12, 27 (1947); L. D. Wright, H. R. Skeggs, and E. L. Cresson, *Proc. Soc. Exp. Biol. Med.*, **64**, 150 (1947); N. M. Green, *Biochem. J.*, **89**, 599 (1963).
(32) R. E. Rosenfield, Jr., and R. Parthasarathy, *J. Am. Chem. Soc.*, **96**, 1925 (1974).

(33) D. B. Boyd, *J. Phys. Chem.*, **78**, 1554 (1974); *Ibid.*, **78**, 2604 (1974); *Theor. Chim. Acta*, **30**, 137 (1973), and personal communication to one of us (R.P.).
(34) H. E. Van Wart, L. L. Shipman, and J. A. Scheraga, *J. Chem. Phys.*, in press.

Mechanism of Phosphorylation by N'-Phosphorocreatine. Concurrent Formation of Creatine and Creatinine[1,2]

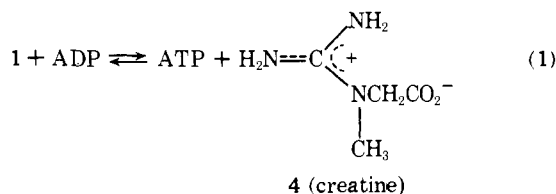### Gary W. Allen and Paul Haake*

Contribution from the Department of Chemistry, Wesleyan University, Middletown, Connecticut 06457. Received November 24, 1975

Abstract: The mechanism of phosphorylation by phosphorocreatine and the mechanism of creatinine formation have been investigated in aqueous solution. The hydrolysis of phosphorocreatine (PC) was found to occur via two pathways: pathway A leads to the production of creatine and inorganic phosphate; pathway B leads to the production of creatinine and inorganic phosphate. At pH values above 1.0, pathway A accounts for greater than 90% of the hydrolysis; pathway B becomes predominant only in strongly acidic solutions. For pathway A, the bell-shaped, pH–rate profile with a rate maximum at pH 1–2 (k_{max} = 1.77×10^{-2} min^{-1}), ΔS^* = -2 eu, and $k(H_2O)/k(D_2O)$ = 0.86 strongly support a metaphosphate mechanism for phosphorylations by phosphorocreatine. The biological implications with regard to the action of ATP:creatine N-phosphotransferase (creatine kinase, E.C. 2.7.3.2) and the origin of urinary creatinine are discussed.

N'-Phosphorocreatine (**1**, PC), a phosphoroguanidine,[3] plays an essential role by phosphorylating ADP (eq 1) during periods of rapid utilization of ATP in muscle. Our studies of the mechanism of phosphorylation by the simple phosphoroguanidines, **2** and **3**, appears to involve proton transfer leading to unimolecular cleavage to the protonated guanidine and the reactive intermediate, monomeric metaphosphate ion, PO_3^-,[2,4] which is the actual phosphorylating agent. Phosphoroguanidines appear to be the most reactive precursors of metaphosphate.[4] The presence of the carboxylate group in PC presents possibilities for functional group and ionization effects which could lead to special reactivity in this natural phosphorylating agent. Therefore, in order to assess the significance of the metaphosphate mechanism with respect to the in vivo phosphorylation reaction involving PC, the in vitro mechanism of phosphorylation of water (hydrolysis) by PC has been studied. This paper is addressed to the chemistry of hydrolysis of **1**, the implications for biological phosphorylation by **1**, and the mechanism of formation of creatinine (**5**).
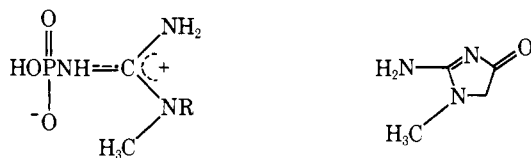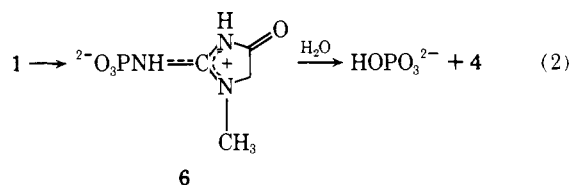
1, R = CH$_2$CO$_2$H (N'-phosphorocreatine)
2, R = CH$_3$
3, R = CH$_2$C$_6$H$_5$

5 (creatinine)

$$1 + ADP \rightleftharpoons ATP + H_2N\text{===}C\begin{matrix} \nearrow NH_2 \\ + \\ \searrow NCH_2CO_2^- \\ | \\ CH_3 \end{matrix} \quad (1)$$

4 (creatine)

Previous investigations on PC are inconclusive. PC has been shown to be stable in alkaline solution[5] but to undergo rapid hydrolysis in acid with a rate maximum at pH 1–3.[6,7] It has also been reported that PC hydrolyzes in acid some 30 times faster than an unsubstituted phosphoroguanidine,[8] therefore,

indicating possible intramolecular catalysis by the carboxylate group.

The products of hydrolysis of PC are reported to be creatine (**4**) and a small amount of creatinine (**5**).[9] A mechanism for formation of **5** involving cyclization to **6** followed by P–N cleavage and ring opening (eq 2) has been proposed;[10] evidence for this mechanism includes the report of an induction period.[11] However, hydrolysis in ^{18}O-enriched water has demonstrated that this mechanism cannot be correct since no label was found in the creatine isolated from the reaction.[12] Recently, it has also been shown that **5** hydrolyzes to creatine more slowly than **1** does.[13]

$$1 \longrightarrow {}^{2-}O_3PNH\text{===}C\begin{matrix} H \\ N \\ + \\ N \\ | \\ CH_3 \end{matrix}\!\!\!\nearrow\!\!O \xrightarrow{H_2O} HOPO_3^{2-} + 4 \quad (2)$$

6

Results

Potentiometric Titration. A potentiometric titration was carried out on the disodium salt of PC under the same conditions as those employed in the kinetic studies above pH 0.78, that is, T = 30.5 °C and μ = 0.20 N in added NaCl. The overlapping dissociation constants were calculated by the method of Noyes.[14] The dissociation constants (Table I) are assigned in eq 3; the bases for the assignments have been discussed.[4,15]

Products of the Hydrolysis of PC. The rates of hydrolysis of PC were measured at 30.47 ± 0.05 °C in the pH range of 0.78–5.58 in buffered solutions with the ionic strength maintained constant at 0.20 N by the addition of NaCl or NaClO$_4$, and in 0.40–4.00 N HClO$_4$. On hydrolysis, PC produces inorganic phosphate and both **4** and **5**. Both products were detected by paper chromatography for the hydrolysis of PC in 1.0 N HClO$_4$. The Jaffé–Folin determination[16] demonstrated conclusively that **5** is ca. 5% of the products of the hydrolysis of PC at pH 5.22 and 5.88.

