of the CCl_3 group between the two systems could be explained by a greater ability of the purine ring structure, compared to benzene, to contribute to the double bond–no bond resonance[14] of the molecule, thus leading to stronger C–Cl bonds.



(14) J. Hine, *J. Am. Chem. Soc.*, **85**, 3239 (1963).

Examples of resonance stabilization resulting from the presence of an electron-withdrawing group on the same carbon atom with a resonance electron-donating group have been given by Hine and Rosscup.[15]

The purine ring system may be unusual in that it is able to act both as electron acceptor and electron donor; in the present case, the latter effect may predominate. Generally, however, purine is considered as a π-excessive heteroaromatic because it embodies an electron releasing setting (=CH—NH—CH=).[16]

Acknowledgment. The authors wish to thank Dr. E. D. Bergmann and Dr. A. Bendich for their continued interest in this work.

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Reactions of Nucleophilic Reagents with Phosphoramidate[1]

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The rates of the reactions of a number of compounds with the phosphoramidate monoanion show only a small dependence upon the basicity of the nucleophilic reagent: the Brønsted β-value is 0.22 for substituted pyridines, and other amines react at similar rates. In concentrated solutions there is a significant accumulation of the phosphorylated tertiary amine in the reactions of phosphoramidate with pyridine, 4-methylpyridine, triethylenediamine, and N-methylimidazole. The rate of hydrolysis of the phosphorylated 4-methylpyridine is independent of pH and occurs with a half-time of about 5 min. at 25°; the product of the reaction with N-methylimidazole is much more stable. The reaction with hydroxylamine gives predominantly the O-phosphorylated product, which is probably the same as the compound which is formed from adenosine triphosphate and hydroxylamine in the presence of pyruvate kinase and bicarbonate. Fluoride reacts predominantly with the species of phosphoramidate which carries no net charge, but also reacts slowly with the monoanion. Several phosphorylated diamines which have two available sites for protonation on the leaving group undergo rapid hydrolysis at slightly acidic pH.

In recent studies of the reactions of nucleophilic reagents with acetyl phosphate, it was found that glycine, aniline, imidazole, N-methylimidazole, and morpholine attack the carbonyl group, while pyridine, fluoride, triethylenediamine, and trimethylamine attack at phosphorus.[2,3] Although there appears to be a tendency for primary amines to attack preferentially at carbon and for tertiary amines to attack at phosphorus, this distinction is not clear-cut and there is no obvious reason for the order of nucleophilic reactivities. In the hope of clarifying this situation, we have examined the reactivity of a series of nucleophilic reagents toward phosphoramidate, which is not subject to the complication of nucleophilic attact at positions other than the phosphorus atom.

There is a considerable amount of information available regarding nucleophilic reactivity toward fully substituted phosphate compounds,[4] but there are only scattered data for ionized phosphates, which are the phosphate compounds of principal biological interest.[2,3,5–8] Phosphoramidates have been widely utilized for synthetic work, especially for the synthesis of pyrophosphates,[9] and the synthesis of a substituted pyrophosphate has been shown to be a bimolecular reaction.[10] Phosphoramidate has been shown to undergo nucleophilic reactions with imidazole, pyridines, and fluoride, and the reactions with pyridine and 4-methylpyridine proceed at rates which differ by only 30%.[5–8,11] An enzyme preparation from *E. coli* has been shown to catalyze phosphoryl transfer from a

(1) Supported by grants from the National Science Foundation and the National Institute of Child Health and Human Development of the Public Health Service (HD-01247).
(2) G. Di Sabato and W. P. Jencks, *J. Am. Chem. Soc.*, **83**, 4393, 4400 (1961).
(3) J. H. Park and D. E. Koshland, Jr., *J. Biol. Chem.*, **233**, 986 (1958).
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(5) J. D. Chanley and E. Feageson, *J. Am. Chem. Soc.*, **85**, 1181 (1963).
(6) M. Halmann, A. Lapidot, and D. Samuel, *J. Chem. Soc.*, 1299 (1963).
(7) T. Rathlev and T. Rosenberg, *Arch. Biochem. Biophys.*, **65**, 319 (1956).
(8) T. Müller, T. Rathlev, and T. Rosenberg, *Biochim. Biophys. Acta*, **19**, 563 (1956).
(9) (a) V. M. Clark, G. W. Kirby, and A. Todd, *J. Chem. Soc.*, 1497 (1957); (b) R. W. Chambers and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 3749 (1958); (c) J. G. Moffatt and H. G. Khorana, *ibid.*, **80**, 3756 (1958); (d) R. W. Chambers, P. Shapiro, and V. Kurkov, *ibid.*, **82**, 970 (1960).
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(11) W. P. Jencks, *Brookhaven Symp. Biol.*, **15**, 134 (1962).

number of phosphoramidates to hexoses.[12] Knowledge of the characteristics of nucleophilic reactions with ionized phosphate compounds may be helpful in elucidating the mechanisms by which enzymes accelerate the rates of reactions of these compounds.

Experimental

Materials and methods were essentially the same as described previously.[13] Potassium phosphoramidate was prepared by the method of Stokes.[9b,14] N-Methylhydroxylamine hydrochloride was kindly supplied by Dr. J. Reimann. N,N-Dimethylhydroxylamine hydrochloride was prepared by the method of Hepworth.[15] Glycine hydroxamic acid, m.p. 142.5–143.5 dec., was prepared by the method of Safir and Williams.[16] Hydrazine dihydrochloride was recrystallized from ethanol–hydrochloric acid. Other reagents were commercial products and were generally redistilled or recrystallized before use.

The methods[13] used for analysis of different phosphate compounds were as follows: The modified Martin–Doty procedure measures inorganic phosphate and the products of the reaction of phosphoramidate with pyridine, 4-methylpyridine, and triethylenediamine, but gives only approximately 5% reaction with phosphoramidate with 5 sec. of shaking of the isobutyl alcohol and aqueous layers. Analyses of the reactions with pyridine, 4-methylpyridine, and triethylenediamine in which an intermediate accumulated were carried out with shaking for 10 sec.; this resulted in a more complete decomposition of the phosphorylated tertiary amine at the expense of an increase to approximately 10% in the hydrolysis of phosphoramidate. This method, while not of high precision for analytical purposes, was found to give results which were satisfactory for the measurement of pseudo-first-order kinetics; the decomposition of a small and constant fraction of the material being analyzed does not affect the determination of a first-order rate constant. The Fiske and Subbarow method, with 0.1% Elon (Eastman Kodak Co.) in 0.3% sodium bisulfite as reducing agent, measures inorganic phosphate and the various phosphoramidates (after 10- or 20-min. color development), but phosphorofluoridate, pyrophosphate, and O-phosphorylhydroxylamine do not react as inorganic phosphate under these conditions. These three compounds were determined as inorganic phosphate by the Fiske and Subbarow method after hydrolysis in 1 *M* hydrochloric acid at 100° for 10 min. The formation of phosphorofluoridate from the reaction of phosphate compounds with aqueous fluoride has been shown previously by other methods.[2] The ethylene glycol method is specific for inorganic phosphate, except for O-phosphorylhydroxylamines, which undergo partial or complete decomposition to inorganic phosphate upon heating in ethylene glycol. For determination of first-order rate constants, aliquots of up to 0.1 ml. were heated with 0.9 ml. of ethylene glycol for 5 min. in a boiling water bath; for analytical purposes a 0.02-ml. aliquot was added to 1.0 ml. of ethylene glycol. Under these conditions, unstable phosphate compounds are converted to the stable ethylene glycol esters. The remaining inorganic phosphate is determined by the Fiske and Subbarow method. The ethylene glycol method is unreliable in the presence of excess mineral acid or if the sample to be analyzed is sufficiently alkaline to prevent the decomposition of phosphoramidate. For analysis of the course of reactions carried out in alkaline solutions, therefore, sufficient acetic acid (usually 0.05 *M*) was added to the ethylene glycol to neutralize the excess base.

