Mechanism of Urea Participation in Phosphonate Ester Hydrolysis. Mechanistic and Stereochemical Criteria for Enzymic Formation and Reaction of Phosphorylated Biotin[1]

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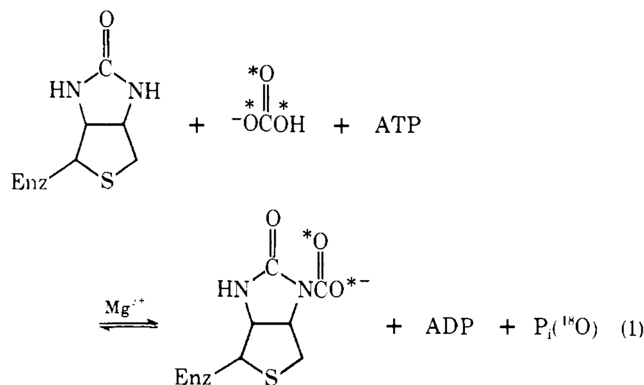
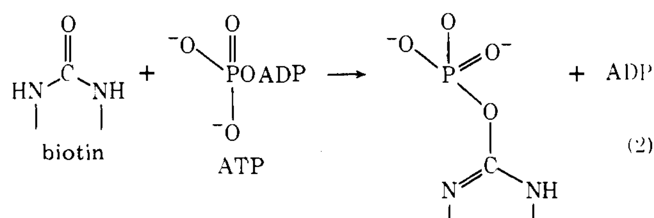
Abstract: The mechanism of participation of a urea group in hydrolysis of a phosphonate ester in acidic solution was investigated. Participation of the oxygen center of the ureido group of **1** and **3** at the phosphonate center is consistent with (1) the high degree of facilitation of ester hydrolysis compared to molecules lacking the adjacent functional group, (2) the observation that species resulting from attack of proximal nitrogen atoms of **1** and **3** are more stable than their precursors, and (3) the observation that acidic, anhydrous media containing alcohols cause rapid transesterification. A detailed mechanistic examination suggests that the participation reaction involves initial formation of a pentacovalent phosphorus intermediate with the rate-determining step overall being breakdown of the intermediate to expel the leaving alcohol. The potential involvement of *O*-phosphobiotin in adenosine 5′-triphosphate (ATP) dependent carboxylations in enzymic systems is considered with regard to these results, enzymic results from other laboratories, and stereochemical restrictions. It is concluded that all available experimental evidence is consistent with, but does not require, intermediate formation of *O*-phosphobiotin. Enzymic carboxylation of *O*-phosphobiotin, leading to formation of *N*-carboxybiotin and phosphate, is considered in terms of the stereochemistry of displacement at the phosphorus center of phosphobiotin. An "adjacent" mechanism, not involving free carboxyphosphate, and an "in-line" mechanism, involving free carboxyphosphate are possible. Reactions not involving *O*-phosphobiotin are likely to involve "in-line" displacement by bicarbonate at the terminal phosphate residue of ATP, requiring inversion of configuration at phosphorus. Since formation of *O*-phosphobiotin should occur with inversion at phosphorus, and transfer to bicarbonate with inversion or retention, the route via *O*-phosphobiotin can account for net inversion or net retention at phosphorus.

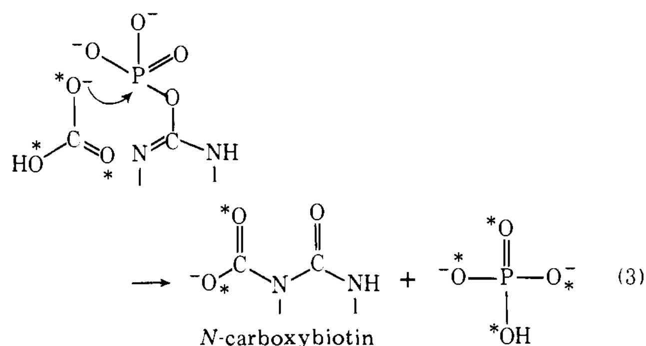
Biotin serves as a coenzyme in enzymic reactions involving transfer of the elements of carbon dioxide from dissolved bicarbonate to a carbanionic acceptor.[2] An initial reaction involving adenosine 5′-triphosphate (ATP), bicarbonate, and biotin to yield *N*-carboxybiotin, adenosine 5′-diphosphate (ADP), and phosphate (P_i) occurs with the oxygen labeling pattern of eq 1.[3-5] *N*-Carboxybiotin serves as the source of the