A quantitative measure of the product composition is afforded by the absorbance at 225.0 nm at the end of the hy-
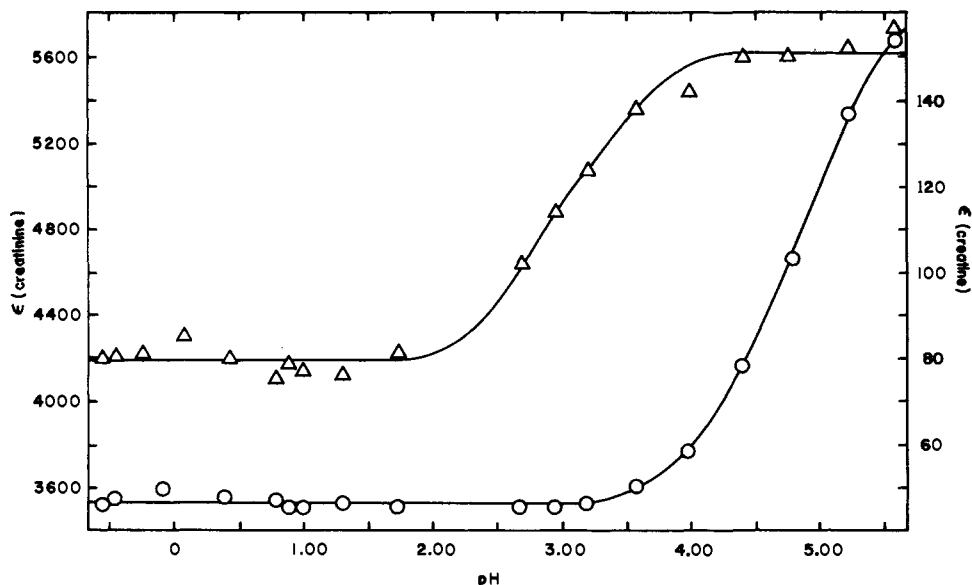
Figure 1. The molar absorptivities (ϵ) of creatinine (5) and creatine (4) at 225.0 nm as a function of pH: O, creatinine; $\triangle$, creatine.
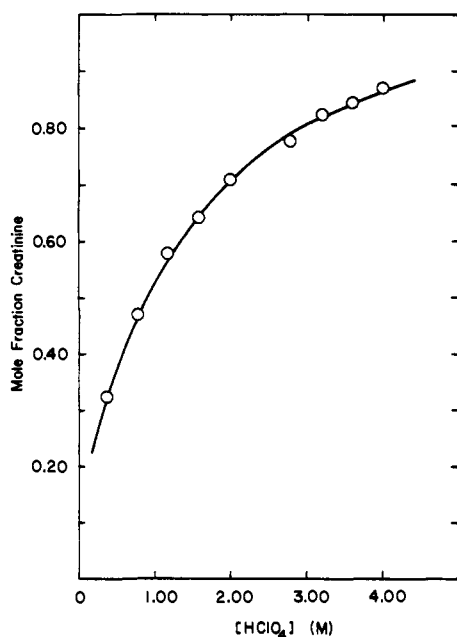

Figure 2. The mole fraction of creatinine produced by hydrolysis of PC at 30.5 °C (μ = 4.00 N) as a function of the concentration of $HClO_4$. The curve was calculated from the constants (at the appropriate ionic strength) listed in Tables II and III.

Table I. pK_a Values for the Ionization of Phosphorocreatine (1)

Dissociable group	$pK_a{}^a$	$pK_a{}^b$
First H of P moiety ($pK_a{}^I$)	−0.41[c]	<1.9
−COOH ($pK_a{}^{II}$)	2.84	2.7
Second H of P moiety ($pK_a{}^{III}$)	4.46	4.5
Guanidine ($pK_a{}^{IV}$)		11.2

[a] This work, T = 30.5 °C, μ = 0.20 N in added NaCl. [b] Determined at 25 °C by O. Meyeroff and K. Lohmann, *Biochem. Z.* **196**, 49 (1928), and by W. D. Kumler and J. J. Eiler, *J. Am. Chem. Soc.*, **65**, 2355 (1943). [c] Determined kinetically.

drolysis of PC. The extinction coefficients, ϵ (4) and ϵ (5) at 225.0 nm and 30.5°, were determined as a function of pH (Figure 1); the variations with pH are suggestive of titration curves, indicating approximate pK_a values of 2.7 and 4.7 for
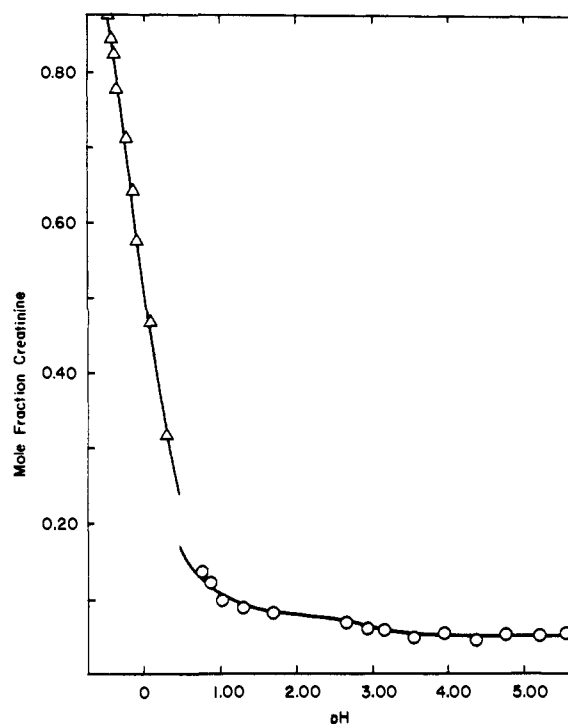

Figure 3. The mole fraction of creatinine produced by hydrolysis of PC at 30.5 °C as a function of pH: O, μ = 0.20 N; $\triangle$, μ = 4.00 N. The curves were calculated from the constants (at the appropriate ionic strength) in Tables II and III.

creatine and creatinine, respectively. Such behavior is expected since the pK_a values of creatine and creatinine are reported to be 2.63 and 4.75, respectively, at 30°.[17] The hydrolysis of PC in acid and in buffered solutions gave the product compositions displayed in Figures 2 and 3.