Ammonia release was determined by titration after Conway diffusion of aliquots of the reaction mixture to which had been added excess potassium carbonate.

Phosphorylimidazole and phosphoryl-N-methylimidazolium were prepared by the reaction of 0.1 *M* potassium phosphoramidate with a 0.25 *M* solution of the appropriate base for 1–3 hr. at 39°. The N-methylimidazole was 20% in the form of the hydrochloride. Aliquots of these solutions were added to 0.1 *M* acetate, imidazole (for phosphorylimidazole), or N-methylimidazole (for phosphoryl-N-methylimidazolium) buffers at 39°, and the rate of inorganic phosphate appearance was followed by the modified Martin–Doty procedure. It was shown that the rate was not affected by a two- to fivefold increase in the concentration of imidazole or N-methylimidazole buffer. The hydrolysis experiments were carried out with dilute (5×10^{-3} *M*) solutions of phosphorylimidazole in order to avoid a reaction of this compound with itself.[7]

The pK_a' values of phosphoramidate at 25° at an ionic strength of 1.0, maintained with potassium chloride, were found by titration to be 8.03 and 2.83. At an ionic strength of approximately 0.015 the higher pK_a' was found to be 8.25. The pK_a' at 39°, ionic strength 1.0, was estimated to be 7.87 by determination of the change in pH observed upon rapidly warming a solution from 25 to 39°. Titrations were carried out by rapid titration of the stable dianion with hydrochloric acid in order to avoid hydrolysis of the phosphoramidate. Back-titration gave results which were experimentally indistinguishable from those of the forward titration. The results for both dissociation constants are in satisfactory agreement with the pK_a' values of 2.8 and 8.2 reported by Meyerhoff and Lohmann[17] and similar values reported by other workers[5,7,9b,18,19]; however, they do not agree with the values of 4.6 and 7.7 at 10° reported by Halmann, *et al.*[6]

Pseudo-first-order rate constants were determined at 39 ± 0.1°, ionic strength maintained at 1.0 with potassium chloride, with the nucleophilic reagent present in large excess unless noted otherwise. Second-order rate constants, k_2, were obtained from the relationship $k_2 = (k_{obsd} - k_{hydrol})/[\text{nucleophile}]$.

Product of the Reaction of Phosphoramidate with Hydroxylamine. In a typical experiment, 1.3 mmoles of potassium phosphoramidate was dissolved in 1.0 ml. of 1.0 *M* hydroxylamine, pH 7.0, which had been

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(13) W. P. Jencks and M. Gilchrist, *J. Am. Chem. Soc.*, **86**, 1410 (1964).
(14) N. H. Stokes, *Am. Chem. J.*, **15**, 198 (1893); R. Klement and K. H. Becht, *Z. anorg. Chem.*, **254**, 217 (1947).
(15) H. Hepworth, *J. Chem. Soc.*, **119**, 251 (1921).
(16) S. R. Safir and J. H. Williams, *J. Org. Chem.*, **17**, 1298 (1952).
(17) O. Meyerhof and K. Lohmann, *Biochem. Z.*, **196**, 22 (1928).
(18) R. R. Irani and C. F. Callis, *J. Phys. Chem.*, **65**, 934 (1961).
(19) R. Klement, G. Biberacher, and V. Hille, *Z. anorg. Chem.*, **289**, 80 (1957).

Table I. Reactions of 1.5–2.5 × 10^{3} *M* Phosphoramidate with Heterocyclic and Primary Amines at 39°, Ionic Strength 1.0

Compound	pK_a	Fraction free base	pH	No. of runs	Concn.[a] range, *M*	k_2',[b] M^{-1} min.$^{-1}$	k_2,[c] M^{-1} min.$^{-1}$
3-Chloropyridine	2.8	0.8	3.8	8	0.02–0.10	0.22	0.22
3-Acetylpyridine	3.2	0.8	4.2	6	0.026–0.32	0.24	0.24
Nicotinamide	3.4	0.8	4.2	6	0.026–0.32	0.24	0.24
Pyridine	5.2	0.8	6.1	5	0.072–0.96	0.71	0.71
		0.5	5.4	3	0.016–0.05	0.67	0.67
		0.5		3	0.016–0.05	0.69[d]	0.69[d]
		0.25	5.0	3	0.008–0.025	0.66	0.66
3-Methylpyridine	5.7	0.8	6.6	10	0.028–0.96	0.71	0.74
4-Methylpyridine	6.0	0.8	6.9	8	0.028–0.96	0.77	0.96
2,6-Dimethylpyridine	6.8	0.5	6.9	3	0.1–0.3	[e]	<0.003
2,4,6-Trimethylpyridine	7.5	0.5	7.5	3	0.05–0.15	[e]	[e]
Imidazole	7.0	0.97	8.6	2	0.2	0.043[f]	0.28
N-Methylimidazole	7.1	0.93	8.3	1	0.1	0.10[f]	0.37
		0.65	7.4	1	0.1	0.23[f]	0.31
Triethylenediamine	9.0	0.25	8.7	1	0.1	0.8	4.0
		0.5	9.2	3	0.03–0.09	0.24	4.1
Monocation Ethylenediamine	3.6	0.5	3.6	4	0.05–0.30	0.40	0.40
Monocation	7.0	0.95	8.3	2	0.2	0.175[f]	0.70
Glycylglycine	8.2	0.5	8.2	8	0.05–0.30	0.194[g]	0.48
Glycine	9.7	0.1	8.8	5	0.13–0.40	0.20[g]	1.7

[a] As free base. [b] Observed k_2', from the initial slope of plots of k_{obsd} against the concentration of amine as the free base. Inorganic phosphate appearance followed by the Martin–Doty or ethylene glycol method. [c] For the reaction of phosphoramidate as the monoanion; $k_2 = k_2'$/(fraction of phosphoramidate as the monoanion). [d] In D_2O. [e] No detectable reaction. [f] Measured with Radiometer TTTlc automatic titrator, with 0.025 *M* phosphoramidate. [g] Phosphoramidate concentration 5 × 10^{-3} *M*. Rate of ammonia appearance determined by the Conway diffusion method.

prepared by the neutralization of hydroxylamine hydrochloride with potassium hydroxide. The pH was maintained at 7.0 ± 0.2 by the addition of concentrated acid or base. After incubation for 40 min. at 39°, analysis for hydroxylamine[20] showed 93% disappearance, and 73% of the total phosphate was unreactive by the Fiske and Subbarow method. The product was stable upon storage at −15° for at least several weeks.

For spectrophotometric examination of the reaction of this product with furfural, the solution was diluted with four volumes of water and the diluted solution was incubated at room temperature with an equal volume of 0.175 *M* furfural and 0.2 volume of a buffer containing 1 *M* sodium acetate and 1 *M* acetic acid. Aliquots were removed after 12 and 60 min., were diluted 100-fold, and were examined spectrophotometrically, with a 0.5-mm. path length. Control experiments were carried out with hydroxylamine, N-methylhydroxylamine, and methoxylamine. Identical absorption maxima were obtained after 12 and 60 min., and it was shown that the product did not undergo hydrolysis to inorganic phosphate during the incubation with furfural. In each case there was observed an increase in the extinction coefficient of the principal absorption band and a disappearance of the furfural absorption maximum at 227 mμ. The absorption maximum obtained upon incubation of the product of the reaction of phosphoramidate and hydroxylamine with furfural is at 270 mμ, while the maxima of furfural oxime, furfural oxime methyl ether, and the nitrone formed from furfural and N-methylhydroxylamine are at 268, 275, and 300 mμ, respectively; the principal absorption maximum of furfural is at 276 mμ.

(20) S. Frear and R. C. Burrell, *Anal. Chem.*, 27, 1664 (1955).