carbon dioxide equivalent which is added to the substrate.[4] The partial reactions have not been explicated in terms of detailed reaction mechanisms, although numerous propositions exist.[2]

Our interest in biotin reactions has been centered on reaction 1, formation of carboxybiotin.[6] Evidence has been presented that a trapped enol of biotin would be likely to be carboxylated by a carbonate monoester.[7-9] Early proposals, based on the general characteristics of the reactions, included suggestions that covalent interaction of ATP and biotin could produce a trapped enol which could also serve as a means of converting bicarbonate to a derivative that would react similarly to a monoester.[9-11] In (2), the ureido oxygen atom of biotin attacks the terminal phosphate of ATP. Prior to our observation of the possibility of an intramolecular attack of a urea upon a phosphonate ester,[6] chemical reactions between these functional groups were unknown. We reasoned that, in an enzymic sys-





tem, binding overcomes most translational entropic barriers that obscure many biomolecular reactions that have low enthalpic barriers. Therefore, we were pleased to observe that the intramolecular reaction of a phosphonate ester and a covalently connected urea occurs readily.

We have now examined the mechanism of participation of a urea at a phosphonate ester in detail. The reactivity and structure of intermediates can be deduced. With this information, and a knowledge of related phosphate ester stereochemical mechanisms, specific requirements for an enzymic reaction proceeding via (2) and (3) can be deduced.

Results

Kinetics of Hydrolyses. The methyl ester lithium salt (**1**) hydrolyzes rapidly in acidic solution, yielding the corresponding diacid (**2**) and methanol.[6] The observed first-order

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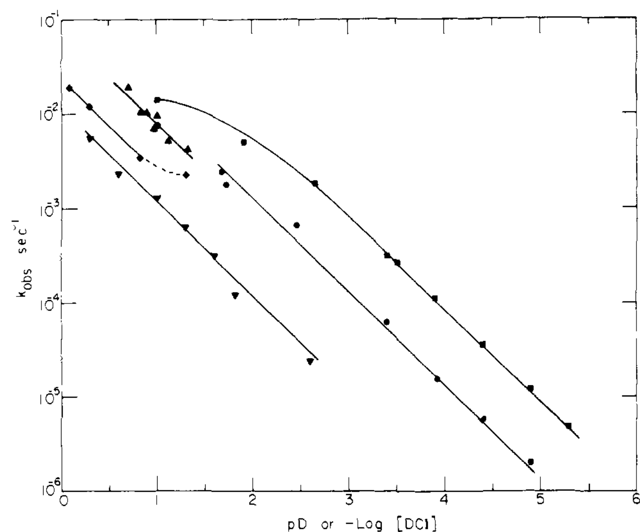
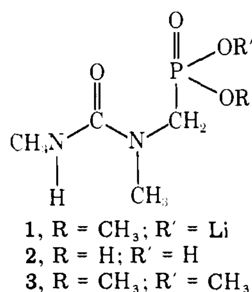


Figure 1. Observed first-order rate constants for ester hydrolysis at 35 °C of **1** (■), **3** (●), and **4** (▼) in deuterium oxide; of **3** (▲) and **5** (◆) in 1:1 (v:v) deuterium oxide/acetone-d_6. Data are from Tables I and II. Lines are drawn according to equations in text.



