Hydrolysis of Phosphoroguanidines. A Model System for Phosphorylation by Phosphorocreatine[1]

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Abstract: The phosphagens are naturally occurring phosphoroguanidinates whose biological role involves the phosphorylation of ADP to give ATP. In an effort to elucidate the mechanism of the *in vivo* phosphorylation, an *in vitro* investigation of the fundamental chemistry of several simple phosphoroguanidines was undertaken. The hydrolyses of *N,N*-dimethyl-*N'*-phosphoroguanidine (DMPG) and *N*-benzyl-*N*-methyl-*N'*-phosphoroguanidine (BMPG) yield inorganic phosphate and the corresponding guanidinium ion. A bell-shaped pH–rate profile with a rate maximum at pH 2 was obtained for both DMPG and BMPG. The rate law describing this behavior is: rate $= k_0[N]$, where [N] is the concentration of the neutral form of the phosphoroguanidine. At 30.5° the values for the first-order rate constant, k_0, are 3.70×10^{-2} and 2.54×10^{-2} min^{-1} for DMPG and BMPG, respectively. The acidity constants for DMPG are: $K_a^{I} = 2.04$ and $K_a^{II} = 4.95 \times 10^{-5}$ (determined kinetically); $K_a^{II} = 4.9 \times 10^{-5}$ and $K_a^{III} = 5.0 \times 10^{-12}$ (determined potentiometrically). The values for the solvent deuterium isotope effect, $k(H_2O)/k(D_2O)$, are 0.90 and 0.86 and the values for the entropy of activation, ΔS^*, are +0.4 and −1.2 eu for DMPG and BMPG, respectively. The cationic and anionic forms were found to be at least 10^4 times less reactive than the neutral forms, and the monobenzyl ester of neutral DMPG was found to hydrolyze at least 10^4 times more slowly than neutral DMPG. These results support a mechanism of hydrolysis with the neutral form of phosphoroguanidines as the reactive species and hydrolysis *via* a unimolecular decomposition to metaphosphate ion, PO_3^-, and guanidinium ion. The most likely mechanism appears to involve proton transfer and formation of a highly reactive double zwitterion which very rapidly produces PO_3^- in the rate-determining step. Although proton transfer may be part of the rate-determining step, the proton must be nearly completely transferred in the transition state. These results are related to the mechanism of biochemical phosphorylation by phosphorocreatine.

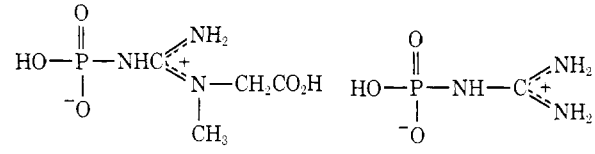
The term phosphagen was introduced in 1927[2] to describe an acid-labile, phosphorus-containing compound detectable in frog skeletal muscle. The phosphagen was isolated and purified by Fiske and Subbarow,[3] and shown to contain phosphate and creatine in a mole ratio of 1:1. These workers correctly formulated the compound as *N*-phosphorocreatine, PC (**1**).

Although other phosphagens are found in certain species, PC is the only phosphagen present in detectable quantities in vertebrates, and it is distributed in detectable amounts in most vertebrate tissue.[4] The amount of PC present in the various tissues varies greatly, and skeletal muscles contain much more PC than any other tissue. PC plays an important role in muscle by "buffering" the concentration of ATP. During muscular contraction,[5] even to the point of rigor, the concentration of ATP remains constant because ADP is phosphorylated by PC in a rapid, enzyme-catalyzed reaction.[5,6]

$$H^+ + ADP + PC \overset{Mg^{2+}}{\rightleftharpoons} ATP + \text{creatine} \qquad (1)$$

The phosphagens are derivatives of *N*-phosphoroguanidine (**2**). In 1938 Zeile and Fawaz[7] synthesized PC and isolated various salts. They reported that in alkaline solution PC was stable, but underwent rapid hydrolysis in dilute acid. Other workers[1,8-10] have shown that the rates of hydrolysis of PC and phosphoroarginine exhibit maxima from pH 1 to 3.

It is the purpose of this work[10] to understand the nature of the phosphorylation reactions of the phosphagens and of phosphoroguanidines in general. With this in mind, we studied the hydrolysis (phosphorylation of water) of two simple phosphoroguanidines, *N,N*-dimethyl-*N'*-phosphoroguanidine, DMPG (**3**), and *N*-benzyl-*N*-methyl-*N'*-phosphoroguanidine BMPG (**4**).



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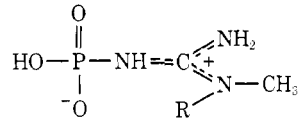


3. DMPG (R = CH_3)
4. BMPG (R = $CH_2C_6H_5$)

Results

pH–Rate Profiles. The rates of hydrolysis of DMPG and BMPG at 30.47 ± 0.05° and at various pH values in buffered solutions (μ = 0.20 *N*) were measured spectrophotometrically by observing the decrease in absorbance of the starting phosphoroguanidine or by determining the amount of inorganic phosphate liber-

(1) Research supported by Grant AM-12743 from the National Institutes of Health; communicated in part in preliminary form in *Proc. Nat. Acad. Sci. U. S.*, **68**, 2691 (1971).
(2) P. Eggleton and G. P. Eggleton, *Biochem. J.*, **21**, 190 (1927).
(3) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **81**, 629 (1929).
(4) A. H. Connor and H. Rosenberg, *Aust. J. Sci.*, **15**, 214 (1953).
(5) (a) H. E. Hurley, *Science*, **164**, 1356 (1969); (b) S. A. Kuby and E. A. Noltmann, *Enzymes, 2nd Ed.*, **6**, 515 (1962).
(6) A. S. Mildvan and M. Cohn, *Advan. Enzymol.*, **33**, 1 (1970); M. Cohn and J. Reuben, *Accounts Chem. Res.*, **4**, 214 (1971).
(7) K. Zeile and G. Fawaz, *Hoppe-Seyler's Z. Physiol. Chem.*, **256**, 193 (1938).
(8) K. Lohmann, *Biochem. Z.*, **194**, 306 (1938).
(9) (a) V. M. Clark, A. R. Todd, and S. G. Warren, *Biochem. Z.*, **338**, 591 (1963); (b) V. M. Clark and A. R. Macrea, private communication.
(10) G. W. Allen, *Diss. Abstr. B*, **32**, 2573 (1971).