Rates of Hydrolysis of PC. The first-order rate constants, k_{obsd}, for the hydrolysis of PC were determined from data collected spectrophotometrically at 225.0 nm and by following the production of inorganic phosphate (Figure 4). The agreement between k_{obsd} values determined by these two methods is excellent. Good first-order kinetics were observed through at least 2 half-lives (75% reaction). In no case was there any evidence for the buildup of an intermediate nor was any induction period observed as had been reported previously.[10]
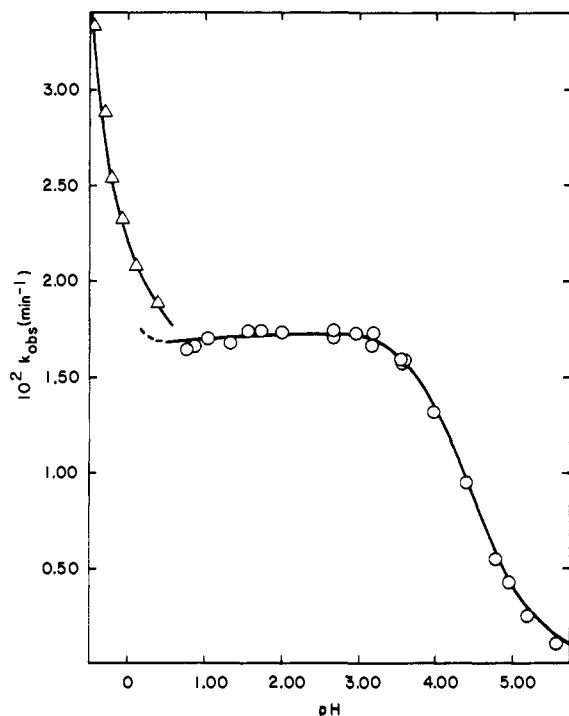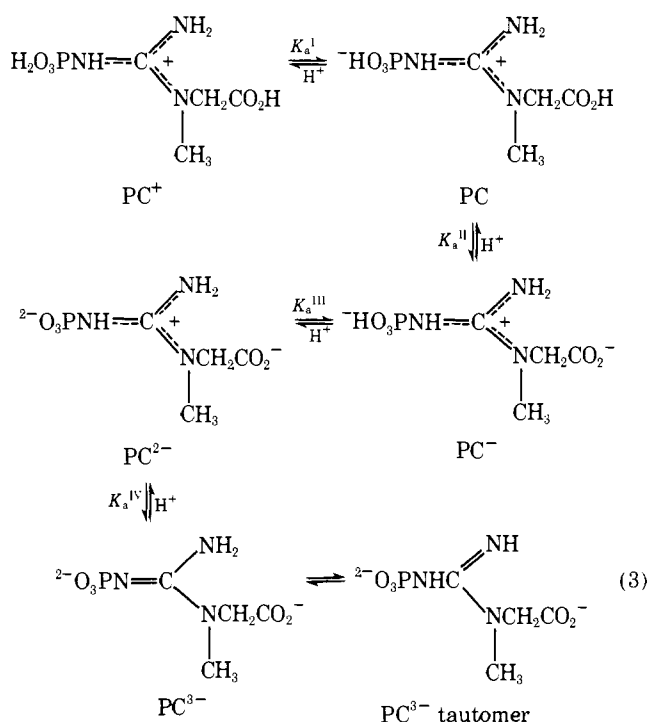
Figure 4. The pH-rate profile for the hydrolysis of PC at 30.5 °C: O, μ = 0.20 N; Δ, μ = 4.00 N. The points are experimental; the lines are calculated from eq 13 and the constants in Tables II and III.

Figure 5. The pH-rate profile for the hydrolysis of PC at 30.5 °C via pathway A to produce creatine: O, μ = 0.20 N; Δ, μ = 4.00 N. The curve was calculated from eq 5 and the constants (at μ = 0.20 N) listed in Table II.

(3)

At any pH value $k_{obsd} = k_A + k_B$ where k_A and k_B are the rate constants for reaction of PC to give creatine (**4**) and creatinine (**5**), respectively. The individual values of k_A and k_B are obtained by multiplying k_{obsd} by X_A and X_B, the mole fractions of **4** and **5** produced at each pH (Figure 3).

Rates of Creatine Formation. The pH dependence of k_A (Figure 5) is similar to that oberved for the hydrolysis of the simple phosphoroguanidines, **2** and **3**;[4] however, the ionization of the carboxylate group in the guanidine portion of PC results in two hydrolyzable species (eq 3), PC and PC⁻, both of which have one proton and one negative charge on the phosphate moiety. The value of k_A then consists of two rate constants (eq
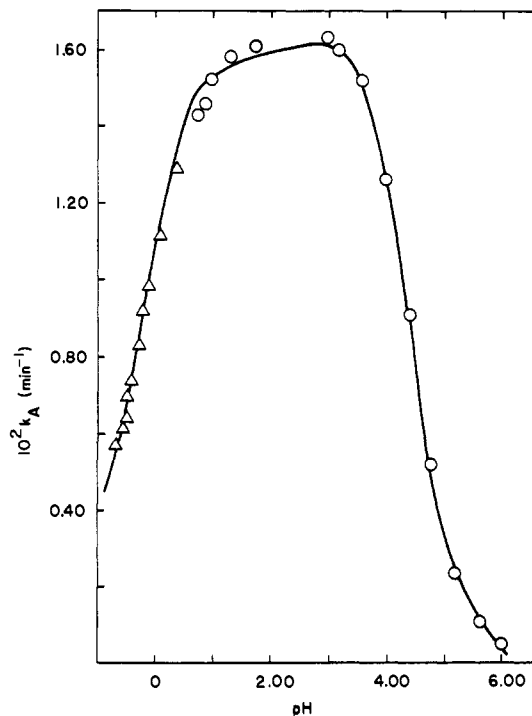
Table II. Kinetic Parameters for the Hydrolysis of PC to Produce Creatine, T = 30.47 ± 0.05 °C

k_1 = 1.47 × 10⁻² min⁻¹ (μ = 4.00 N);
1.58 × 10⁻² min⁻¹ (μ = 0.20 N, based on $K_a{}^I$ = 2.56)
k_2 = 1.70 × 10⁻² min⁻¹ (μ = 0.20 N)
$K_a{}^I$ = 2.56 (μ = 4.00 N)
$K_a{}^{II}$ = 1.45 × 10⁻³ (pot. titn, μ = 0.20 N)
$K_a{}^{III}$ = 3.46 × 10⁻⁵ (pot. titn, μ = 0.20 N);
3.69 × 10⁻⁵ (μ = 0.20 N)[a]

[a] Determined kinetically from plot of eq 6.