Attempts to isolate the product of the reaction of phosphoramidate and hydroxylamine were unsuccessful because of decomposition. However, the product could be separated from inorganic phosphate and other impurities by adsorption from aqueous solutions onto Dowex 1 chloride ion-exchange resin at 5°, followed by elution with 0.05–0.10 *M* triethylammonium bicarbonate.

Results

Reactions with Tertiary Amines. The rate constants for the reactions of phosphoramidate with a series of substituted pyridines, determined by following the rate of appearance of inorganic phosphate, are summarized in Table I. At high concentrations of pyridines, the rates do not increase linearly with pyridine concentration because of self-interaction of the substituted pyridines[21]; the rate constants were, therefore, determined at low concentrations of pyridine, at which this self-interaction does not introduce an appreciable error. The reactions of phosphoramidate with imidazole and N-methylimidazole, determined by automatic titration, are slower than that with pyridine.

The relatively unhindered aliphatic tertiary amine, triethylenediamine, reacts several times faster than the substituted pyridines, but the observed rate of reaction of this compound should be divided by a factor of two for comparison with other amines because of its two reactive sites. The dependence on pH of the rates of reaction of pyridine, N-methylimidazole, and triethylenediamine is consistent with a mechanism which involves reaction of the free base form of the amine with the monoanion of phosphoramidate. Triethylenediamine also reacts at a significant rate as the monocation; the rate of this reaction is similar to that of substituted pyridines of comparable basicity. The

(21) A. J. Kirby and W. P. Jencks, *J. Am. Chem. Soc.*, 87, 3209 (1965).

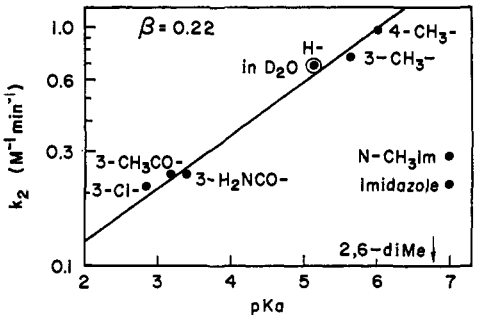


Figure 1. Logarithmic plot of the rates of reaction of substituted pyridines and imidazoles with phosphoramidate monoanion at 39° as a function of the basicity of the amine. The substituents refer to pyridine unless noted otherwise.

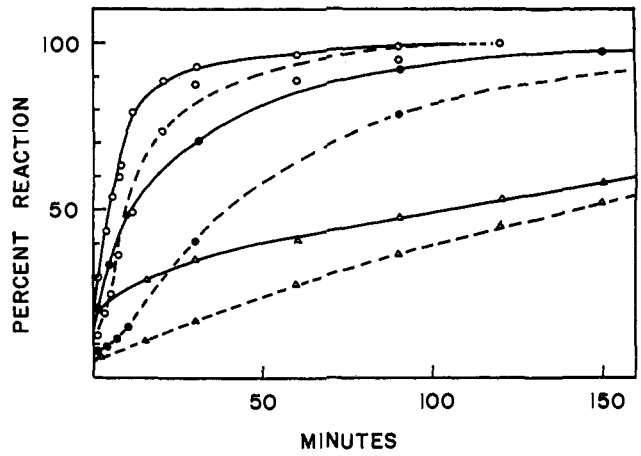
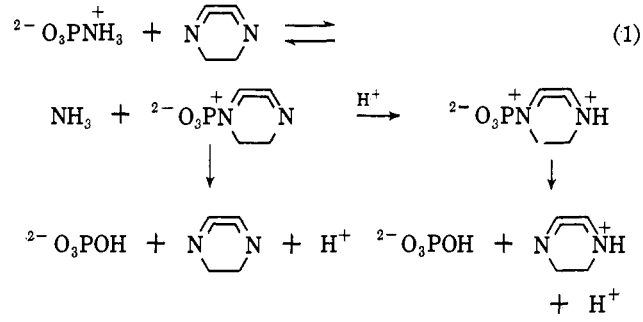


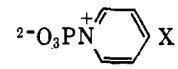
Figure 2. The reaction of 0.05 *M* phosphoramidate with 0.5 *M* triethylenediamine at 39°, ionic strength 1.0. Solid lines: phosphate determination by the modified Martin–Doty method (sum of inorganic phosphate and intermediate); dashed lines: phosphate determination by the ethylene glycol method (inorganic phosphate); open circles: pH 8.7 (triethylenediamine 30% free base); closed circles: pH 9.5 (triethylenediamine 70% free base); triangles: pH 9.6, in the presence of 0.2 *M* ammonia.

rate of the reaction with pyridine is not significantly altered if the reaction is carried out in deuterium oxide. Chanley and Feageson have shown previously that the rate of the reaction with nicotinic acid anion is not altered in deuterium oxide.[5] 2,4,6-Trimethylpyridine and, after careful purification, 2,6-dimethylpyridine do not react with phosphoramidate at a measurable rate. The logarithms of the rate constants for the reactions with substituted pyridines are plotted against the pK_a values of the conjugate acids of the pyridines in Figure 1. The rates show only a small dependence on basicity, with slope $\beta = 0.22$.

At high concentrations of tertiary amines there is evidence for the accumulation of an intermediate reaction product, presumably $\geqslant N^+PO_3^{2-}$. In the case of the pyridine, 4-picoline, and triethylenediamine reactions, this intermediate is not stable and reacts as inorganic phosphate under the conditions of the modified Martin–Doty method for phosphate determination. Its accumulation can, therefore, be observed by comparison of phosphate determinations by this method and by the ethylene glycol method, which measures only inorganic phosphate. The results of some representative experiments with triethylenediamine are shown in Figure 2, in which the solid lines show the sum of the concentrations of inorganic phosphate and intermediate and the dashed lines show the concentration of inorganic phosphate; the difference between the dashed and solid lines is, therefore, an approximate measure of the accumulation and hydrolysis of the intermediate (it is not an exact measure, because phosphoramidate itself reacts slightly and the intermediate does not react completely under the conditions of the modified Martin–Doty method). Both the rate of formation and the rate of hydrolysis of the intermediate increase with decreasing pH. This indicates that triethylenediamine reacts with the phosphoramidate monoanion and that the intermediate undergoes hydrolysis more readily if a proton adds to the second nitrogen atom of triethylenediamine. No accumulation of an intermediate could be detected in the reaction of triethylenediamine with phosphoramidate at pH 3.7. The accumulation of the intermediate and the appearance of inorganic phosphate are strongly inhibited in the presence of ammonia (Figure 2, triangles). This indicates that the formation of the intermediate is an equilibrium reaction, which is repressed by the back reaction which occurs with added ammonia. The reaction mechanism suggested by these results is summarized in eq. 1.

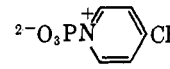


Similar evidence for the accumulation and hydrolysis of an intermediate



was obtained with pyridine and 4-methylpyridine. However, the formation of this intermediate is not inhibited by ammonia at pH 6.2, presumably because ammonia is protonated and unreactive at this pH. The rate of hydrolysis of 1-phosphoryl-4-methylpyridinium ion can be measured without interference from the hydrolysis of phosphoramidate at alkaline pH, at which phosphoramidate is in the form of the unreactive dianion. Phosphoramidate was allowed to undergo reaction with concentrated aqueous 4-methylpyridine until the intermediate had accumulated, and the solution was then diluted with sodium carbonate buffer or sodium hydroxide, and the rate of hydrolysis of the intermediate was followed by the ethylene glycol method. The intermediate undergoes hydrolysis with a half-time of just under 5 min. at 25°, hydrolysis occurs in alkaline solution (in contrast to phosphoramidate itself), and the rate of hydrolysis is independent of pH between pH 10.0 and 12.2 (Table II). The reaction with pyridine is similar to that with 4-methylpyridine except that the accumulation of inter-

mediate is less and its hydrolysis is faster in the pyridine reaction.