1, R = CH_3; R′ = Li
2, R = H; R′ = H
3, R = CH_3; R′ = CH_3

rate constants for the hydrolysis of **1** are summarized in Table I. The observed rate constant appears to be linear in acid concentration for acid solutions more dilute than pD 2.5. As the acidity increases beyond that point, the observed rate constant approaches independence of acid concentration. Since the expected pK_a of the conjugate acid of **1** is ~1.7,[12] the active species is likely to be the conjugate acid of **1**. The expression for the observed rate constant that fits the points (drawn in Figure 1) assumes that k_1 is the rate constant for hydrolysis of the conjugate acid of **1**.

$$k_{obsd} = k_1(1 + K_a/[H^+]) \quad (4)$$

A plot of $1/k_{obsd}$ vs. $1/[H^+]$ gives a straight line for the same data, yielding values of k_1 of 1.7×10^{-2} s^{-1} and K_A of 1.9×10^{-2} (pK_a = 1.7). These values were used to give the curve in Figure 1.

For comparison, a solution of monomethylmethylphosphonic acid (0.05 M) and 1,3-dimethylurea (0.05 M) gave no observable reaction when kept at 35 °C in 0.5 M DCl for 1 month. Since we could detect production of methanol resulting from 2% reaction by ^{1}H NMR, the rate constant for hydrolysis of **1** implies an "apparent molarity"[13,14] of the internal urea as a neighboring group in a nucleophilic mechanism of more than 10^6 M.

The dimethyl ester (**3**) of diacid (**2**) hydrolyzes rapidly in acid to give **2** and 2 equiv of methanol. The data in Table I, plotted in Figure 1, indicate that the observed first-order rate constant for hydrolysis is linear in acid concentration over the entire range studied. The rate law is

$$v = k_{obsd}[\mathbf{3}] = k_3[H^+][\mathbf{3}] \quad (5)$$

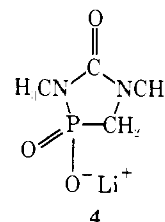
The value derived from the unit slope plot in Figure 1 for k_3 is 1.2×10^{-2} s^{-1} M^{-1}. The *rate* of hydrolysis of **3** in dilute acid

Table I. Observed First-Order Rate Constants for Hydrolysis (35 °C, Deuterium Oxide, NMR Method)

pD (or −log [DCl] if <2)	$10^5 k_{obsd}$, s^{-1}		
	1	4	3
0.30		550	
0.60		230	
1.0	1400	130	770
1.3		64	
1.6		32	
1.68			250
1.72			180
1.82		12	
1.89	510		
2.46			68
2.59		2.43	
2.65	180		
3.4	32		6.4
3.5	27.5		
3.9	11.2		1.57
4.14		0.073	
4.4	3.6		0.57
4.9	1.2		0.2
5.3	0.48		

is slower than observed for **1** so that monoester **1** cannot build up as an intermediate.

The cyclic phosphonamide salt (**4**) hydrolyzes to **2** more



slowly than **1** is converted to **2** under the same reaction conditions (Figure 1 and Table I). Therefore the ureido group does not accelerate hydrolysis of **1** by nitrogen participation since an intermediate that is kinetically incompetent, **4**, would form.

The observed first-order rate constant for hydrolysis of **4** shows no curvature from linearity through the expected pK_a region in Figure 1, which would be expected if the only hydrolysis route involved the conjugate acid of **4** reacting with water. If an acid-catalyzed reaction of the conjugate acid of **4** also occurs with a second-order rate constant of k_4^H in addition to the uncatalyzed reaction (first-order rate constant k_4), then if $k_4^H = k_4/K_A$ an apparent straight line with unit slope as in Figure 1 should result. The rate-constant relationships are

$$k_{obsd} = (k_4 + k_4^H[H^+])/(1 + K_A/[H^+]) \quad (6)$$

$$k_{obsd} = k_4(1 + [H^+]/K_A)/(1 + K_A/[H^+]) \quad (7)$$

At $[H^+] = K_A$ (pK_a = pH), $k_{obsd} = k_4$. At low acid concentrations, $k_{obsd} = k_4[H^+]/K_A$. At high acid concentrations, $k_{obsd} = k_4[H^+]/K_A$, because $k_4^H = k_4/K_A$. By comparison, where (4) holds (no acid catalysis), at pH = pK_a, $k_{obsd} = k_1/2$. The value obtained for k_4 depends on the exact value of K_A. Using a pK_a value of 1.7, the value of k_4 is 2.5×10^{-4} s^{-1}, about 80 times less than k_1 (for **1**).