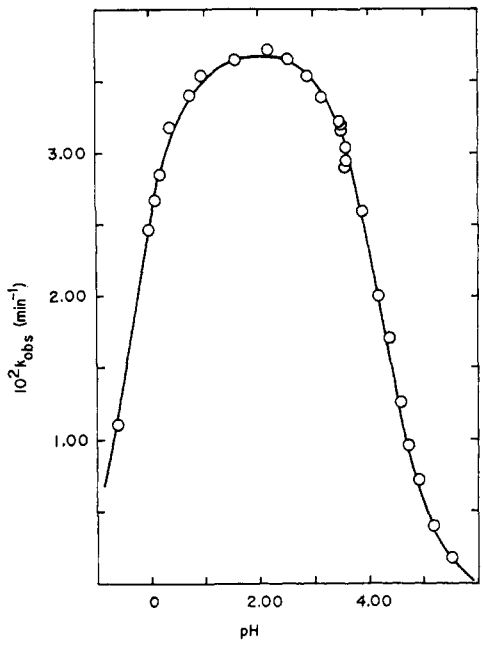


Figure 1. The pH–rate profile for the hydrolysis of DMPG at 30.5°. The curve was calculated (see text); the points are experimental.

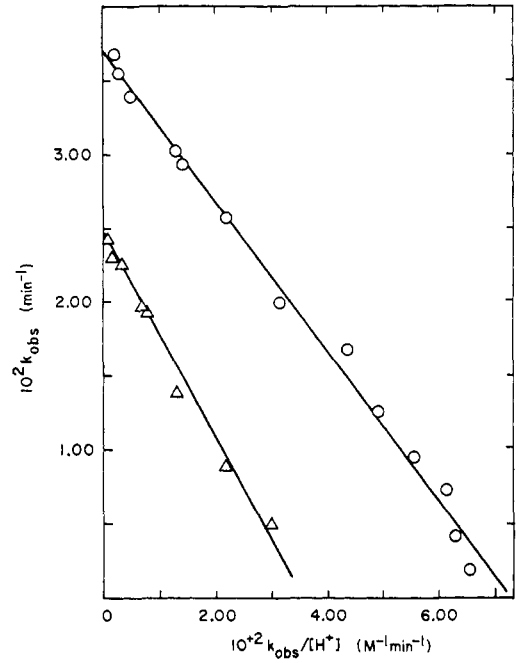


Figure 2. Plots of k_{obsd} against $k_{obsd}/[H^+]$ for the hydrolysis of DMPG and BMPG at 30.5° (μ = 0.20 N) for pH values greater than 2.5: (○) DMPG; (△) BMPG.

ated. The only products of the hydrolyses were the corresponding guanidinium phosphates. The observed first-order rate constants for the hydrolysis of DMPG and BMPG demonstrate that the rate of hydrolysis at a given pH is independent of: (1) buffer concentration, (2) ionic strength, and (3) analytical method used.[10]

The observed first-order rate constants are plotted against pH in Figure 1 for DMPG (similar plot, same pH_{max} for BMPG).[10] The bell-shaped curve that these points define is characteristic of a reaction involving two ionizations,[11] as shown in eq 2, with AH being the reactive species.

$$AH_2^+ \overset{K_a^I}{\rightleftharpoons} AH + H^+ \overset{K_a^{II}}{\rightleftharpoons} A^- + 2H^+ \qquad (2)$$

The rate equation for this scheme is

$$\text{rate} = k_{obsd}[A]_{total} = k_0[AH] \qquad (3)$$

thus[11]

$$k_{obsd} = \frac{k_0}{1 + [H^+]/K_a^I + K_a^{II}/[H^+]} \qquad (4)$$

At pH values greater than 2.5, $K_a^I \gg [H^+]$, and eq 4 can be rewritten as

$$k_{obsd} = k_0 - k_{obsd}/[H^+]K_a^{II} \qquad (5)$$

A plot of k_{obsd} against $k_{obsd}/[H^+]$ should be linear and yield K_a^{II} as the negative slope and k_0 as the ordinate intercept (Figure 2).

At pH values less than 1.5, $K_a^{II} \ll [H^+]$, and eq 4 can be rewritten as[11]

$$k_{obsd} = k_0 - k_{obsd}[H^+]/K_a^I \qquad (6)$$

A plot of k_{obsd} against $k_{obsd}[H^+]$ should be linear and yield K_a^I as the negative inverse of the slope, and k_0 as the ordinate intercept (Figure 3).

(11) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, Chapter 11.

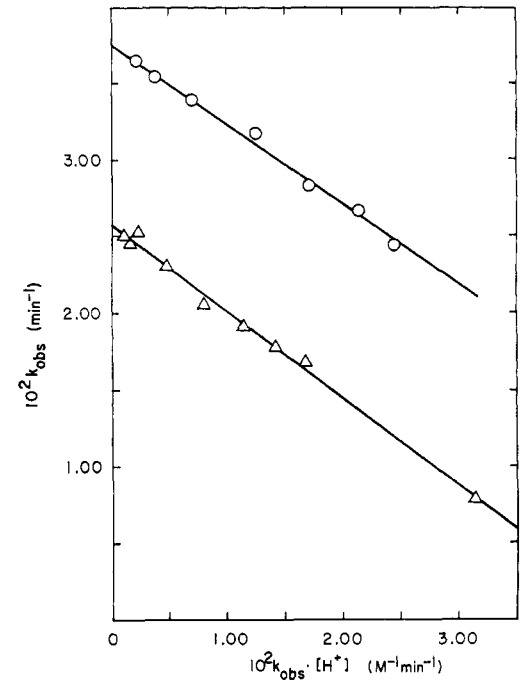


Figure 3. Plots of k_{obsd} against $k_{obsd}[H^+]$ for the hydrolysis of DMPG and BMPG at 30.5° (μ = 0.20 N) for pH values less than 1.5: (○) DMPG; (△) BMPG.

The constants obtained from the above treatment are collected in Table I. The k_0 values obtained at pH <1.5 and at pH >2.5 agree favorably. The K_a's correspond to $pK_a^I = -0.31$ and $pK_a^{II} = 4.31$ for DMPG, and $pK_a^I = -0.26$ and $pK_a^{II} = 4.16$ for BMPG. The values in Table I were used with eq 4 to construct the theoretical curve in Figure 1. The agreement between the experimental points and the theoretical curves is excellent. The hydrolysis scheme of DMPG and BMPG can therefore be formulated as shown in eq 7.

Table I. Constants Derived from Kinetic Treatment

Compd	K_a^I	$10^5 K_a^{II}$	$10^2 k_0$, min^{-1}[a] at pH >2.5	at pH <1.5	Av
DMPG	2.04 ± 0.25[a]	4.95 ± 0.43	3.66 ± 0.03	3.74 ± 0.04	3.70 ± 0.03
BMPG	1.81 ± 0.60	7.00 ± 2.64	2.52 ± 0.03	2.55 ± 0.02	2.54 ± 0.02

[a] Errors given as average deviation.

$$\underset{5}{HDMPG^+} \overset{K^I}{\rightleftharpoons} DMPG \overset{K^{II}}{\rightleftharpoons} \underset{6}{[DMPG - H^+]^-} \quad (7)$$

$$\downarrow$$

$$H_2PO_4^- + \underset{7}{[(H_2N)_2CN(CH_3)_2]^+}$$

Potentiometric Titrations. In order to check the validity of the kinetic treatment, the ionizations of DMPG and BMPG were determined potentiometrically. The kinetic analysis indicates that DMPG and BMPG should exhibit pK_a's of 4.31 and 4.16, respectively, in the range where ionization constants can be determined potentiometrically.