4) and when the concentrations are all expressed in terms of total [PC], eq 5 results.

$$\text{rate} = k_A[\text{PC}]_T = k_1[\text{PC}] + k_2[\text{PC}^-] \qquad (4)$$

$$k_A = (k_1 K_a{}^I[\text{H}^+]^2 + k_2 K_a{}^I K_a{}^{II}[\text{H}^+])/([\text{H}^+]^3 + K_a{}^I[\text{H}^+]^2 + K_a{}^I K_a{}^{II}[\text{H}^+] + K_a{}^I K_a{}^{II} K_a{}^{III}) \qquad (5)$$

At pH values greater than 3.5, $K_a{}^I \gg [\text{H}^+]$, $K_a{}^{II} \gg [\text{H}^+]$, and $K_a{}^I K_a{}^{II} \gg [\text{H}^+]^2$, so eq 5 reduces to eq 6.

$$k_A = k_2/(1 + K_a{}^{II}/[\text{H}^+]) = k_2 - k_A K_a{}^{III}/[\text{H}^+] \qquad (6)$$

A plot of k_A against $k_A/[\text{H}^+]$ at pH >3.5 is linear and gives $K_a{}^{III}$ as the negative slope and k_2 as the ordinate intercept (Table II);[2] $K_a{}^{III}$ obtained in this manner is in good agreement with that determined potentiometrically.

At pH values less than ca. 1.0, $K_a{}^{II} \ll [\text{H}^+]$, $K_a{}^{III} \ll [\text{H}^+]$, and $K_a{}^I K_a{}^{II} \ll [\text{H}^+]^2$, so eq 5 reduces to eq 7; a plot of k_A vs. $k_A[\text{H}^+]$ gives a straight line[2] and enables determination of k_1 and $K_a{}^I$ (Table II) at μ = 4.0 N. A similar treatment utilizing the Hammett acidity function[18] was clearly nonlinear. The fit of the data in Figure 5 with the theoretical curve provides evidence for the validity of the constants in Table II.

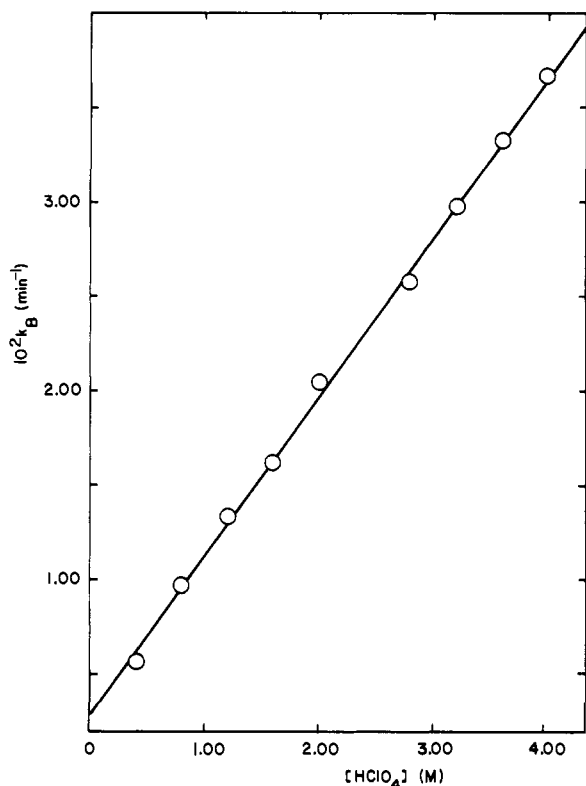$$k_A = k_1 - k_A[\text{H}^+]/K_a{}^I \qquad (7)$$

Figure 6. The first-order rate constants (k_B) for the hydrolysis of PC at 30.5 °C (μ = 4.00 N) via pathway B to produce creatinine as a function of the concentration of $HClO_4$; the intercept = k_5 (eq 10).

Rates of Formation of Creatinine. The first-order rate constant for PC $\rightarrow$ creatinine is k_B. Creatinine (5) is the major product of the hydrolysis of PC only in strongly acidic solutions, but it is a detectable product of the hydrolysis of PC at all pH values studied (Figures 2 and 3). Values of k_B in $HClO_4$ (μ = 4.00 N) and in the pH range 0.78–5.58 (μ = 0.20 N) are shown in Figures 6 and 7, respectively. A kinetic scheme encompassing all possible modes of PC $\rightarrow$ creatinine is given in eq 8. Expressing all concentrations in terms of [PC] total gives eq 9.

$$\text{rate} = k_B[PC]_{total} = k_3[H^+][PC^+] + k_4[PC^+]$$
$$+ k_5[PC] + k_6[PC^-] \quad (8)$$

$$k_B = \frac{k_3[H^+]^4 + k_4[H^+]^3 + k_5K_a^I[H^+]^2 + k_6K_a^IK_a^{II}[H^+]}{[H^+]^3 + K_a^I[H^+]^2 + K_a^IK_a^{II}[H^+] + K_a^IK_a^{II}K_a^{III}}$$
$$(9)$$

At pH's less than 0.5, dropping negligible terms from eq 9 and rearranging gives eq 10. A plot of k_B against H^+ (Figure 6) is nearly linear; extrapolation to [H^+] = 0 gives k_5 as the intercept (Table III).

$$k_B = (k_3[H^+]^2 + k_4[H^+] + k_5K_a^I)/([H^+] + K_a^I) \quad (10)$$

Rearrangement of eq 10 gives eq 11; the value of k_5 was used to plot k' against [H^+] giving a straight line with k_3 as the slope and k_4 as the intercept (Table III).[2]

$$k' = (k_B[H^+] + k_BK_a^I - k_5)/[H^+] = k_3[H^+] + k_4 \quad (11)$$

At pH's greater than 3.5, eq 9 reduces to eq 12; a plot of k_B vs. $k_B/[H^+]$ is linear yielding K_a^{III} as the slope and k_6 as the intercept (Table III). K_a^{III} agrees well with the value found by potentiometric titration.

$$k_B = k_6 - K_a^{III}(k_B/[H^+]) \quad (12)$$

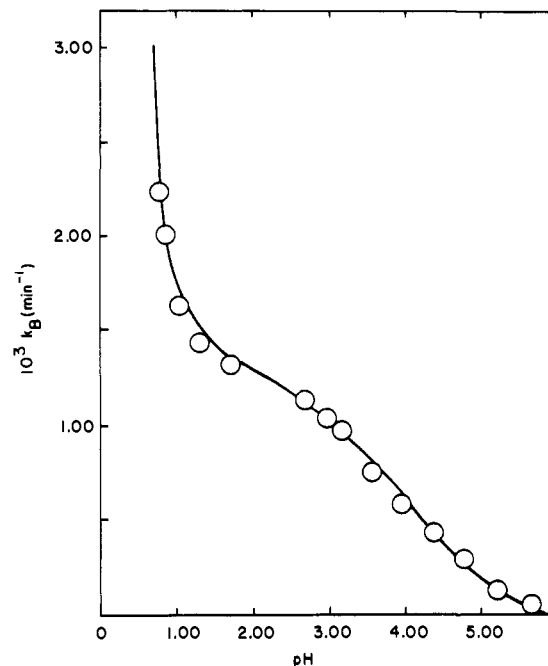The constants in Table III have been used to construct the

Figure 7. The pH–rate profile for the hydrolysis of PC at 30.5 °C (μ = 0.20 N) via pathway B to produce creatinine. The curve was calculated from eq 9 and the constants (at μ = 0.20 N) are listed in Table III.