The methyl ester (**5**) of cyclic phosphonamide **4** is the intermediate that would result if ureido participation in the hydrolysis of diester **3** occurred by participation of the nitrogen center. The kinetic validity of **5** as an intermediate was ruled out by the finding that its rate of hydrolysis is slower than that of **3**. Since **5** is insufficiently soluble in water to conduct a hydrolysis study at concentration levels necessary for ^{1}H NMR analysis, 1:1 (v:v) acetone-d_6/D_2O (DCl) was used as a re-

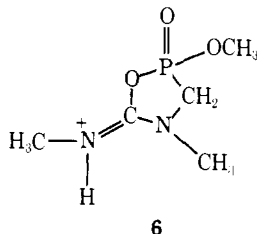
Table II. Observed Rate Constants for Hydrolysis of **3** and **5** 1:1 (v:v) D_2O/Acetone-d_6, 35 °C

compd	[DCl]	$10^3 k_{obsd}$, s^{-1}
5	0.05	2.3
	0.15	3.5
	0.50	12
	0.80	19
3	0.05	4.5
	0.08	5.2
	0.105	9.9
	0.107	7.2
	0.13	11
	0.15	10.6
	0.20	19

action medium. The utility of this medium has been presented by Kluger and Chan.[15] Since buffer salts decrease the solubility of **5**, acid concentrations above 0.05 M were used to avoid their necessity.

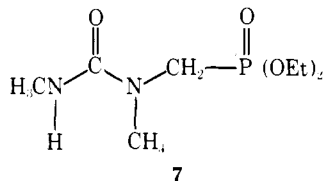
Both the diester, **3**, and cyclic phosphonamide methyl ester, **5**, were hydrolyzed under comparable conditions (Table II and Figure 1). The cyclic phosphonamide monoester **5** hydrolyzed more slowly than the diester **3**. Hence, **5** is not a kinetically competent intermediate for the reaction of **3** to produce **1** or **2**. The dashed line in Figure 1 indicates the contribution of a possible neutral hydrolysis route.

Transesterification Reactions. The participation of the oxygen center of the ureido moiety of **3** at its phosphorus center should produce **6**, which should react rapidly with any nucleophile present (see Discussion).



Using a solution of **3** in $CDCl_3$ containing toluenesulfonic acid hydrate (1 equiv) we observed a ^1H NMR spectrum consistent with structure **6**.[6] However, further analysis using ^{31}P NMR reveals that the spectrum is identical with that of monoester monoacid **1** (or a mixture of diester **3** and diacid **2**). Apparently 1 equiv of water reverses the equilibrium completely. Under scrupulously anhydrous conditions (trifluoroacetic acid and trifluoroacetic anhydride in $CDCl_3$), only the spectrum of **3** is observed. Therefore, although **6** probably forms readily, its concentration is very low because the second-order reactions are fast.

If **6** does form, then the presence of an alcohol other than methanol in the anhydrous reaction medium should produce acyclic esters derived from the other alcohol. Thus, when dimethyl ester **3** was dissolved in $CDCl_3$ containing anhydrous 1.6 M HCl in ethanol, the spectrum indicated production of diethyl ester **7** and methanol.