Titrations were carried out under the same conditions as the kinetic runs. Since neutral DMPG and BMPG are labile, the titrations were done starting with the corresponding dianions. There are two ionizations apparent for both DMPG and BMPG. The data were analyzed by the methods outlined by Albert and Serjeant[12] and the results are presented in Table II.

Table II. Potentiometric pK's[a]

Compd	pK_a^{III}	pK_a^{II}
DMPG	11.30 ± 0.10	4.31 ± 0.03
BMPG	11.5 ± 0.17	4.33 ± 0.01

[a] The ± values are average deviations.

There is excellent agreement with the kinetic determination in Table I.

Activation Parameters. The rates of hydrolysis of DMPG and BMPG at pH 3.61 were determined as a function of temperature. Although the variation of the pK_a's of DMPG and BMPG with temperature is not known, the pK_a's of H_3PO_4 are virtually invariant over the temperature range used in this work;[13] therefore, we assumed that pK_a^{II} is constant over the temperature range employed, so Table I and eq 4 were used to evaluate k_0. The parameters are in Table III.[14] Note that six rates over a 25° interval were used to obtain the parameters for DMPG.

Solvent Deuterium Isotope Effect. Since the solutions were prepared somewhat differently (Experimental Section) from the solutions used in other kinetic runs, two solutions, one containing H_2O and the other D_2O, were identically prepared. The k_{obsd}'s were converted to the specific rate constants, k_0's, by use of eq 4, the dissociation constants in Table I, and the following assumptions.[15] (1) Since the second pK_a of H_3PO_4 increases by +0.56 on going from H_2O to D_2O, the same increase is assumed for pK_a^{II} of DMPG and BMPG on going from H_2O to D_2O, so that p$K_a^{II}(D_2O)$ = p$K_a^{II}(H_2O)$ + 0.56. (2) pD = pH + 0.40. The results are presented in Table IV.

Table III. Activation Parameters for the Hydrolysis of DMPG and BMPG[a]

Compd	E_a, kcal/mol	ΔH^*, kcal/mol	ΔF^*, kcal/mol	ΔS^{**}, eu
DMPG[b]	22.9	22.3	22.2	0.4
BMPG[c]	22.7	22.1	22.4	−1.2

[a] Standard state taken as 1 M and 25°. [b] Measurements at the following temperatures (°C) with $10^4 k_{obsd}$ (sec^{-1}) in parentheses: 44.8 (27.89), 39.9 (15.55), 35.3 (9.15), 30.5 (4.86), 24.9 (1.55), and 20.5 (1.39). [c] Measurements at the following temperatures (°C) with $10^6 k_{obsd}$ (sec^{-1}) in parentheses: 51.6 (37.6, 37.2), 41.4 (11.95), 30.5 (3.22).

Table IV. Solvent Deuterium Isotope Effect for the Hydrolysis of DMPG and BMPG in H_2O and D_2O, 0.10 M Acetate Buffer, T = 30.47 ± 0.05°, μ = 0.20 N in NaCl

Compd	Solvent	pH[a]	pD	$10^2 k_{obsd}$, min^{-1}	$10^2 k_0$, min^{-1}	$k_0^{H_2O}/k_0^{D_2O}$
DMPG	H_2O	3.95		2.54	3.66	0.90
	D_2O[b]	4.04	4.44	3.00	4.10	
BMPG	H_2O	3.61		1.93	2.48	0.86
	D_2O[b]	3.75	4.15	2.29	2.91	

[a] Measured at 30.5°. [b] 99.0% D_2O.

Hydrolysis of DMPG in the Presence of Cysteine. It has been reported that the hydrolysis of *N*-phosphoroguanidinic acid is subject to "marked acceleration" in the presence of cysteine at pH 6.5 and 50°.[9] Since there is a free sulfhydryl group at the active site of ATP, creatine phosphotransferase,[5] this report could be significant. Therefore, the hydrolysis of DMPG in the presence of cysteine was studied at several pH's and temperatures (Table V). The k_{obsd} values (Table V) are within experimental error of those calculated for no catalysis.

Stability of DMPG Monoanion. In order to determine the stability of DMPG monoanion, a 1.00×10^{-3} M solution of DMPG was titrated to pH 9.5 and kept at 30.5°. The absorbance was measured at t = 0 and t = 30 days. The change in absorbance indicated a 12% decomposition of DMPG. This corresponds to a first-order rate constant of 3×10^{-6} min^{-1}. This is within experimental error of the constant expected at pH 9–9.5 for hydrolysis through the neutral species and indicates that the monoanion hydrolyzes at least 10^4 times more slowly than the neutral species.

Stability of the Monobenzyl Ester of DMPG. The stability of the monobenzyl ester of DMPG (**8**) in a solution of dilute H_2SO_4 was determined. At pH 1.87

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

(13) W. M. Latimer, "Oxidation Potentials," 2nd ed, Prentice-Hall, Englewood Cliffs, N. J., 1952, p 106.

(14) Calculated according to S. W. Benson, "The Foundations of Chemical Kinetics," McGraw-Hill, New York, N. Y., 1960, Chapter 4.

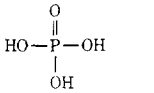
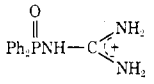
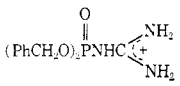
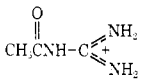
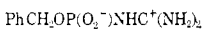
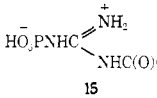
(15) A. O. McDougall and F. A. Long, *J. Phys. Chem.*, **66**, 429 (1962); P. Haake and L. P. Bausher, *J. Amer. Chem. Soc.*, **91**, 1113 (1969).

Table V. Hydrolysis of DMPG in the Presence of Cysteine, μ = 0.20 *N* in NaCl

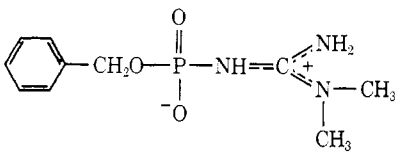
T, °C	pH[b]	Buffer	$10^2 k_{obsd}$, min^{-1}	$10^2 k_{obsd}$, min^{-1}, calcd[a]
30.47	3.00	0.01 *M* Cysteine	3.36	3.50 ± 0.02
30.47	7.20	0.10 *M* Cysteine	0.0067	0.0047 ± 0.0005
50.12	6.17	0.04 *M* Cysteine	0.57	0.49 ± 0.05
50.12	6.01	0.04 *M* Imidazole	0.86	0.70 ± 0.06

[a] Errors given as average deviations, calculated assuming no catalysis. [b] Measured at appropriate temperature.