Table II. Hydrolysis of  at 25°, Ionic Strength 1.0[a]

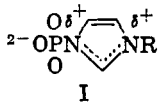
pH	k_{obsd}, min.$^{-1}$
10.0	0.142
10.3	0.151
10.7	0.151
12.2	0.148

[a] Dipotassium phosphoramidate, 0.1 *M*, was incubated for 5 min. with 1 *M* 4-methylpyridine, 20% as the hydrochloride; the solution was then added to four volumes of carbonate buffer or sodium hydroxide and the rate of hydrolysis of the reaction product was followed by the ethylene glycol method.

Further evidence for the high reactivity of this intermediate at alkaline pH was obtained from its reaction with methylamine. Phosphoramidate itself does not react with methylamine, because it is converted to the unreactive dianion at alkaline pH. A solution containing the intermediate formed from phosphoramidate and 4-methylpyridine was added to 0.5 *M* methylamine at pH 11.7. Within 1 min. the intermediate was completely converted to a product which underwent no further hydrolysis at this pH, presumably N-methylphosphoramidate dianion.

In the presence of 2 *M* potassium fluoride the reaction of phosphoramidate with pyridine at pH 8.3 was found to give 49% phosphorofluoridate; the reactions with 0.5 *M* triethylenediamine at pH 8.8 and with 0.3 *M* triethylenediamine monocation at pH 5.9 were found to give 48 and 58% phosphorofluoridate, respectively. A solution containing the intermediate formed from phosphoramidate and 4-methylpyridine which was brought to pH 9.3 with carbonate buffer and allowed to decompose in the presence of 2 *M* potassium fluoride was found to contain 30% phosphorofluoridate. It was shown that fluoride did not react directly with phosphoramidate to a significant extent under the conditions of these experiments. Tertiary amines, therefore, cause nucleophilic catalysis of transfer reactions as well as the hydrolysis of phosphoramidate.

The initial product of the reaction of phosphoramidate with N-methylimidazole is phosphoryl-N-methylimidazolium ion (I, R = CH_3). This compound



I

undergoes hydrolysis at a very slow rate, slower than that of phosphoramidate itself, and the rate constant is constant at approximately 1.2×10^{-3} min.$^{-1}$ at 39° and ionic strength 1.0 over the pH range 5 to 8 (Figure 3). Over the same range of pH, the rate of hydrolysis of phosphorylimidazole (1-imidazolylphosphonate, I, R = H) decreases, because of the loss of a proton to form the unreactive dianion according to a dissociation curve with a pK_a' of approximately 7.0. The dependence on pH of the hydrolysis of a phosphoryl-

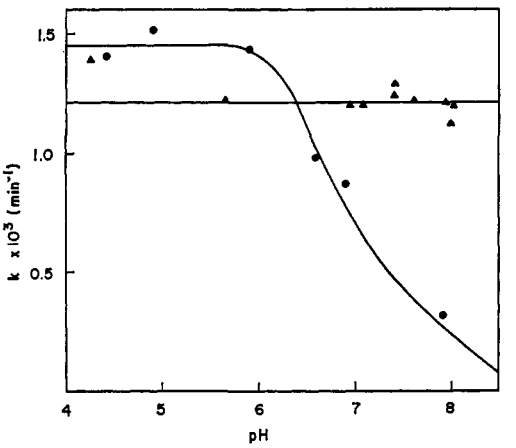


Figure 3. Dependence on pH of the rate of hydrolysis of phosphorylimidazole (circles) and phosphoryl-N-methylimidazolium (triangles) in 0.05–0.1 *M* acetate, imidazole, and N-methylimidazole buffers at 39°, ionic strength 1.0.

imidazole derivative at 77° has been reported previously by Boyer and co-workers.[22]

Reaction with Hydrazine. In slightly acidic solutions, near pH 6, the rate of appearance of inorganic phosphate from phosphoramidate in the presence of hydrazine and N,N-dimethylhydrazine increases with increasing hydrazine concentration and pH (Table III). This is the behavior which is expected if phos-

Table III. Rate of the Reactions of Hydrazine and N,N-Dimethylhydrazine with Phosphoramidate at 39°[a]

Total hydrazine, *M*	pH	$k_{obsd} \times 10^3$, min.$^{-1}$	k_2,[b] M^{-1} min.$^{-1}$
	Hydrazine		
0.1	5.81	6.4	(2.6)
0.2	5.81	8.7	3.3
0.3	5.82	10.2	3.1
0.4	5.83	12.8	3.4
0.5	5.84	15.1	3.4
0.4	6.03	16.5	2.8
		Av.	3.2
	N,N-Dimethylhydrazine		
0.2	5.99	12.3	0.65
.3	5.98	16.7	0.69
.4	5.99	22.4	0.77
.5	5.98	27.9	0.81
		Av.	0.73

[a] Succinate and glycerophosphate buffers, 0.2 *M*. Ionic strength maintained at 1.4 ± 0.1 (hydrazine) or 1.0 (dimethylhydrazine) with KCl. Inorganic phosphate appearance measured by the ethylene glycol method. [b] $(k_{obsd} - k_{hyd})/[\text{hydrazine}]_{\text{free base}}$ based on pK_a values of 8.07 and 7.21 for $N_2H_5^+$ and $(CH_3)_2NNH_3^+$, respectively: R. L. Hinman, *J. Org. Chem.*, **23**, 1587 (1958).

phorohydrazide is formed in the rate-determining step and undergoes hydrolysis to inorganic phosphate at a rate which is fast compared to the initial step (eq. 2).

$$O_3PNH_3^- + N_2H_4 \longrightarrow O_3PNHNH_2^{2-} + NH_4^+ \xrightarrow{H^+} O_3POH^{2-} + N_2H_4 \quad (2)$$

The second-order rate constant for the reaction of N,N-dimethylhydrazine as the free base is about one-fourth

(22) C. H. Suelter, M. DeLuca, J. B. Peter, and P. D. Boyer, *Nature*, **192**, 43 (1961).

of that for hydrazine. In more basic solutions, near pH 8, the rate of inorganic phosphate formation in the presence of hydrazine at a concentration of 0.1 *M* or more is independent of hydrazine concentration. From the rate constants obtained in more acidic solution it would be expected that the formation of phosphorohydrazidate would be fast under these conditions, so that the rate of inorganic phosphate appearance represents the rate of hydrolysis of phosphorohydrazidate. These conclusions were confirmed by preparing a solution of phosphorohydrazidate by incubation of concentrated hydrazine and phosphoramidate at pH 8.7 and measuring the rate of hydrolysis of portions of this solution added to buffer solutions at different pH values (Table IV). The data are not of sufficient accuracy for the determination of the rate constants for the hydrolysis of the different ionic species of phosphorohydrazidate, but they do show that the dianion is stable at alkaline pH, that there is a progressive increase in hydrolysis rate with decreasing pH, which appears to be an acid-catalyzed reaction of the monoanion, and that there is an indication of a pK_a near 8, the expected pK of phosphorohydrazidate. Because of the relatively rapid formation and acid-catalyzed hydrolysis of the hydrazide, hydrazine acts as an effective nucleophilic catalyst for the hydrolysis of phosphoramidate.

Table IV. Hydrolysis of Phosphorohydrazidate at 39°

pH	Hydrazine, *M*	k_{obsd}, min.$^{-1}$
11.3	*a*	<0.002
8.13	0.4[b]	0.019
7.60	0.05[b]	0.022
7.68	0.1[b]	0.028
7.70	0.3[b]	0.030
7.69	0.5[b]	0.031
7.71[c]	*a*	0.033
7.06[c]	*a*	0.072
6.60[c]	*a*	0.154
6.28[c]	*a*	0.257
5.52[d]	*a*	>1.0
4.58[d]	*a*	>1.0

[a] Phosphorohydrazidate was prepared by the reaction of 0.2 *M* phosphoramidate and 0.8 *M* hydrazine at pH 8.7 ± 0.05 for 20 min. at room temperature. Aliquots of this solution were added to buffers for measurement of hydrolysis rates by the ethylene glycol method. [b] Rate of inorganic phosphate appearance from phosphoramidate in the presence of the indicated concentration of hydrazine. Ionic strength maintained at 1.0 with KCl. [c] 0.2 *M* glycerophosphate buffer. [d] 0.1 *M* acetate buffer.