Similarly, when 40 mg (0.1 mmol) of **7** was dissolved in 0.5 mL of $CDCl_3$, upon addition of 0.1 mL of anhydrous 1 M HCl/CH_3OH, the ^1H NMR spectrum revealed the presence of **3**, **7** (or methyl ethyl ester), methanol, and ethanol: δ 1.22 (t, J = 7 Hz, CH_3 of EtOH), 1.33 (t, J = 7 Hz, $POCH_2CH_3$ of **7**), 2.82 (s, NCH_3 of **3** and **7**), 3.05 (s, NCH_3 of **3** and **7**), 3.43 (s, CH_3 of CH_3OH), 3.61 (q, J = 7 Hz, CH_2 of EtOH), 3.80 (d, J = 11 Hz, $POCH_3$ of **3** and **7**), 3.82 (d, J = 10 Hz, PCH_2 of **3** and **7**), 4.17 (quintet, J = 7 Hz, $POCH_2CH_3$ of **7**).

Attempts to trap alcohol produced, and thus prevent reaction of **6**, have not yet been successful. However, structural modification appears to be a promising way of "taming" the intermediate.

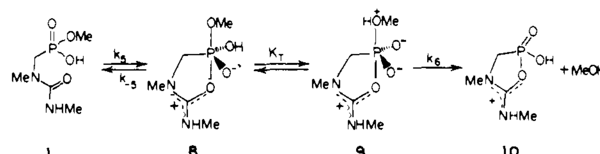
Discussion

Pathway of Participation Reaction. We have suggested that the urea moieties of phosphonate esters **1** and **3** facilitate reaction by nucleophilic attack at the phosphorus center, generating O-phosphorylated oxyamidinium ions (e.g., **6**) which react rapidly with water. We now have demonstrated that oxygen participation rather than nitrogen participation occurs since the nitrogen-cyclized species (phosphonamides **4** and **5**) are less reactive than their necessary precursors. Furthermore, transesterification reactions confirm that reaction occurs via a highly reactive intermediate generated from attack at phosphorus.

It is clearly established that nucleophilic substitution reactions of phosphonates in acidic solutions occur through intermediate formation of pentacoordinated phosphorus species.[16-18] Analysis of the structures resulting from application of the Westheimer rules for formation and decomposition of these intermediates[16-18] gives information about the identity of the rate-determining step in the kinetic mechanism when combined with our experimental observations.

The intermediate involved in the reaction of **1** should have structure **8** to conform to Westheimer's rules regarding strain minimization, electronic preferences, and attack in trigonal bipyramids (Scheme I).

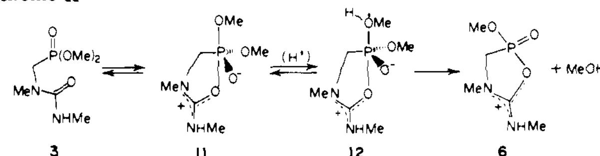
Scheme I



The moiety derived from the ureido portion of **1** is a C-substituted amidinium ion. The pK_a of ethoxyamidinium ion is 10.0.[19] In the acidic solutions used for this study, **8** would be protonated as shown. The identity of the rate-determining step depends on the identity of the loser in the rate competition between steps designated k_{-5} and k_6. Since K_T = [**9**]/[**8**], its value is very small, requiring a prototropic shift in the direction that is unfavorable by a factor of at least 10^6 (see ref 17 for estimates of K_A's of related intermediates). From intermediate **8**, expulsion of neutral urea will be much faster than expulsion of the strongly basic methoxide ion (Δ log k/ΔpK_a(leaving group) has not been determined but we estimate the value to be near unity). The competition in leaving from **9** should favor methanol by a factor (pK_a(CH_3OH) = −2.0,[20] pK_a(urea) = −0.5[21]) which is not nearly sufficient to overcome the unfavorable value of K_T. Therefore, k_6 must be rate determining in production of **2**. External acid catalysis has not been indicated because the rate of hydrolysis appears to level with increasing acid. The phosphorane oxygen proton is shown as the source of acid since diester **3** (vide infra) requires external acid and the only difference is this proton.