Table VI. pK_a Values

Compd		pK_a	Ref
	I	2.12	
	II	7.21	12
	III	12.67	
	I	−0.31	
5	II	4.31	This research
	III	11.3	
7		13.6	*a*
		4.8 (50% MeOH-H_2O)	This research
9			
		4.7 (50% MeOH-H_2O)	9
10			
		8.33	*b*
11			
		7.4	9
14			
		3.62 8.88	This research
15			

[a] S. J. Angyal and W. K. Warburton, *J. Chem. Soc.*, 2492 (1951). [b] A. Albert, R. Goldacre, and J. Phillips, *ibid.*, 2240 (1948).



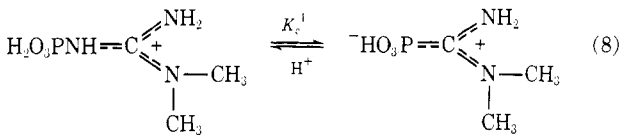
8

and 30.5°, the initial absorbance of a 1.00 × 10^{-3} *M* solution of **8** was unchanged after 7 days. A phosphate analysis of this same solution after 7 days indicated no inorganic phosphate had been liberated. Allowing for experimental error (see Experimental Section), the maximum amount of P_i that could have been present is 5 × 10^{-6} *M*, which would correspond to 0.5% decomposition. This gives a maximum rate constant of 5 × 10^{-6} min^{-1} for the hydrolysis of **8** at pH 1.87 and 30.5°. In fact, the rate constant for P–N cleavage of **8** is probably much smaller, because this is a limit and any phosphate that is produced probably ensues after initial cleavage of the benzyl ester.

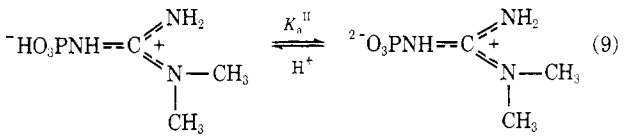
Discussion

Ionization Behavior. The study of phosphoroguanidines and, in general, all monofunctional phosphate derivatives is complicated by multiple ionizations. In the case of phosphoroguanidines, the basicity of the guanidine moiety can cause zwitterion formation. Experimentally, three acid dissociations of DMPG and BMPG are evident. The pK_a's for DMPG are −0.31, 4.31, and 11.3 (Table VI). The first pK_a was determined kinetically while the other two were determined potentiometrically. In view of the pH–rate profile (Figure 1), it is important to assign these experimental pK_a's to the three ionizable protons: two P–OH protons and a guanidinium NH proton.

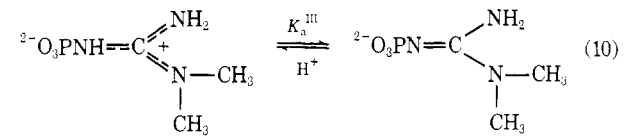
The first ionization, pK_a^I = −0.31, must be due to a P–OH ionization (eq 8). The difference from pK_a^I



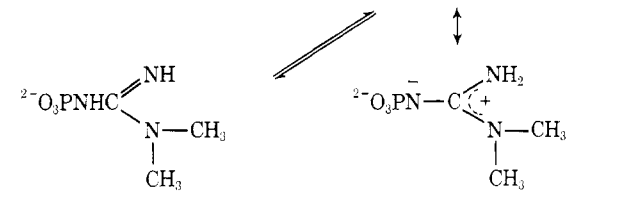
5 3 (8)



3 6 (9)



6 12 (10)



13 12a

for H_3PO_4, ΔpK = 2.4, is a reasonable acid-strengthening effect due to the guanidinium cation substituent on phosphorus. The pK_a (Table VI) of **10** demonstrates that K_a^I for **5** cannot be dissociation of a P–NH proton. For these reasons the structures of all neutral phosphoroguanidines are written as in **3** and **4**.

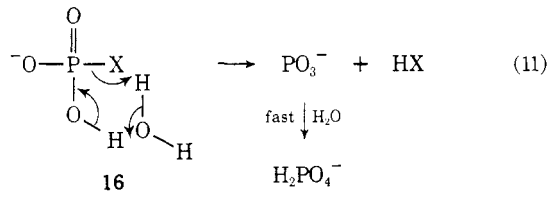
The second dissociation of DMPG (pK_a^{II} = 4.31) must also correspond to a P–OH → P–O^- (eq 9) dissociation for the following reasons. (1) The difference between pK_a^I and pK_a^{II} (ΔpK = 4.6) is about that expected due to the difference in charge in the two equilibria, in H_3PO_4, ΔpK_a = 5. (2) If it were an NH proton that dissociated, the charge difference from **10** and the pK_a of **14** would lead one to expect $pK_a^{II} > 7$ for DMPG. This is clearly too divergent from the observed pK_a^{II} to be due to dissociation of an NH proton. (3) The ΔpK_a^I = 2.4 for the guanidinium substituent ($pK_a^I(H_3PO_4)$ − pK_a^I(DMPG) = 2.4) is approximately

valid for pK_a^{II} as well and indicates that pK_a^{II} represents dissociation of the second P–OH group.

Since pK_a^{I} and pK_a^{II} represent P–OH dissociations, pK_a^{III} must represent dissociation of an NH proton (eq 10). This also fits the other data in Table VI; most notably pK_a^{III}(DMPG) − pK_a (**14**) = 4 is about what one expects for the electrostatic difference between the anion of DMPG and neutral **14** on the basis of the electrostatic effect on the pK's of H_3PO_4. It is probably **12** rather than **13** that predominates in eq 10, since the electronic interaction between phosphorus and nitrogen, with its contribution from **12a**, would make **12** more stable than **13**. However, although P–NH dissociation must predominate in **11**, **9**, and **10**, **13** could be the favored tautomer for $[DMPG - 2H^+]^{2-}$ due to the double negative charge on the phosphate residue which would provide an unfavorable electrostatic repulsion with **12** ↔ **12a**.

Reactive Species. It is apparent from the maxima in the pH–rate profiles at pH 2 for the hydrolyses (Figure 1) that the neutral zwitterion is the most reactive form of these phosphoroguanidines.