Table III. Parameters for Hydrolysis of Phosphorocreatine to Produce Creatinine, T = 30.5 °C

μ	4.00 N	0.20 N
k_3, M^{-1} min^{-1}	8.35×10^{-3}	
k_4, min^{-1}	2.48×10^{-2}	1.35×10^{-2}
k_5, min^{-1}	0.29×10^{-2}	0.11×10^{-2}
k_6, min^{-1}		0.86×10^{-3}
K_a^I	2.56	
K_a^{II}		1.45×10^{-3} (by titn)
K_a^{III}		3.46×10^{-5} (by titn), 3.61×10^{-5} (eq 11)

theoretical line in Figure 7; there is good agreement with experimental data. The values of k_4 and k_5 (Table III), which are required to give the theoretical line in Figure 7 (at μ = 0.20 N), are less than those at μ = 4.00 N. This positive salt effect is comparable to others that have been observed.[19]

The total rate equation for hydrolysis of PC is eq 13 which was used to construct the theoretical curves in Figure 4 using the values of K_a^{II} and K_a^{III} determined by titration. The mole fraction of creatinine, given by eq 14, was calculated using the constants in Tables II and III and the theoretical curve in Figure 3 was generated.

$$k_{obsd}$$
$$= \frac{\begin{array}{c}(k_3[H^+]^4 + k_4[H^+]^3 + (k_1 + k_5)K_a^I[H^+]^2 \\ + (k_2 + k_6)K_a^IK_a^{II}[H^+])\end{array}}{([H^+]^3 + K_a^I[H^+]^2 + K_a^IK_a^{II}[H^+] + K_a^IK_a^{II}K_a^{III})}$$
$$(13)$$

$$X_B = k_B/k_{obsd} \quad (14)$$

Activation parameters for the hydrolysis of PC were determined over a temperature range of 21° (Table IV). At pH 3.61, k_B is a small fraction of the total rate so no separation of k_{obsd} into k_A and k_B has been made. In addition, in calculating ΔS^*, we have corrected for ionization via K_a^{III} by the equation, $k' = k_{obsd}(1 + K_a^{III}/[H^+])$. The ΔH^* value rests on the as-

Table IV. Activation Parameters for Hydrolysis of
Phosphorocreatine, pH 3.61, $\mu = 0.20$ N[a]

| T, °C	k_{obsd}, s^{-1}	
51.61	3.03×10^{-3}	$E_a = 22.5$ kcal/mol
41.39	9.72×10^{-4}	$\Delta H^* = 21.9$ kcal/mol
30.47	2.67×10^{-4}	$\Delta G^* = 22.6$ kcal/mol
		$\Delta S^* = -2$ cal/mol

[a] Standard state taken as 25 °C and 1 M.

sumption of a negligible change in K_a's with temperature. The values of k_{obsd} give an excellent straight line vs. $1/T$ but, as is usually true in such determinations, we expect that ΔH^* has a *possible deviation* of ± 0.5 kcal/mol and ΔS^* has a possible deviation of ± 5 eu. Therefore, ΔS^* is within experimental error of zero as expected for a unimolecular reaction.

The solvent deuterium isotope effect was determined at 30.5°, $\mu = 0.20$ N in 0.10 M acetate buffer. The pH values were measured and corrected in D_2O as previously described.[4] Because of the complications of mixed products and mixed pathways of hydrolysis, the results are highly approximate, but using $k' = k_1 + k_2 + k_B$, $k'(H_2O)/k'(D_2O) = 0.89$. Deuterium isotope effects have been discussed by Allen and Haake;[4] effects can occur on both concentrations of reactive species and their rates of reactions.

Discussion

Mechanism of Creatine Formation from PC. The results demonstrate that the neutral (carboxyl protonated) and anionic (carboxyl unprotonated) forms of PC both hydrolyze at pH's near 2 (Figure 5) by pathways similar to that studied in detail for **2**, that is, via a metaphosphate mechanism.[4] The bell-shaped, pH–rate profile, the requirement for a PO_3H^- phosphate moiety, and the ΔS^* all support this conclusion. The small difference between k_1 and k_2 and the faster rate of **2** indicate that the carboxyl group plays no catalytic role in the cleavage of the P–N bond as had been suggested.[10,11] Instead, the slower rates observed for PC than for **2** indicate that the inductive effect of the carboxyl slows the rate by decreasing the basicity of the P–N nitrogen to which a proton must be transferred in order to give the double zwitterion (**7**) which is the intermediate (eq 15) which generates metaphosphate ion, PO_3^-.[4]

$$ 7, \text{R} = CH_2CO_2H \text{ or } CH_2CO_2^- $$
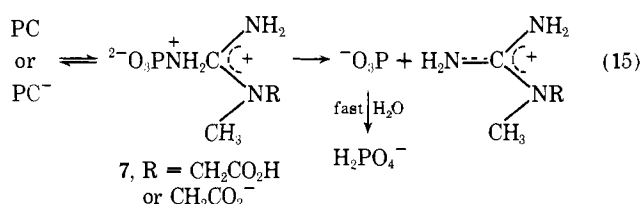
Mechanism of Enzymic Phosphorylation by Phosphorocreatine. Creatine is the sole guanidine-containing product of the enzyme-catalyzed phosphorylation of ADP by PC;[20-22] the mechanism of generation of **4** from PC and PC$^-$ at pH's near 2 appears to be applicable to the enzyme-catalyzed reaction,[23] namely, phosphorylation of ADP by metaphosphate ion, which would be generated by cleavage of **7**.[24] Enzymes generally catalyze pathways for reactions that are already favored in substrates and in PC the metaphosphate mechanism is clearly very favorable (Figure 5). Therefore, our fundamental chemistry enables us to predict these important features of the active site of the enzyme, creatine kinase (creatine:ATP transphosphorylase, E.C. 2.7.3.2). (1) PC will be bound in the active site so that the P–N nitrogen of PC is near an acid catalyst which will either donate a proton to produce **7** or function as a general acid catalyst transferring a proton as the P–N bond breaks. (2) As PO_3^- is produced, it must be in intimate