Reactions with Simple Primary Amines. Most primary amines do not react at an easily detectable rate with phosphoramidate because there is no pH value at which the amine exists as the free base and phosphoramidate exists as the monoanion. Rathlev and Rosenberg were unable to detect a reaction of phosphoramidate with amino acids.[7] However, a reaction can be observed with amines of relatively low basicity. Rate constants were measured for the reactions with ethylenediamine monocation, glycylglycine, and glycine by automatic titration or by measurement of the rate of ammonia release (Table I). These amines react only slightly faster than the less basic heterocyclic tertiary amines.

Reaction with Fluoride. Phosphoramidate reacts readily with fluoride,[6,11] but measurement of the dependence on pH of the rate of the reaction is difficult for technical reasons. The experiments shown in Table V, in which the pH of the buffer solutions was measured in duplicate reaction mixtures which did not contain fluoride, demonstrate clearly that the rate of the reaction increases with decreasing pH so that in the rate law of eq. 3, $k_1 >> k_2$. Between pH 7 and 8

$$\text{rate} = k_1[H_3{}^+NPO_3{}^-H][F^-] + k_2[H_3{}^+NPO_3{}^{2-}][F^-] \quad (3)$$

the rate of solvolysis of phosphoramidate is approximately doubled in the presence of 2 *M* potassium fluoride, and the reaction gives a constant yield of approximately 50% phosphorofluoridate. This indicates that the k_2 term of eq. 3 is significant under these conditions; however, the difficulty of pH determinations in these solutions prevented a quantitative evaluation of k_2. The previously reported leveling off of the rate with increasing fluoride concentration[11] may be attributed to an increase in the pH of the reaction mixtures at high fluoride concentrations. Under all experimental conditions the formation of phosphorofluoridate was found to increase regularly with the increase in rate of the reaction with fluoride, and there is no evidence for any formation of phosphorofluoridate which cannot be accounted for by the rate increase and might be attributed to trapping of a reactive intermediate.

Table V. Reaction of Phosphoramidate with 0.16 *M* Potassium Fluoride at 39°

Buffer pH	k_{obsd} × 10^3, min.$^{-1}$	(k_{obsd} − k_{hydrol}) × 10^3, min.$^{-1}$	Fraction phosphorofluoridate at end[a]
5.95[b]	8.5	3.5	24
5.45[b]	10.7	5.7	45
5.33[c]	11.6	6.6	46
4.69[c]	29.5	24.5	69
4.12[c]	61	56	83
3.65[d]	96	91	82

[a] Uncorrected for approximately 5% inorganic phosphate in the phosphoramidate. [b] 0.2 *M* succinate buffer. [c] 0.2 *M* acetate buffer. [d] 0.2 *M* formate buffer.

Reaction with Hydroxylamine. The following evidence indicates that the principal initial product of the reaction of phosphoramidate with hydroxylamine is O-phosphorylhydroxylamine, $^{2-}O_3PONH_2$. The reaction of hydroxylamine with phosphoramidate is thus analogous to that with activated acyl groups, in which a large fraction of the initial product is O-acylhydroxylamine rather than the hydroxamic acid.[23]

(a) In contrast to known phosphoramidates, but in agreement with the properties of phosphate esters, the product does not undergo rapid hydrolysis in the presence of molybdate and acid. The rate of product formation may, therefore, be determined by the rate of disappearance of material which reacts as inorganic phosphate in the ordinary Fiske and Subbarow phosphate determination. On prolonged incubation in

(23) W. P. Jencks, *J. Am. Chem. Soc.*, **80**, 4581, 4585 (1958).

acid molybdate, hydrolysis to inorganic phosphate does take place. Under the conditions of the Fiske–Subbarow phosphate determination, color development in the presence of this product at about 25° was found to follow first-order kinetics with a half-time of 68 min. and a rate constant of 0.0102 min.$^{-1}$.

(b) In contrast to phosphoramidates, but in agreement with the properties of phosphate esters, the product is stable at neutrality and in dilute acid, as well as in alkali, at room temperature. Between pH 4 and 7.4 the rate constant for hydrolysis of the product is less than 10^{-4} min.$^{-1}$ at 25°. In 0.1 *M* hydrochloric acid and in dilute alkali it undergoes decomposition over several days, at an irreproducible rate which is decreased by the presence of 10^{-4} *M* ethylenediaminetetraacetic acid.

(c) A product with the same properties is formed at a very similar rate from phosphoramidate and N-dimethylhydroxylamine (see below).

(d) In ethylene glycol at 100° the principal decomposition product is inorganic phosphate. In contrast, phosphoramidates undergo P–N cleavage to give a phosphate ester under these conditions. The mechanism of this decomposition is not known, but it appears that it proceeds with cleavage of the O–N bond of $^{2-}O_3PONH_2$ to give inorganic phosphate. The product from the reaction with N-dimethylhydroxylamine was found to give 85% inorganic phosphate in 90% ethylene glycol after 5 min. at 100°; the hydroxylamine product reacts more slowly, but was found to give inorganic phosphate even in 98% ethylene glycol.

(e) The presence of the unsubstituted NH_2 group of the hydroxylamine moiety of the product was demonstrated spectrophotometrically by reaction with furfural in aqueous solution. The product of this reaction exhibits an absorption maximum at 270 mμ, which is similar to the maxima of furfural oxime and furfural oxime methyl ether, at 268 and 275 mμ, respectively, but is different from the absorption maximum at 300 mμ of the nitrone formed from N-methylhydroxylamine and furfural.

Attempts to isolate $^{2-}O_3PONH_2$ were unsuccessful because of decomposition. The compound underwent decomposition in the presence of calcium or barium salts and upon lyophilization. However, aqueous solutions could be prepared by incubation of concentrated solutions of hydroxylamine and phosphoramidate at pH 7 and could be purified by chromatography on Dowex 1 ion-exchange resin.

The kinetics of the reaction of phosphoramidate with hydroxylamine were followed by measurement of the rate of disappearance of material (phosphoramidate) which reacts as phosphate in the Fiske and Subbarow method for phosphate determination. The readings were found to decrease to a constant level, but occasionally showed a subsequent slow increase, apparently caused by decomposition of the initial product. A given experiment was found to follow pseudo-first-order kinetics if the constant reading was taken as the end point. The pseudo-first-order rate constants increase with increasing hydroxylamine concentration and with an increase in the fraction of hydroxylamine present as the free base (Table VI). Similar results were obtained with N,N-dimethylhydroxylamine, which

Table VI. Initial Reaction of Hydroxylamine with Phosphoramidate at 25° and Ionic Strength 1.6

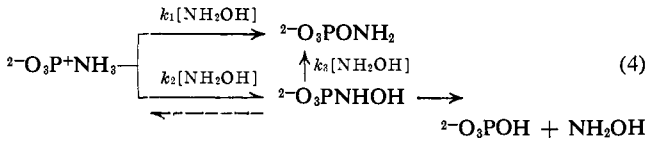
Total hydroxylamine, *M*	Fraction free base	k_{obsd},[a] min.$^{-1}$	k_2,[b] M^{-1} min.$^{-1}$
		NH_2OH	
1.6	0.1	0.24	0.15
1.6	0.2	0.38	0.24
1.6	0.4	0.77	0.48
1.6	0.6	0.75	0.47
0.4	0.8	0.28	0.70
0.8	0.8	0.46	0.57
1.6	0.8	0.79	0.50
		$(CH_3)_2NOH$	
0.2	0.5	0.11	0.52
0.4	0.5	0.19	0.48
1.0	0.5	0.37	0.37
0.2	0.8	0.11	0.57
0.4	0.8	0.24	0.61
0.8	0.8	0.47	0.58
1.2	0.8	0.70	0.58
		0.64	0.54