The occurrence of a maximum in the pH–rate profile is characteristic of many monofunctional derivatives of phosphoric acid. This phenomenon was first noted by Bailly and Desjobert[16,17] for the hydrolysis of monoalkyl phosphates. These workers suggested that the maximum at pH 4 was due to some facile reaction of the monoanion, which is the predominant ionic species of most phosphate esters between pH 2 and 6. This view has since received considerable support[18–23] in terms of a metaphosphate intermediate, PO_3^- (eq 11),



(11)

which is very reactive toward water. Acyl phosphates,[24] phosphoramidate monoanions,[25] and *N*-acyl phosphoramidates[26] have also been postulated to hydrolyze *via* this metaphosphate mechanism, which, as eq 11 indicates, requires a dissociable P–OH proton and a negative charge on the phosphate portion of the molecule. The pH–rate profiles for DMPG and BMPG support a metaphosphate mechanism because only the species with structure **16** is reactive. Phosphoroguanidines appear to be among the most reactive phosphates which hydrolyze by a metaphosphate mechanism.[9,24–28]

Requirement for a Dissociable Proton on the Phosphoryl Moiety. In the case of alkyl phosphate monoanions, the requirement for a dissociable proton is absolute. The replacement of the proton of the monoanion of methyl phosphate (**16**, X = OMe) by a methyl group results in at least a 10^4-fold decrease in the rate of hydrolysis.[20,21] The dianion of methyl phosphate is very stable, being hydrolyzed only in very strong alkali.[20,21] The dianion of 2,4-dinitrophenyl phosphate, however, is hydrolyzed at a faster rate than the corresponding monoanion.[28] This behavior is attributed to the fact that the leaving group, 2,4-dinitrophenoxide, is an easily expelled, stable anion. Similar behavior is observed in the hydrolysis of acyl phosphates.[24] The monoanion of acetyl phosphate is more reactive than its dianion, while the reverse is true for *p*-nitrobenzoyl phosphate. In general, as the pK_a of the conjugate acid of the leaving group decreases, the rate of hydrolysis of the dianion increases. The hydrolysis of aryl phosphate dianions is characterized by a Brønsted $\beta = 1.23$.[23] The rate of hydrolysis of the corresponding monoanions is relatively insensitive to the pK_a of the leaving group. Presumably, an electron-withdrawing group which would aid in the expulsion of the leaving group will hinder proton transfer to the leaving group, and *vice versa*.[29]

Replacement of the dissociable P–OH proton on the phosphoryl moiety of DMPG with a benzyl group (**8**) decreases the rate of hydrolysis by at least a factor of 10^4. In contrast to this behavior, the monomethyl ester of neutral phosphoramidate undergoes hydrolysis at a rate comparable to that of neutral phosphoramidate, $^-HO_3PNH_3^+$. This fact, along with the $\Delta S^{\pm}$ of −18.2 eu, is evidence that phosphoramidate hydrolyzes *via* a bimolecular mechanism.[30] This behavior would be expected; since there is no free electron pair on the N atom of $^-HO_3PNH_3^+$, intramolecular proton transfer, as required by eq 11, cannot take place. The monoanion of phosphoramidate, however, is postulated to hydrolyze *via* a metaphosphate mechanism, and it is consistent that the corresponding monomethyl ester hydrolyzes at least 10^4 times more slowly.

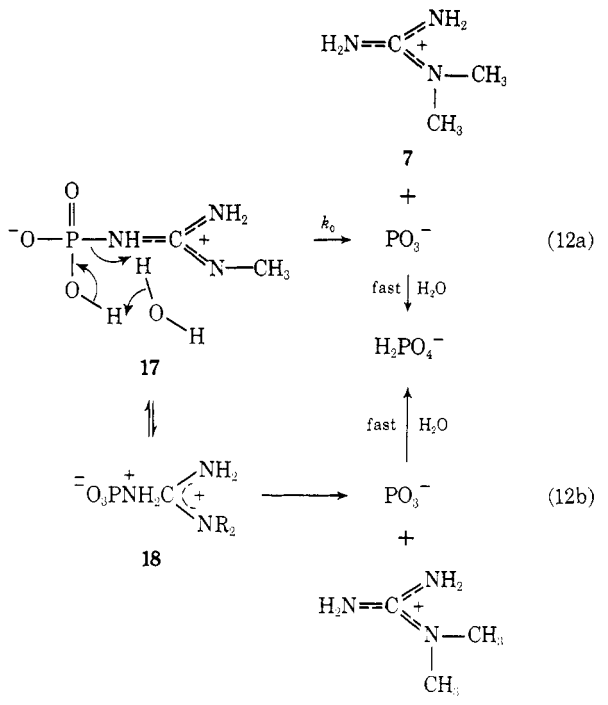
The monoanion and dianion of DMPG, **6** and **12**, are stable, being hydrolyzed at least 10^4 times more slowly than neutral DMPG. This behavior is expected since a metaphosphate mechanism would require expulsion of either neutral dimethylguanidine or dimethylguanidine anion—both are too basic to be reasonable as leaving groups.

Within the range of acidity studied, 4 *M* $HClO_4$ to pH 7, there is no evidence for the hydrolysis of the cation of DMPG (**5**), or of the cation of BMPG, or of acid catalysis of their neutral form. In contrast, phosphate monoesters, acyl phosphates,[24] and phosphoramidates[26] undergo both uncatalyzed and acid-catalyzed hydrolysis of their neutral forms.

Mechanism of Phosphorylation by Phosphoroguanidines. The fact that the hydrolysis of simple phosphoroguanidinates, BMPG and DMPG, occurs *via* the neutral zwitterion, and the fact that the cation,

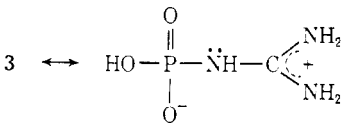
(16) M. C. Bailly, *Bull. Soc. Chim. Fr.*, **9**, 340 (1942).
(17) A. Desjobert, *ibid.*, **14**, 809 (1947).
(18) W. W. Butcher and F. H. Westheimer, *J. Amer. Chem. Soc.*, 77, 2420 (1955).
(19) J. Kumamoto and F. H. Westheimer, *J. Amer. Chem. Soc.*, 77, 2515 (1955).
(20) C. A. Bunton, D. R. Llewellyn, K. G. Oldman, and C. A. Vernon, *J. Chem. Soc.*, 3574 (1958).
(21) C. A. Bunton, M. M. Mhala, K. G. Oldman, and C. A. Vernon, *J. Chem. Soc.*, 3293 (1960).
(22) J. R. Cox, Jr., and O. B. Ramsay, *Chem. Rev.*, **64**, 317 (1964).
(23) C. A. Bunton, *Accounts Chem. Res.*, **3**, 257 (1970).
(24) G. DiSabato and W. P. Jencks, *J. Amer. Chem. Soc.*, **83**, 4400 (1961).
(25) (a) J. D. Chanley and E. Feageson, *J. Amer. Chem. Soc.*, **85**, 1181 (1963); (b) *ibid.*, 77, 4002 (1955).
(26) M. Halmann and A. Lapidot, *J. Chem. Soc.*, 419 (1960).
(27) (a) S. J. Benkovic and K. J. Schray, *Biochemistry*, **7**, 4090 (1968); (b) S. J. Benkovic and E. J. Sampson, *J. Amer. Chem. Soc.*, **93**, 4009 (1971).
(28) A. J. Kirby and A. G. Varvoglis, *J. Amer. Chem. Soc.*, **89**, 415 (1967).
(29) W. P. Jencks, *Brookhaven Symp. Biol.*, **15**, 143 (1962).
(30) I. Oney and M. Caplow, *J. Amer. Chem. Soc.*, **89**, 6972 (1967).

anion, and monoester of these compounds are unreactive toward hydrolysis are consistent with one of the two mechanisms shown in eq 12. Whether or not a

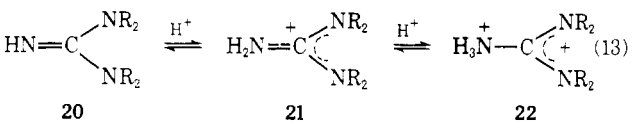


water molecule is involved in mechanism 12a is open to question—a direct proton transfer might be possible.