association with ADP so that the β-phosphate group traps the PO_3^- to give ATP. The binding in the active site near the PO_3^- transfer area must exclude any water molecules which could compete with ADP for the highly reactive PO_3^-. We have shown that PO_3^- is highly indiscriminate toward oxygen nucleophiles;[2] in the enzyme, reaction with water would form inorganic phosphate and constitute an inefficiency in utilization of PC. (3) Both the rate of P–N bond cleavage to give PO_3^- and the basicity of the P–N nitrogen will be greatest if PC is bound to the active site with two negative charges on the phosphate moiety. (4) Since binding to exclude water will be essential for enzymic efficiency, one should expect to find, after crystallography is completed, binding sites for the phosphate dianion (possibly hydrogen bonded to lysine or arginine), the guanidinium cation (possibly hydrogen bonded to a carboxylate), and the carboxylate anion (possibly hydrogen bonded to an arginine).[23] Other phosphagens[2] differ in structure from PC but all have a group, such as the carboxyl in PC, which can be negatively charged. We suggest that the reason for this evolutionary theme in phosphagen structure is the need for highly oriented binding of the phosphagen in order to provide selective phosphorylation of ADP.

Mechanism of Creatinine Formation from PC. Creatinine (**5**) appears to be formed by four different pathways (Table III). At pH values above ca. 1.0, the rate of hydrolysis of PC to produce creatinine contributes less than 10% to the total rate of hydrolysis. Below pH 1.0, the rate of creatinine formation increases with acid concentration to become the predominant hydrolysis pathway. Kinetic indistinguishability must be considered in discussing possible mechanisms associated with each term in eq 9. For example, the term k_5[PC] could represent neutral PC as the substrate but it is kinetically equivalent to k_5'[PC$^-$][H$^+$], where $k_5' = k_5/K_a^{II}$ which represents an acid-catalyzed reaction of monoanionic PC. Another possibility is k_5''[PC$^+$][$^-$OH], which represents the reaction of hydroxide ion with cationic PC. However, this possibility is excluded because the value of k_5'' would be 2.6×10^{11} M^{-1} s^{-1}, greater than the diffusion controlled limit for such a reaction.[25]

Creatinine (**5**) could be formed by three pathways: directly from PC, through **4**, and through **6**. Although at all pH values studied **4** is converted to **5**,[26] the rate of this reaction is considerably less than the rate of hydrolysis of PC. For example, in 0.40 N $HClO_4$ at 30.5° the first-order rate constant, k_{obsd} (**4** $\rightarrow$ **5**) is 2.3×10^{-4} min^{-1},[25] while k_{obsd} for the hydrolysis of PC is 1.88×10^{-2} min^{-1}. Therefore, the creatinine present at the end of the hydrolysis of PC does not appear to be formed through **4** as an intermediate. The hydrolysis of phosphorocreatinine (**6**) has a very different rate profile from PC with a maximum near pH 6 and acid-catalyzed hydrolysis at low pH,[27] so **6** may be an intermediate in all or some of the pathways for creatinine formation. A concerted conversion of PC to creatinine may be possible if the carboxyl group were protonated and functioned as an electrophilic catalyst for cleavage of the P–N bond. More research on the mechanism of PC $\rightarrow$ creatinine will be required to solve these problems.

It is likely that acid-catalyzed reactions (e.g., Figure 6) do not proceed by a metaphosphate mechanism; a likely possibility involves nucleophilic attack by water on a ctionic phosphate species similar to that found in other acid-catalyzed reactions of phosphorus esters.

Origin of Urinary Creatinine. Creatinine (**5**) is a normal constituent of the urine of vertebrates, the amount depending on species and body weight. In 1938, Bloch and Schoenheimer[28] proved conclusively, by isotope tracer studies, that creatine is the sole precursor of urinary creatinine. It was shown that creatinine was the only urinary constituent containing any significant amount of creatine nitrogen. The site and mechanism of creatinine formation and the role, if any, of PC in the

process were not known. The amount of creatinine normally excreted in the urine is greater than can be accounted for by the spontaneous (i.e., nonenzymic) dehydration of creatine at physiological temperature and pH.[9]

Myers and Fine[29] showed that creatine was converted to creatinine three times as fast in the presence of finely ground muscle than in pure solution, and they proposed that an enzyme was responsible for the increased rate. Carpenter et al.[30] reported evidence for such an enzyme but subsequent work casts doubt.[31]

Borsook and Dubnoff[9] reported that the hydrolysis of PC at physiological temperature and pH (38 °C and pH 7) produced small amounts of creatinine as well as creatine and, furthermore, that this rate of prodution of creatinine, based on the amounts of creatine and PC present in the skeletal muscles, was sufficient to account for the rate of excretion of urinary creatinine. These workers proposed that urinary creatinine is a product of the slow, nonenzymic breakdown of PC in the muscle tissue.

The study of the rates of the hydrolysis of PC and product composition by Borsook and Dubnoff, however, was limited to a small pH range (6.0-7.5). The results of the work presented here substantiate the original proposal of Borsook and Dubnoff; that is, creatinine is a product of the hydrolysis of PC, and the rate of' production of creatinine via this pathway is greater than the production of creatinine from the dehydration of creatine. Our calculated first-order rate constant obtained for the production of creatinine via the hydrolysis of PC at pH 6.0 and 30.5 °C, $k = 2.4 \times 10^{-5}$ min^{-1}, is in good agreement with that obtained by Borsook and Dubnoff at 38 °C, $k = 4.3 \times 10^{-5}$ min^{-1}, in view of the temperature difference.

Experimental Section

N'-Phosphorocreatine was obtained from Nutritional Biochemical Co., Cleveland, Ohio, as the disodium hexahydrate salt. It was purified by dissolving 600 mg of the compound in 50 ml of distilled, CO_2-free H_2O, filtering, and adding ethanol until the solution became slightly turbid (ca. 100 ml). The solution was allowed to stand several days at room temperature until crystals could be seen on the side of the flask and kept several days at 5 °C; the product was collected by filtration and washed with ice-cold ethanol. The entire procedure was repeated, and the resulting crystals were washed with anhydrous ether following the ethanol washing. The long, needle-like crystals were dried overnight in a desiccator at 10 mmHg. The amount of inorganic phosphate released on the hydrolysis of this material was consistent with the tetrahydrate. Anal. Calcd for $Na_2(C_4H_9N_3O_5P)\cdot4H_2O$: P, 9.48. Found: P, 9.52.