[a] Pseudo-first-order rate constant for the disappearance of material which reacts as inorganic phosphate in the Fiske and Subbarow determination. [b] $k_{obsd}/[NH_2OH]_{tot}$.

reacts at a rate very similar to that of hydroxylamine in spite of its lower basicity (0.8 p*K* unit[24]) and the possibility of steric hindrance, which would be expected from the presence of two methyl groups (Table VI). Extrapolation of the apparent second-order rate constants to 100% free base gives rate constants of approximately 0.8 and 0.65 M^{-1} min.$^{-1}$ for the reactions with phosphoramidate monoanion of free hydroxylamine and N,N-dimethylhydroxylamine, respectively. Extrapolation to 100% hydroxylammonium ion suggests the existence of a slower reaction which occurs at a rate proportional to the concentrations of hydroxylammonium ion and phosphoramidate monoanion with a rate constant of approximately 0.1 M^{-1} min.$^{-1}$. This reaction is probably better described mechanistically in terms of the kinetically equivalent reaction of free hydroxylamine with neutral phosphoramidate. These rate constants should not be accepted without reservation, because there are several complications in the kinetics. There is a tendency for the apparent second-order rate constants to decrease with increasing hydroxylamine concentration at a given fraction of hydroxylamine as the free base. Furthermore, the disappearance of Fiske and Subbarow reactive material does not go to completion at any hydroxylamine concentration, and at lower concentrations of hydroxylamine less O-phosphorylhydroxylamine is formed. The discrepancy is too large to be accounted for by the known rate of concurrent hydrolysis of phosphoramidate to inorganic phosphate. For example, the final values were found to be 37 and 15% of the original values in the reactions with 0.2 and 1.6 *M* hydroxylamine (80% as the free base), respectively. Experiments carried out in the presence of added ammonium ion showed that the incomplete reaction at low hydroxylamine concentrations is not an equilibrium. Analyses by the modified Martin–Doty method showed that the Fiske

(24) T. C. Bissot, R. W. Parry, and D. H. Campbell, *J. Am. Chem. Soc.*, 79, 796 (1957).

and Subbarow reactive material present at the end of the reaction is either inorganic phosphate or a compound which reacts as inorganic phosphate by both of these methods. These results are consistent with the mechanism shown in eq. 4. According to this mech-



(4)

anism, both the N- and the O-phosphorylated products are formed in the initial reaction of phosphoramidate with hydroxylamine. The N-phosphorylated product can undergo hydrolysis or can react further with hydroxylamine to form the stable O-phosphorylhydroxylamine. At low hydroxylamine concentration, the hydrolytic reaction would be expected to be of greater significance than at high hydroxylamine concentration and would account for the observed incomplete formation of O-phosphorylhydroxylamine. It is of interest that the relative stabilities of the O- and N-phosphorylhydroxylamines appear to be in the opposite order from those of the O- and N-acylhydroxylamines, presumably because of the much larger resonance stabilization of acyl- than of phosphoryl-amines.

The rate constants for the disappearance of phosphoramidate in the presence of 0.12 and 0.24 *M* glycine hydroxamic acid at pH 7.5 were found to be 0.0099 and 0.0165 min.$^{-1}$, respectively. Half of the rate increase was accounted for by the formation of a product which does not react as inorganic phosphate by the ethylene glycol or Fiske and Subbarow methods. The second-order rate constant calculated from these results for the reaction of phosphoramidate with the dipolar form of glycinehydroxamic acid, $H_3N^+CH_2CONHO^-$, is 0.10 M^{-1} min.$^{-1}$. These results suggest that at least part of the reaction of this compound involves attack of oxygen on phosphoramidate to give the O-phosphorylated hydroxamic acid.

Uncatalyzed Hydrolysis. The rates of hydrolysis of phosphoramidate in acetate buffer, pH 5.5 at 0, 25, and 39°, ionic strength 1.0, are given in Table VII. These rate constants are in reasonable agreement with those reported previously.[5-7] The thermodynamic activation parameters calculated from the data of Table VII are ΔH^* = 22,600 cal./mole and ΔS^* = −4.8 e.u.; these may be compared to the values of ΔH^* = 23,600 cal./mole and ΔS^* = −1.6 e.u. reported by Chanley and Feageson.[5]

Table VII. Uncatalyzed Hydrolysis of Phosphoramidate at Ionic Strength 1.0[a]

Temp., °C.	$k \times 10^3$, min.$^{-1}$
0	0.022
25	.78
39	5.0
ΔH^*	22,600 cal./moles[b]
ΔS^*	−4.8 e.u.[b]

[a] Acetate buffer, 0.1 *M*, pH 5.5. [b] Calculated from the equations $\Delta H^* = E_a - RT$; $\Delta F^* = -RT \ln kh/k_BT$; $\Delta S^* = (\Delta H^* - \Delta F^*)/T$; where E_a is the Arrhenius activation energy, h is Planck's constant, and k_B is Boltzmann's constant.

Other Compounds. No reactions could be detected with any of the compounds shown in Table VIII under the experimental conditions indicated. The reason for the absence of an observed reaction with several of these compounds is that the concentration of the reactive ionic species is too small to give a detectable rate of reaction at pH values at which phosphoramidate exists as the reactive monoanion. The rate of inorganic phosphate appearance was found to be increased severalfold in the presence of 0.1 and 0.5 *M* morpholine at pH 8.2, but we were unable to devise a satisfactory method to obtain accurate rate constants for the reaction of morpholine with phosphoramidate. Irregular small increases in the rate of inorganic phosphate formation from phosphoramidate were observed in the presence of methoxylamine, but the possibility was not ruled out that these were caused by traces of decomposition products of methoxylamine. The results indicated that the second-order rate constant for the reaction with methoxylamine is less than 0.1 M^{-1} min.$^{-1}$. Calcium or magnesium ions, 0.2 *M*, do not increase the rate of phosphoramidate hydrolysis. Chanley and Feageson have previously reported the absence of a detectable reaction of phosphoramidate with aniline, 2-methylpyridine, 2,6-dimethylpyridine, phosphate dianion, and picolinic acid anion.[5]

Table VIII. Compounds Which Do Not Undergo a Detectable Reaction with Phosphoramidate at 39°, Ionic Strength 1.0[a]

Compound	*M*	pH
Sodium azide	0.2	7.3
Phenol	0.3	7.6
Mercaptoethanol	0.2	5.5
Sodium sulfite	0.5	6.4
Trimethylamine N-oxide	0.5	5.9
Pyridine-2-aldoxime methiodide	0.12	7.6
Chloral hydrate	0.5	5.4
Sodium thiosulfate	0.33	4.6
Hydrogen peroxide	1.0	7.5, 5.1
Trifluoroethanol	1.4	7.6
Aniline	0.18	5.9
t-Butylamine	1.0	7.5
Methylamine	1.0	7.5
Sodium acetate	0.75	5.5
Magnesium chloride[b]	0.2	5.7
Calcium chloride[b]	0.2	5.4

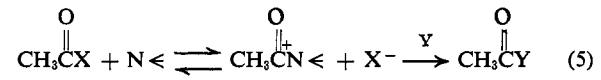
[a] No effect on the observed rate of hydrolysis of phosphoramidate. [b] Does not accelerate the rate of phosphoramidate hydrolysis.