Because all the N electron pairs are involved in the π-bonding of the guanidinium system, proton transfer preceding breaking of the P–N bond might seem unlikely in phosphoroguanidines (eq 12b). However, MO calculations demonstrate that the two highest filled π orbitals of guanidinium ions are nonbonding; therefore, a pair of electrons may be available for protonation to form **18**, because structures such as **19** con-



tribute strongly to phosphoroguanidines. The conversion **19** → **18** may violate chemical intuition less than **3** → **18**. It is also relevant that **18** is a double zwitterion; therefore, the positive charge on the nitrogen bonded to phosphorus will be electrostatically stabilized by the two negative charges on the phosphate portion of the molecule. Watson[31] has demonstrated that it is possible to protonate a guanidinium ion (eq 13): tetramethylguanidine (**20**, R = CH_3) adds the



first proton with pK_a = 13.6 to give the delocalized guanidinium ion (**21**) which can be protonated again in H_2SO_4 solutions to produce **22** (in equilibrium with the other two tautomers) which has $pK_a = -11$.[31]

(31) (a) P. Haake and J. W. Watson, *J. Org. Chem.*, **35**, 4063 (1970); (b) S. Limatibul and J. W. Watson, *J. Org. Chem.*, **36**, 3805 (1971).

The electrostatic stabilization in **18** will cause it to be much less acidic than **22**. One can estimate the electrostatic effect of the two negative charges in **18** using the ionization of H_3PO_4 (Table VI) as a model: $pK_a^{III} - pK_a^{I} = 10.5$. This would cause one to estimate pK_a (**18**) to be approximately −1. This is probably slightly too high because we have ignored the nonelectrostatic substituent effect involved in replacing an H of **22** with the phosphorus atom of **18**. However, it certainly indicates that appreciable amounts of **18** could be present at $pK = 2$ where the rate maxima for **3** and **4** occur.

Entropy of Activation. In general, reactions which occur *via* a unimolecular mechanism are characterized by a ΔS^* which is positive or near zero, while reactions which occur by a bimolecular pathway are characterized by a ΔS^* which is large and negative.[32] In the hydrolysis of **3** and **4**, ΔS^* is near zero (Table III) as is true in other reactions postulated to proceed through a metaphosphate intermediate.[24-28] If a water molecule is involved (eq 12a) in proton transfer, it must be present in the ground state in order to satisfy the ΔS^* values observed here.

Solvent Deuterium Isotope Effect. Since the postulated metaphosphate mechanism involves the acidic proton on the phosphoryl moiety and this proton is rapidly exchanged with the protic solvent, both deuterium isotope effects on equilibria and deuterium isotope effects on proton transfer could be involved. Phosphate derivatives postulated to hydrolyze *via* a metaphosphate mechanism show small isotope effects. This behavior might not be expected if a proton transfer occurs in the rate-determining step of the mechanism (12a), particularly since it is likely that the proton transfer would take place through one or more molecules of water since water would be expected to strongly solvate the compounds (**16**), which are either anionic or zwitterionic. The lack of a large primary ^2H isotope effect can be discussed in terms of the degree of proton transfer in the transition state. Although the detailed theoretical interpretation of the mechanism of primary deuterium isotope effects is a problem of great complexity and considerable uncertainty,[33,34] primary isotope effects are expected to be small when there is either very little proton transfer in the transition state or the proton is essentially completely transferred in the transition state.[33] In view of the lack of reactivity of the monoanion, dianion, and benzyl ester of DMPG, it is unlikely that there is little proton transfer in the transition state. Therefore, the isotope effect supports mechanism 12b or a transition state for (12a) in which proton transfer is nearly complete.

Kirby and Varvoglis[28] have proposed a mechanism for the hydrolysis of monoanions of phosphate monoesters in which the proton transfer to the leaving group is complete in the transition state, that is, a mechanism involving a preequilibrium proton transfer. Benkovic and Sampson[27] have discussed the question of the timing of proton transfer, and conclude that proton transfer precedes P–N bond breaking if the parent amine

(32) F. A. Long and H. A. Scheraga, *Advan. Phys. Org. Chem.*, **1**, 1 (1962).

(33) (a) F. H. Westheimer, *Chem. Rev.*, **61**, 265 (1961); (b) J. Bigeleisen, *J. Chem. Phys.*, **17**, 675 (1949); (c) J. Bigeleisen and M. Wolfsberg, *Advan. Chem. Phys.*, **1**, 15 (1958).

(34) C. A. Bunton and V. J. Shiner, Jr., *J. Amer. Chem. Soc.*, **83**, 3214 (1961).

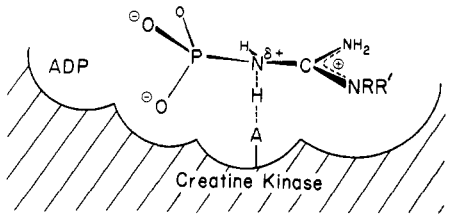


Figure 4. Proposed mechanism of action of creatine kinase.

has $pK_a > 8$; this also supports (12b) as the favored mechanism.

Conclusion

The hydrolysis of the simple phosphoroguanidines, DMPG and BMPG, takes place *via* the neutral zwitterion. The ΔS^* values of 0 and −1 eu for DMPG and BMPG, the lack of reactivity toward hydrolysis of the monobenzyl ester of DMPG and the anions and cations of DMPG and BMPG, and the k_H/k_D values near 1 are consistent with mechanism 12b or with 12a involving a transition state where proton transfer is nearly complete. Metaphosphate intermediates will be examined in more detail in a paper which is being prepared. However, it seems clear that the preferred mechanism for phosphorylation by phosphoroguanidine involves unimolecular decomposition of the neutral species to form a metaphosphate ion which is rapidly converted into a new phosphate derivative.[35]

The labilization of P–N bonds under conditions giving acid catalysis is not limited to phosphate derivatives such as phosphoramides and phosphoroguanidines. We have shown[36a] that phosphinamides, $R_2P(O)NR'R''$, hydrolyze nearly 10^6 times more rapidly than corresponding carboxylic amides at the same acidity. This reactivity of P–N bonds in acid is particularly demonstrated by our study[36b] of $(C_6H_5)_2P(O)NHAr$. With this anilide, acid catalysis causes sufficient instability so that an A_1 reaction ensues. Since the A_1 reaction generates a phosphinylium ion, $(C_6H_5)_2P(O)^+$, which we have shown[36c] to be a very unstable intermediate, it is clear that protonation of nitrogen is a potent mode of catalysis for cleavage of P–N bonds.