Kinetics. The rates of hydrolysis of PCwere determined by following he release of inorganic phosphate with respect to time and spectrophotometrically by the change in absorbance at 225.0 nm. The extinction coefficients are in Figure 1. The value of ϵ (4) below pH 1.5 was taken to be 79 ± 2 (average of ten measurements). Similarly, ϵ (5) was 3550 ± 10 from measurements below pH 3.0. These deviations were used in assessing experimental error over the entire pH range used in our experiments. Depending on the amount of creatinine produced the net change in absorbance was either positive (pH <0.5) or negative (pH >2.5). Between pH 0.5 and 2.5, the amount of creatinine produced was such that the net change in absorbance was quite small (less than 0.05 absorbance unit) and, as a consequence, the rates could not be determined spectrophotometrically within this pH range.

In strongly acidic solutions (pH <0.5), the kinetics were complicated by the conversion of the creatine produced on hydrolysis of PC to creatinine. At a given concentration of acid this reaction is reported to be first order in creatine.[25] Although the rate of this reaction is at least ten times less than the rate of hydrolysis of PC, it was observable and it caused a steady increase in absorbance after all the PC initially prsent had been hydrolyzed. Consequently, an indirect method for the determination of A_∞ had to be employed. A_∞ is the absorbance at infinite time, $t_\infty = 10\tau$ ($\tau = 1$ half-life), due to creatine *and* creatinine. Figure 8 depicts a typical example of the observed change in absorbance with time (heavy line). The slope of the linear change in
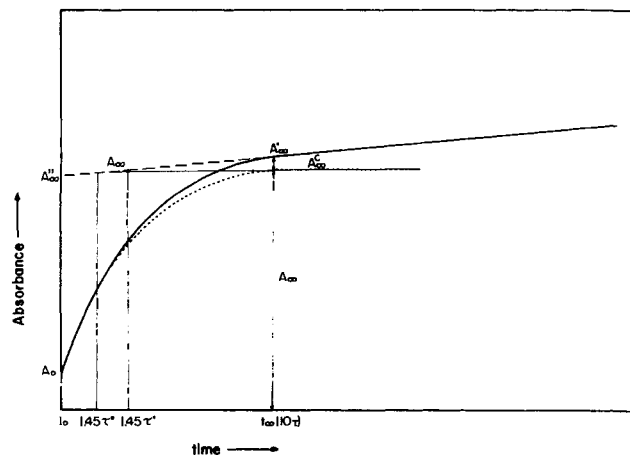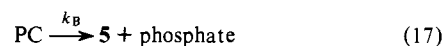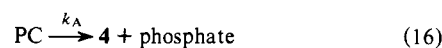

Figure 8. Schematic plot of absorbance against time for the hydrolysis of PC in strongly acidic solution where creatinine is formed from creatine at a significant rate.

absorbance after t_∞ is exaggerated for clarity. The dotted line represents the expected change in absorbance with time if the creatine produced were not converted to creatinine at a detectable rate. The absorbance at the point where the initial change in absorbance becomes linear, A'_∞, is greater than the true value of A_∞, since A'_∞ contains a contribution from the creatinine formed from creatine. The following method was used to calculate A_∞.

$$PC \xrightarrow{k_A} 4 + \text{phosphate} \tag{16}$$

$$PC \xrightarrow{k_B} 5 + \text{phosphate} \tag{17}$$

$$4 \xrightarrow{k_C} 5 \tag{18}$$

As defined in Figure 8, our problem is to find

$$A_\infty = A'_\infty - A_\infty{}^C \tag{19}$$

where A^C = absorbance due to eq 18. Since $[PC] = [PC]_0 e^{-kt}$ where $k = k_A + k_B = k_{obsd}$, and $k_A \gg k_C$

$$[4] \simeq f[PC]_0 (1 - e^{-kt}) \tag{20}$$

where $f = k_A/k$. We may then obtain an expression for A^C

$$dA^C/dt = \epsilon_4 k_C[4] \tag{21}$$

where ϵ_4 = extinction coefficient for **4**. Therefore, substituting eq 21 into eq 20 and integrating

$$A_\infty{}^C = \epsilon_4 k_C f[PC]_0 \int_0^{10\tau} (1 - e^{-kt})dt$$

$$= \epsilon_4 k_C f[PC]_0[+ e^{-kt}/k - 1/k]_0^{10\tau}$$

Since $k = 0.693/\tau$

$$A_\infty{}^C = \epsilon_4 k_C f[PC]_0 8.55\tau \tag{22}$$

Because **4** → **5** is a very slow reaction, the change of absorbance after 10τ (Figure 8) is approximately a straight line which describes A^C as a function of time.

$$\Delta A^C = \epsilon_4 k_C f[PC]_0 \Delta t$$

Therefore, we may find $A_\infty{}^C$ (eq 22) by measuring ΔA^C over $\Delta t = 8.55\tau$, and A_∞ is obtained by subtracting this value from the absorbance at $t = 10\tau$ (eq 19 and Figure 8). e examined the possible errors inherent in the approximations in this method, and we estimate that the maximum possible error from this procedure is less than 0.1% when applied to our experiments.

The values of A_∞ determined by the above treatment were used to calculate the k_{obsd} values in the usual manner. In all cases excellent first-order kinetics were obeyed through at least 2 half-lives (75% reaction). At $t > 2\tau$ deviations from first-order kinetics were evident. These deviations are due to the fact that no corrections were applied to the absorbance values at time t, which contain a contribution due to reaction 18. At $t < 2\tau$, however, this contribution is negligible.

Allen, Haake / Phosphorylation by N'-Phosphorocreatine

Product Composition. Creatine (**4**) and creatinine (**5**) are the only guanidine products of the hydrolysis of PC, so $[4] + [5] = C_{tot}$. All kinetic runs were initiated by the addition of 50 μl of the same PC stock solution ([PC] = 0.03 M) from a Grunbaum micropipet to 3.00 ml of the kinetic solution in a 1-cm quartz cuvette to give a solution ca. 5×10^{-4} M in PC. The value of C_{tot} was accurately determined for the hydrolysis of PC in strongly acidic solution (0.40–4.00 M $HClO_4$). At the end of the hydrolysis reaction, the solutions were heated at 95 °C for several hous, converting all creatine to creatinine. Creatinine was shown to be stable under these conditions. The absorbance at 225.0 nm of these solutions so heated gave $[5] = C_{tot} = (3.94 \pm 0.05) \times 10^{-4}$ M. Since the same stock solution and the same micropipet were used in all runs, this value was taken as C_{tot} for all product determinations. The mole fractions, X (**4**) and X (**5**), were determined from A_∞ and the extinction coefficients (Figure 1).