Discussion

The order of nucleophilic reactivity toward phosphoramidate is generally similar to that toward the phosphorus atom of *p*-nitrophenyl phosphate; this topic will, therefore, be discussed with reference to both compounds in the following paper,[20] and the present discussion will be restricted to individual reactions with phosphoramidate.

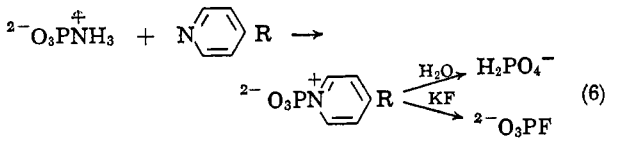
Tertiary Amines. Acyl transfer reactions from such activated acyl compounds as acetic anhydride, *p*-nitrophenyl acetate, and acylated phosphate esters are catalyzed by tertiary amines, such as pyridine and N-methylimidazole, with the intermediate formation of

the acylammonium cation (eq. 5).[25] This inter-



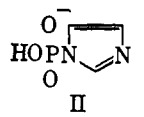
(5)

mediate is very unstable: acetyl-N-methylimidazolium cation has a half-life of 15 sec. at 25° and that of acetylpyridinium cation is too short to measure.[26] Synthetic reactions of phosphate compounds are often carried out in pyridine solutions, pyridine and other tertiary amines are known to catalyze phosphate transfer reactions, and a labile phosphorylpyridinium intermediate has often been postulated in such reactions but, except for the formation of some poorly characterized precipitates which contain pyridine and phosphate, the accumulation of phosphorylated pyridines analogous to acylpyridines has not been demonstrated previously.[2,3,5,7,27] The reactions of pyridines and triethylenediamine with phosphoramidate result in nucleophilic catalysis of phosphate transfer as well as hydrolysis, as shown by the formation of phosphorofluoridate in the presence of fluoride ion. The experiments reported here show that phosphorylpyridinium and phosphoryl-4-methylpyridinium ions are formed (eq. 6) and accumulate in the reactions



(6)

with phosphoramidate in aqueous solution; the half-time for the hydrolysis of phosphoryl-4-methylpyridinium ion is approximately 5 min. at 25° and is independent of pH in alkaline solution. A similar intermediate accumulates in the reaction with triethylenediamine. Phosphoryl-N-methylimidazolium ion is also formed from phosphoramidate and is much more stable, with a half-time for hydrolysis which is greater than that of the phosphoramidate monoanion. The formation of relatively stable phosphoryl tertiary amine compounds should not really be surprising, because the nitrogen atom is positively charged in phosphoramidate itself in neutral or slightly acidic solutions.[13,28] The rapid hydrolysis of the compounds formed from pyridines is presumably the result of the relatively electrophilic character of the weakly basic pyridine molecule and, possibly, of steric strain from the tertiary amine. The rapid hydrolysis of the phosphoryltriethylenediamine compound may be ascribed to steric strain and to the facile protonation of the second nitrogen atom of this compound to give a better leaving group (eq. 1). The stability of phosphoryl-N-methylimidazole may be ascribed to the distribution of the positive charge over two nitrogen atoms in this compound (I), which greatly decreases the leaving tendency of the imidazole group. The rate of hydrolysis of this compound is essentially the same as that of phosphorylimidazole in slightly acidic solution, in which the imidazole group of phosphorylimidazole is protonated; however, the rate of hydrolysis of phosphoryl-N-methylimidazole is independent of pH, while that of phosphorylimidazole approaches zero as a proton is lost from the imidazole group in alkaline solution. The facts that the rates of hydrolysis of the monoanions of phosphorylated imidazole and N-methylimidazole are essentially the same is evidence that the structure of the two compounds is similar (I); *i.e.*, it is evidence that the proton on the imidazole compound is on the heterocyclic ring (I) and not on one of the phosphate oxygen atoms (II). It is of interest that the kinetically determined pK of phos-



II

phorylimidazole is essentially the same as that of imidazole itself, which shows that, in contrast to acylimidazoles, there is no significant electron-withdrawing effect of the phosphate group on the imidazole ring. The facts that phosphorylimidazoles and even phosphorylimidazolium ions are formed so readily from phosphoramidate and undergo hydrolysis less rapidly than phosphoramidate indicate that there is no large resonance stabilization of the phosphoramidate by electron donation from the amide nitrogen atom. This is in marked contrast to acylamides, which have a free energy of hydrolysis some 11,000 cal./mole less negative than that of acetylimidazole[29] and which undergo hydrolysis several orders of magnitude less rapidly than acylimidazoles because of the large resonance stabilization of acylamides.

Phosphorylimidazole has been prepared previously and has been shown to react slowly with nucleophilic reagents; esters of phosphorylimidazole are, as might be expected, considerably more reactive toward nucleophilic attack and have been used as phosphorylating agents in synthetic work.[7,8,30] It is of some interest that a protein has been shown to become phosphorylated on the imidazole group of a histidine residue during oxidative phosphorylation; recent experiments indicate that the protein is succinate thiokinase, and that reversible phosphate transfer to this imidazole group may be part of the normal catalytic mechanism of action of this enzyme.[21,31] An enzyme which catalyzes phosphoryl transfer from phosphoenolpyruvate to hexose reacts to form an intermediate phosphoryl-

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(27) (a) T. Wagner-Jauregg and B. E. Hackley, Jr., *ibid.*, **75**, 2125 (1953); (b) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **81**, 4660 (1959); (c) R. Letters and A. M. Michelson, *J. Chem. Soc.*, 71 (1962); (d) W. F. Gerrard, *ibid.*, 1464 (1940); (e) W. Gerrard, *ibid.*, 106 (1945); (f) W. E. Wehrli, D. L. M. Verheyden, and J. G. Moffatt, *J. Am. Chem. Soc.*, **86**, 1254 (1964); (g) B. M. Zeffert, P. B. Coulter, and R. Macy, *ibid.*, **75**, 751 (1953).

(28) E. Hobbs, D. E. C. Corbridge, and B. Raistrick, *Acta Cryst.*, **6**, 621 (1953); D. E. C. Corbridge and E. J. Lowe, *J. Chem. Soc.*, 493 (1954); E. W. Crunden and R. F. Hudson, *ibid.*, 3591 (1962).

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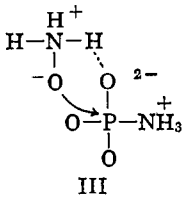
(31) (a) M. DeLuca, K. E. Ebner, D. E. Hultquist, G. Kreil, J. B. Peter, R. W. Moyer, and P. D. Boyer, *Biochem. Z.*, **338**, 512 (1963); (b) R. A. Mitchell, L. G. Butler, and P. D. Boyer, *Biochem. Biophys. Res. Commun.*, **16**, 545 (1964); (c) G. Kreil and P. D. Boyer, *ibid.*, **16**, 551 (1964).

enzyme, and phosphorylhistidine has been isolated from hydrolysates of the phosphoryl-enzyme.[32]

Phosphorylated tertiary amines may be useful phosphorylating agents for synthetic work because of their high reactivity and, in particular, the fact that they remain reactive in alkaline solution under conditions in which other phosphoramidates lose a proton and become unreactive. Diphosphorylimidazole may be regarded as a compound of this general type and has been shown to be an effective phosphorylating agent for basic amines, which do not react readily with phosphorylimidazole.[27a] The high reactivity of N-phosphoryl-4-methylpyridinium ion in alkaline solution is shown by its complete reaction with 0.5 *M* methylamine in less than 1 min. at pH 11.7.