Biological Phosphorylation by Phosphagens. In the biologically important phosphorylation of ADP by phosphorocreatine, this mechanism would also be expected. The primary function of the enzyme must be protonation of the guanidine nitrogen. In a preliminary summary of this work,[1] we drew a mechanism in which an enzymic functional group serves as an agent for proton transfer. We pointed out, as an alternative, that "if phosphorocreatine is bound in the $-PO_3^{2-}$ state, the enzyme must function as a general acid catalyst." Although either mechanism is possible, the latter mechanism seems more likely because (a) phosphoroguanidines exist predominantly in the $-PO_3^{2-}$ charge state at pH 7 and would, therefore, be expected to bind to the enzyme in this state; (b) a very simple mode of catalysis is then possible—an acidic group need only position a proton near the guanidine nitrogen to cause very rapid cleavage of the P–N bond and generation of metaphosphate ion, PO_3^-, which would rapidly be attacked by an ADP properly positioned nearby, thereby generating ATP.[6] This mechanism is shown in Figure 4.

It is known that a divalent cation is essential to the proper functioning of creatine kinase. In view of our results, the most likely role of this ion is in binding of the ADP moiety. If this divalent cation were interacting with the phosphate group which is transferred, it would inhibit the rate of formation of metaphosphate. The reactions in which metaphosphate is generated all involve a $^{2-}O_3PX$ system and can be interpreted in terms of electron donation from the oxygen atoms to phosphorus assisting cleavage of the P–X bond which occurs readily because X is a good leaving group. Coordination of a metal ion to the phosphate would inhibit O → P electron donation and result in a slower rate of formation of metaphosphate. Therefore, it seems likely that the configuration of the active site of creatine kinase enables interaction of M^{2+} with ADP but not with the phosphate of phosphorocreatine.

Experimental Section

H_2O was distilled and freed of CO_2 by boiling and cooling under N_2. D_2O (99.8%) was obtained from Diaprep, Inc. Microanalyses were performed by Spang Microanalytical Lab., Ann Arbor, Mich. Chemical shifts are reported relative to TMS as the internal standard, with the exception of those recorded in D_2O, in which DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) was used as the internal standard. Infrared spectra were recorded on a Perkin-Elmer 457. All melting points are corrected.

Preparation of Compounds. Dibenzyl *N,N*-dimethyl-*N'*-phosphoroguanidine (23) was prepared by the method of Cramer and Vollmar.[37] *N,N*-Dimethylguanidinium sulfate (13.6 g, 0.050 mol) was suspended in 55 ml of H_2O, and brought into solution by the addition of 8.0 g of NaOH in 20 ml of H_2O. Dibenzyl phosphite (26.2 g, 0.10 mol) in 80 ml of CCl_4 was added slowly with vigorous stirring and ice cooling. After 1 hr the organic phase was separated, washed with dilute H_2SO_4, a $NaHCO_3$ solution, and H_2O, and dried over $MgSO_4$. The solvent was removed and the resulting product was recrystallized from benzene–cyclohexane: mp 62–65° (lit.[37] mp 65°); yield 17.1 g (47%); ir (Nujol mull) 3360, 3230, 1640, 1560, 1510, 1300, 1160, 1010, 910, 880, and 700 cm^{-1}; nmr (CCl_4) τ 7.18 (s, 6 H), 5.12 (d, 4 H, J = 7.5 Hz), 3.18 (s, 2 H), and 2.78 (m, 10 H).

***N,N*-Dimethyl-*N'*-phosphoroguanidine, DMPG (3).** The dibenzyl ester (**23**) (3.92 g, 0.010 mol) was dissolved in 100 ml of methanol containing 0.25 g of Pd/C (5% Pd) and hydrogenated. After the calculated amount of H_2 had been absorbed, the catalyst was removed by filtration, and the filtrate was cooled to 0°. Acetone was slowly added with stirring until crystallization began. The product was recrystallized from methanol–acetone: mp 157–159° dec; yield 1.6 g (95%); ir (Nujol mull) 3310, 3150, 2310, 1660, 1530, 1400, 1230, 1180, 1050, and 950 cm^{-1}; nmr (D_2O) τ 6.92 (s).

Anal. Calcd for $C_3H_{10}N_3O_3P$: C, 21.56; H, 6.04; N, 25.15. Found: C, 21.70; H, 5.96; N, 24.77.

***N*-(Dibenzylphosphoro)-*S*-methylisothiourea (24)** was prepared from *S*-methylisothiourea sulfate.[37] The product was recrystallized from benzene–petroleum ether (bp 40–60°): mp 86–88° (lit.[37] mp 89°); yield 27 g (77%); ir (Nujol mull) 3360, 3280, 3210, 3120, 1630, 1570, 1350, 1300, 1210, 1150, 1040, 1000, 970, 890, 740, and 700 cm^{-1}; nmr ($CDCl_3$) τ 7.75 (s, 3 H), 4.98 (d, 4 H, J = 8.0 Hz), and 2.40 (m, 12 H).

Dibenzyl *N*-benzyl-*N*-methyl-*N'*-phosphoroguanidine (25) was prepared by a procedure used for preparation of similar compounds.[37] To a solution of **24** (2.51 g, 0.010 mol) in 50 ml of absolute ethanol was added HgO (1.08 g, 0.005 mol) and benzylmethylamine (1.31 g, 0.011 mol). The resulting mixture was refluxed for 1.5 hr, and, after standing 3 hr at room temperature, it was worked up[37] and

(35) P. Haake and G. W. Allen, *Proc. Nat. Acad. Sci. U. S.*, **68**, 2691 (1971).

(36) (a) P. Haake and T. Koizumi, *Tetrahedron Lett.*, 4845 (1970); T. Koizumi and P. Haake, *J. Amer. Chem. Soc.*, **95**, 8073 (1973); (b) P. Haake and D. A. Tyssee, *Tetrahedron Lett.*, 3513 (1970); D. A. Tyssee, L. P. Bausher, and P. Haake, *J. Amer. Chem. Soc.*, **95**, 8066 (1973); (c) P. Haake and P. S. Ossip, *ibid.*, **93**, 6924 (1971).

(37) (a) F. Cramer and A. Vollmar, *Chem. Ber.*, **91**, 911 (1958); (b) *ibid.*, **91**, 919 (1958).

the product was recrystallized from benzene–hexanes: mp 69–70°; yield 3.70 g (88%); ir (Nujol mull) 3360, 3320, 2330, 1640, 1560, 1510, 1200, 1160, 1050, 1000, 920, 730, and 700 cm^{-1}; nmr ($CDCl_3$) τ 7.12 (s, 3 H), 5.43 (s, 2 H), 5.05 (d, 4 H, J = 8.0 Hz), 3.90 (s, 2 H), and 2.75 (m, 15 H).