Paper Chromatography. All paper chromatography was run on Whatman No. 1 filter paper. The solvent system used to separate creatine and creatinine was 1-butanol–acetic acid–pyridine–H_2O (4:1:1:2). Creatine and creatinine were detected by spraying the dried chromatograms with alkaline ferricyanide–nitroprusside reagent.[32] The following R_f values and behavior to this reagent were observed: creatine, $R_f = 0.34$, orange; creatinine, $R_f = 0.55$, orange, turning blue after a few minutes. For both compounds the detection limit was 1 μg.

Product Identification. The hydrolysis of PC was carried out at 5.0 M and 1.0 M $HClO_4$ (PC concentration = 0.1 M) and at pH 3.61 (0.10 M acetate, PC concentration = 0.05 M) at 30.5 ± 0.1 °C under the same conditions used for the kinetic runs. After 10 half-lives ten aliquots of each solution were applied to the chromatography paper and the chromatograms developed as described above. In 4.0 M $HClO_4$ the only guanidine-containing product detected was creatinine. At 1.0 M $HClO_4$ both creatine and creatinine were detected. At pH 3.61 only creatine was detected.

Jaffé–Folin Creatinine Determination.[16] A 2.00-ml aliquot of the solution to be analyzed, containing up to 5.0×10^{-4} M creatinine, was added to 3.00 ml of a freshly prepared alkaline picrate solution (25 ml of a saturated picric acid solution and 5 ml of a 10% NaOH solution) in a test tube. After allowing 10 min for color development, 3 ml of the solution was added to a 1-cm cuvette and the absorbance a 525 nm was read against a blank. The procedure was calibrated with standard solutions of creatinine, and the absorbance at 525 nm was found to vary linearly with creatinine concentration up to 5.0×10^{-4} M. The procedure was found to be reproducible to within 5%. When this procedure was used to determine creatinine in the presence of large amounts of creatine, a correction for the slow conversion of creatine into creatinine, catalyzed by the alkaline picrate solution, had to be applied.

References and Notes

(1) This research was supported by Grant AM-12743 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.
(2) A preliminary report of a portion of this research has appeared: P. Haake and G. W. Allen, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 2691 (1971); see also G. W. Allen, Ph.D. Thesis, Wesleyan University, 1971.
(3) Nomenclature: PC has been commonly called "creatine phosphate" which is unfortunate since that implies a salt; *N*-phosphorocreatine correctly indicates a P–N covalent bond.
(4) P. Haake and G. W. Allen, *J. Am. Chem. Soc.*, **95**, 8080 (1973).
(5) K. Zeile and G. Fawaz, *Hoppe-Seyler's Z. Physiol. Chem.*, **256**, 193 (1938).
(6) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **81**, 629 (1929).
(7) K. Lohmann, *Biochem. Z.*, **194**, 306 (1938).
(8) G. Fawaz and K. Zeile, *Hoppe-Seyler's Z. Physiol. Chem.*, **263**, 175 (1940).
(9) H. Borsook and J. W. Dubnoff, *J. Biol. Chem.*, **81**, 629 (1947); the structure of acylguanidines is discussed by K. Matsumoto and H. Rapoport, *J. Org. Chem.*, **33**, 552 (1968).
(10) V. M. Clark and A. R. Macrae, unpublished reults.
(11) V. M. Clark and S. G. Warren, *Nature (London)*, **199**, 657 (1963).
(12) A. Lapidot and D. Samuel, *Biochim. Biophys. Acta*, **111**, 537 (1965).
(13) S. Olezza, R. Moratti, and L. Speranza, *Farmaco, Ed. Prat.*, **21**, 319 (1966); *Chem. Abstr.*, **65**, 7525h (1951).
(14) A. A. Noyes, *Z. Phys. Chem.*, **11**, 495 (1893).
(15) O. Meyerhof and K. Lohmann, *Biochem. Z.*, **196**, 49 (1928).
(16) O. Folin, *J. Biol. Chem.*, **17**, 463 (1914); O. Folin and H. Wu, *ibid.*, **38**, 81 (1919).
(17) S. P. Datta and A. K. Grzybowski, *J. Chem. Soc.*, 6004 (1963), 187 (1964).
(18) M. J. Jorgenson and D. R. Hartter, *J. Am. Chem. Soc.*, **85**, 878 (1963).
(19) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms", Vol. I, W. A. Benjamin, New York, N.Y., 1966, Chapter 1.
(20) A. H. Ennor and H. Rosenberg, *Biochem. J.*, **57**, 203 (1954).
(21) R. K. Morton, *Nature (London)*, **172**, 65 (1953).
(22) J. F. Van Pilsum and B. Hiller, *Arch. Biochem. Biophys.*, **85**, 483 (1959).
(23) The metaphosphate mechanism for creatine kinase appears to have been conceived independently by Allen and Haake on the basis of mechanistic studies,[2,4] by M. Cohn and co-workers on the basis of magnetic resonance and binding [see M. Cohn and J.Reuben, *Acc. Chem. Res.*, **4**, 214 (1972), and T. L. James and M. Cohn, *J. Biol. Chem.*, **249**, 2599 (1974)], and by E. J. Milner-White and D. C. Watts, *Biochem. J.*, **122**, 727 (1971), on the basis of binding and inhibition studies although the latter authors argued more forcefully for a direct displacement mechanism.
(24) Metaphosphate as a reactive intermediate appears to have been suggested first by W. W. Butcher and F. H. Westheimer, *J. Am. Chem. Soc.*, **77**, 2420 (1955), and by A. R. Todd, *Proc. Natl. Acad. Sci. U.S.A.*, **45**, 1389 (1959).
(25) M. Eigen and L. Demaeyer in "Techniques in Organic Chemistry", Vol. VIII (part II), S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Interscience, New York, N.Y., 1963, p 1031.
(26) K. Gaede and R. Grittner, *Naturwissenschaften*, **89**, 39 (1952).
(27) V. Paulus, M. G. Choc, and G. W. Allen, unpublished results.
(28) K. Bloch, R. Schoenheimer, and D. Rittenberg, *J. Biol. Chem.*, **138**, 155 (1941); K. Bloch and R. Schoenheimer, *ibid.*, **131**, 111 (1939).
(29) V. C. Myers and M. S. Fine, *J. Biol. Chem.*, **21**, 583 (1915).
(30) M. Carpenter, P. McCay, and R. Caputto, *Proc. Soc. Exp. Biol. Med.*, **97**, 205 (1958).
(31) A. Marty, R. Nordmann, and J. Nordmann, *Bull. Soc. Chem. Biol.*, **43**, 619 (1961).
(32) R. J. Block, E. L. Durrum, and G. Zweig, "Paper Chromatography and Paper Electrophoresis", Academic Press, New York, N.Y., 1958, p 348.