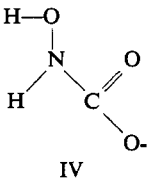
Hydroxylamine. The reaction of hydroxylamine with phosphoramidate, like that with activated acyl compounds,[23] occurs principally at the oxygen rather than the nitrogen atom of hydroxylamine. It is known that the anions of hydroxamic acids, oximes, and related compounds are phosphorylated on oxygen by toxic, fully substituted phosphorylating agents, and may undergo a subsequent rearrangement.[30b,33] Hydroxylamine itself reacts with isopropyl methylphosphonofluoridate and undergoes subsequent decomposition; it has been suggested that this reaction also occurs on oxygen because O-substituted hydroxylamines do not react readily with this compound,[34] but the possibility has not yet been ruled out that the absence of a measurable reaction with O-substituted hydroxylamines may have some other cause, such as the low basicity of such compounds. The rate constant for the reaction of the hydroxyl group of hydroxylamine with phosphoramidate is approximately 0.5 M^{-1} min.$^{-1}$, which is some 5000 times faster than that of the reaction with water if the hydrolysis is treated as a second-order reaction. Although this is smaller than the corresponding rate difference for the reaction with *p*-nitrophenyl acetate,[23] it is still a considerable rate acceleration, in view of the low basicity of the hydroxyl group of hydroxylamine, and suggests that the reaction on oxygen does not proceed by unassisted attack of the free hydroxyl group. A reasonable mechanism involves reaction of the dipolar form of hydroxylamine, $H_3N^+O^-$, with assistance by hydrogen bonding to a phosphoryl oxygen atom (III), as suggested for the analogous reaction with acyl groups.[23,35]



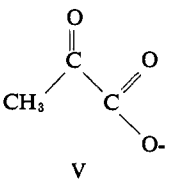
III

A similar mechanism may be suggested for the reaction with glycine hydroxamic acid. Acetate ion, which cannot react by such a mechanism, does not react at a detectable rate with phosphoramidate. Assistance by hydrogen bonding has been suggested for many reactions of phosphate compounds, but certainly does not occur in all cases in which it has been suggested; a fairly strong case may be made for such a concerted mechanism in the reaction of isopropyl methylphosphonofluoridate with catechols.[33c,36]

A phosphorylhydroxylamine is formed from adenosine triphosphate and hydroxylamine in the presence of bicarbonate in a reaction catalyzed by pyruvate kinase.[37] This compound does not hydrolyze in 7 min. in the presence of acid molybdate under the conditions of the Fiske and Subbarow phosphate determination, but does react as inorganic phosphate after 2 hr.[37] The similarity in properties suggests that this compound is O-phosphorylhydroxylamine rather than the N-phosphoryl compound, which would be expected to undergo very rapid hydrolysis in acid. A plausible mechanism for the enzymatic formation of O-phosphorylhydroxylamine involves the nonenzymic formation of the carbamate (IV), which may bind to the pyruvate (V) site of the enzyme and become phosphorylated on oxygen.

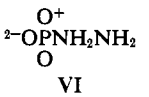


IV

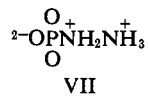


V

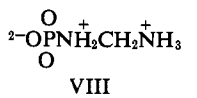
Other Reactions. The phosphorohydrazidate formed from phosphoramidate and hydrazine undergoes hydrolysis near neutral pH in a pH-independent reaction at a rate which is somewhat faster than that of phosphoramidate, but undergoes hydrolysis with increasing rapidity with increasing acidity. These hydrolyses presumably represent reactions of the species VI and VII, respectively. It has previously been reported that



VI



VII



VIII

phosphorohydrazidate itself is unstable in acid, but that the hydrazide of a phosphate ester is relatively stable in acid solution.[38] The hydrolysis of VII is similar to the hydrolysis of phosphoramidate potentiated by formaldehyde and amines, which has been suggested to proceed through the intermediate VIII.[13] In both cases it is probable that the reaction approaches the character of a monomolecular reaction, although free metaphosphate monoanion is not an intermediate.

The reaction of fluoride with phosphoramidate to form phosphorofluoridate at slightly acidic pH, which was previously attributed to a reaction with phosphoramidate monoanion,[6,11] actually involves the species of phosphoramidate which carries no net charge, and

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shows that this species is susceptible to nucleophilic attack. A preferred reaction with this species is not unreasonable, in view of the negative charge of the fluoride ion. However, a slow reaction of concentrated fluoride with phosphoramidate monoanion is detectable at neutral or slightly alkaline pH, and an analogous reaction must account for the formation of phosphorofluoridate from the intermediates in the reactions of phosphoramidate with pyridine and triethylenediamine.

The Reactivity of Nucleophilic Reagents toward the *p*-Nitrophenyl Phosphate Dianion[1]

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The nucleophilic reactivity of a series of amines toward the p-nitrophenyl phosphate dianion displays only a very small sensitivity to the basicity of the amine. Cationic substituents on the amine cause an enhanced reactivity, which is ascribed to an electrostatic effect. Substituents which introduce steric hindrance decrease nucleophilic reactivity. The rate constant for the hydrolysis of p-nitrophenyl phosphate dianion falls on the same line as those of benzoyl phosphate dianions in a logarithmic plot against the pK of the leaving group. It is concluded that the principal driving force for the bimolecular reactions of phosphoramidate monoanion, acetyl phosphate dianion, and p-nitrophenyl phosphate dianion arises from electron donation from the oxygen atoms and electron withdrawal by the leaving group, with little bond formation by the nucleophilic agent.

As a continuation of the experiments described in the previous paper of this series, the reactivity of a number of nucleophilic reagents toward the dianion of *p*-nitrophenyl phosphate has been determined. This substrate has the advantage that its reaction rate may easily be followed spectrophotometrically and its reaction with basic amines may be studied in alkaline solution.

Experimental

Disodium *p*-nitrophenyl phosphate was obtained from the Aldrich Chemical Co., and proved to be a hydrated mixture of the salt with inorganic phosphate. Each lot was assayed by spectrophotometric measurement of *p*-nitrophenol release on complete acid hydrolysis[2] and was found to contain from 65 to 70% of *p*-nitrophenyl phosphate, and less than 0.1% of *p*-nitrophenol. This salt, which is similar to that used in other kinetic investigations,[3] was used without further purification. Amines and their hydrochlorides were redistilled or recrystallized before use. Water and deuterium oxide were glass distilled. Reagent grade inorganic salts were used without further purification.

Initial rates of *p*-nitrophenolate release were followed spectrophotometrically at 400 mμ using a Zeiss PMQ II spectrophotometer equipped with a thermostated cell compartment. Solutions were brought to 39.0° in a water bath before mixing. Reactions were started by the addition of 0.5 ml. of a freshly prepared solution, 0.03–0.06 *M* in *p*-nitrophenyl phosphate, to 2.5 ml. of reaction mixture. The ionic strength was maintained at 1.0 with potassium chloride.

The pH of the reaction mixture was measured at room temperature at the end of each run. With the exception of certain experiments with ethylenediamine cation, the pH of the solutions was always more than one pH unit above the pK_a of *p*-nitrophenol (7.1). However, for all reactions in which the final pH was below 10, the degree of ionization of the product was determined by measuring the absorbance at 400 mμ of 1.0 ml. of a standard solution of *p*-nitrophenol added to a further 5.0 ml. of reaction mixture, and the result was used to calculate the amount of *p*-nitrophenol formation from the spectrophotometric measurements of the rate experiments.

First-order rate constants were calculated from the slopes of the linear plots of optical density against time, by converting to concentration units and dividing by the initial concentration of phosphate ester. (This was calculated using ϵ 18,320 for *p*-nitrophenolate ion at 400 mμ,[4] but the conversion from optical density to concentration units also involves this constant, and the rate constants obtained do not depend directly on a particular value for the extinction coefficient.)

The observed first-order rate constants were corrected for the rates of uncatalyzed hydrolysis, which were obtained from the intercepts of plots of rate against amine concentration or from independent measurements of the rate of hydrolysis in the absence of amine (Figure 1). No precautions were normally taken to exclude light[5] or metal ions, but it was shown that the hydrolysis rates are identical in the dark, in the presence and absence of 10^{-4} *M* ethylenediamine-

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