N-**Benzyl** *N*-**methyl-***N'*-**phosphoroguanidine (4)** was prepared from ester **25** (4.23 g, 0.010 mol), as described for DMPG (**3**). The product was recrystallized from acetone–H_2O: mp 149–151° dec; yield 2.25 g (91%); ir (Nujol mull) 3460, 3130, 2370, 1640, 1600, 1510, 1400, 1180, 1110, 900, 830, and 700 cm^{-1}; nmr (D_2O) τ 6.93 (s, 3 H), 5.37 (s, 2 H), and 2.53 (m, 5 H).

Anal. Calcd for $C_9H_{14}N_3O_3P$: C, 44.50; H, 5.81. Found: C, 44.29; H, 5.83.

Benzyl *N*,*N*-**dimethyl-***N'*-**phosphoroguanidine, Monobenzyl DMPG (26).** The dibenzyl ester of DMPG, **23** (3.47 g, 0.010 mol), in 20 ml of acetone and pyridinium chloride (2.4 g, 0.020 mol) in 8 ml of DMF were mixed together, and the resulting solution was allowed to stand at 25° for 20 hr. The white, crystalline solid which had been produced was collected on a filter and twice recrystallized from ethanol–H_2O: mp 222–223.5° dec; yield 2.35 g (92%); ir (Nujol mull) 3220, 3120, 1660, 1620, 1520, 1400, 1240, 1080, 1020, and 820 cm^{-1}; nmr (D_2O) τ 7.20 (s, 6 H), 4.98 (d, 2 H, J = 12.0 Hz) and 2.53 (s, 5 H).

Anal. Calcd for $C_{10}H_{16}N_3O_3P$: C, 46.69; H, 6.27; N, 16.34. Found: C, 46.66; H, 6.39; N, 16.40.

N,*N*-**Dimethylguanidinium Dihydrogen Phosphate (27).** DMPG (1.70 g, 0.010 mol) was dissolved in 20 ml of H_2O, and the resulting solution was heated on the steam bath for 3 hr. The solution was cooled and 100 ml of acetone was added and the product precipitated. The product was recrystallized from H_2O–methanol: mp 271–275° dec; yield 1.80 g (97%); ir (Nujol mull) 3320, 3100, 2430, 1660, 1530, 1240, 1090, 970, and 880 cm^{-1}; nmr (D_2O) τ 6.97 (s).

Anal. Calcd for $C_3H_{12}N_3O_4P$: C, 19.45; H, 6.54; N, 22.74. Found: C, 19.71; H, 6.30; N, 22.83.

N-**Benzyl-***N*-**methylguanidinium Dihydrogen Phosphate (28).** This compound was prepared from BMPG (2.40 g, 0.010 mol) in an analogous manner to the preparation of **27** from DMPG: mp 180–185° dec; yield 2.50 g (96%); ir (Nujol mull) 3370, 3160, 2430, 1660, 1610, 1240, 1130, 1060, 940, and 750 cm^{-1}; nmr (D_2O) τ 6.93 (s, 3 H), 5.35 (s, 2 H), and 2.53 (m, 5 H).

Anal. Calcd for $C_9H_{16}N_3O_4P$: P, 12.73. Found: P, 12.66 (by the method of Martin and Doty;[38] see below).

N-**Acetyl-***N'*-**phosphoroguanidine (15).** The dibenzyl ester of phosphoroguanidine was prepared[37] and 0.03 mol was acetylated with 0.03 mol of acetic anhydride in 75 ml of pyridine at 75° for 8 hr. The product was isolated by addition of water and recrystallized from benzene–petroleum ether to give a 74% yield of the dibenzyl ester of **15** which showed satisfactory spectral properties. Hydrolysis of 0.005 mol over Pd/C in C_2H_5OH and recrystallization from acetone–water gave a 92% yield of **15**: mp 156–158° dec (lit.[9] mp 158°); ir (Nujol mull) C=O at 1725 cm^{-1}; nmr (H_2O) τ 2.28 (s); electronic absolute spectrum (H_2O) λ_{max} 223.6 nm (ϵ 18,300).

Kinetic Methods. The rates of hydrolysis of DMPG and BMPG were measured spectrophotometrically by recording the decrease in absorbance of starting material at 220 or 225 nm using a Cary Model 16 K spectrophotometer equipped with a thermostated sample compartment and a thermostated cell block through which water from constant-temperature baths was circulated. The *only products* found by electronic spectra, paper chromatography, and isolation *were the corresponding guanidinium phosphates* (prepared above). The pH of the kinetic solutions was maintained constant by the use of buffers or dilute acid at a concentration in excess of substrate which was typically 10^{-3}–10^{-4} M. The runs were initiated by the addition of 20 or 50 μl of substrate stock solution to 3.00 ml of the kinetic solution in a 1-cm quartz cuvette previously equilibrated at the appropriate temperature. The stock solutions were stable for at least 2 months and were prepared for DMPG in the monanion form and BMPG in the dianion form. In all cases excellent first-order plots were obtained, being linear for at least 85% reaction. The k_{obsd} values determined in this manner were found to be reproducible to within 2%. Ionic strength was maintained by addition of NaCl.

The rates of hydrolysis of DMPG and BMPG were also measured by following the release of inorganic phosphate with respect to time. These runs were done in 50-ml volumetric flasks immersed in a constant-temperature bath maintained at 30.5 ± 0.1°. At time t, 1-ml aliquots were removed and analyzed for inorganic phosphate as described below. In all runs the substrate concentration was 5.0×10^{-4} M. The k_{obsd} values determined in this way were in agreement with but less precise than those determined spectrophotometrically, being reproducible to within 5%.

Inorganic phosphate analysis involved a modification of the method of Martin and Doty[38] as utilized by Jencks and Gilchrist.[39] Each day the procedure was checked with standard KH_2PO_4 solutions to ensure that no decomposition of the various stock solutions had occurred. The procedure was found to be reproducible to within ±3%.

Kinetic Runs in Deuterium Oxide. Appropriate amounts of $CH_3CO_2Na \cdot 3H_2O$, CH_3CO_2H, and NaCl were weighed out in a 10.00-ml volumetric flask and D_2O (99.8%) or H_2O added. The isotope dilution by this method of preparation amounted to only 0.8%, so no corrections were applied. The rates were measured spectrophotometrically in the usual manner.

pH Measurements and Titrations. All pH measurements and titrations were done using a Radiometer Type TTTlc pH meter equipped with a G202 B glass electrode and a K401 saturated calomel electrode. Measurements were made in a Radiometer V525 water-jacketed vessel, thermostated at 30.5 ± 0.1°. All titrations were carried out under N_2. The apparatus used is similar to that described by Albert and Serjeant.[12]

Acknowledgments. We thank Dr. Stuart Warren for valuable discussions and Professor V. M. Clark for sending us a copy of the parts of A. R. Macrae's thesis (Cambridge, 1964) which was related to this research. The pK_a of $Ph_2P(O)NHC(NH_2)_2^+$ was determined by Dr. G. Capozzi.

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