The analogous intermediate, **11**, derived from diester **3**, must acquire an external proton to expel methanol (Scheme II) since

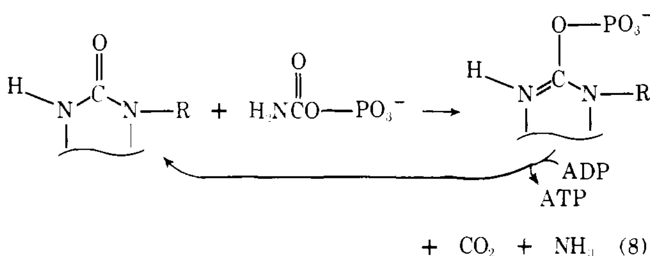
Scheme II



the observed rate depends on acid. These results also suggest that the pK_a of the phosphorane hydroxyl group in **8** is considerably lower than the amidinium ion group whose pK_a is 10,[19] consistent with previous estimates.[17]

Formation of *O*-Phosphobiotin. The rate-determining step in reactions shown in Schemes I and II is expulsion of the leaving group. Enzymic reactions of biotin proceeding via initial formation of *O*-phosphobiotin from ATP and biotin would involve a much better leaving group (ADP) than methoxide of the model (which also generates a strained product and therefore a higher energy transition state). Therefore, an enzymic reaction between bound ATP and biotin should have a low enthalpy barrier since the leaving group is at least 7 pK_a units less basic than methoxide and may be as low as that of the oxyamidinium ion (urea). In the latter case, neither step would be solely rate determining, as in a "perfect" enzyme.[22]

Is *O*-Phosphobiotin an Enzymic Intermediate? *O*-Phosphobiotin is a possible intermediate that may account for the involvement of biotin in the ATP-dependent carboxylation of biotin.[6,9-11] Biotin carboxylase also catalyzes formation of ATP from carbamyl phosphate and ADP in a biotin-promoted reaction.[4] This reaction also occurs if biotin is alkylated or acylated at the nitrogen atom that normally becomes carboxylated (and therefore cannot become carboxylated). This "side" reaction could occur through *O*-phosphobiotin in a manner consistent with the normal mechanism proposed for carboxylation (eq 8). The conclusion that these "structural

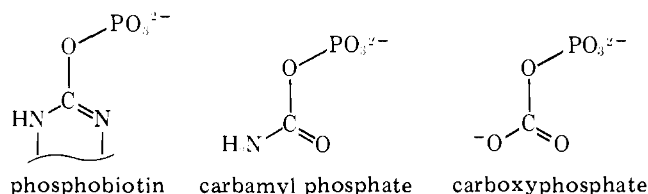


$+ CO_2 + NH_3$ (8)

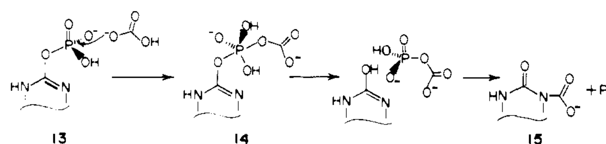
alterations of the biotin molecule would preclude [its] participation in the phosphoryl transfer mechanism"[4] does not take into account the possibility that the sites for carboxylation and phosphorylation are different. Unfortunately, a recent review[23] has cited these experiments as evidence against the involvement of *O*-phosphobiotin; in fact, these experiments do not bear on that question.

The ability of biotin carboxylase to produce ATP from carbamyl phosphate and ADP at a slow rate in the *absence* of biotin (4% of the rate observed in the presence of biotin[4]) has also been cited as evidence against a requirement of the involvement of biotin in ATP cleavage in biotin carboxylase.[4] This would necessarily also eliminate the involvement of *O*-phosphobiotin. The mechanistic significance of this clearly minor reaction is not certain. It has been suggested that biotin induces a conformational change and the slowness of the reaction is due to a control process to minimize waste of ATP.[4] Still, ATP would be wasted. The *O*-phosphobiotin route *requires* biotin to be present and prevents abortive cleavage of ATP.

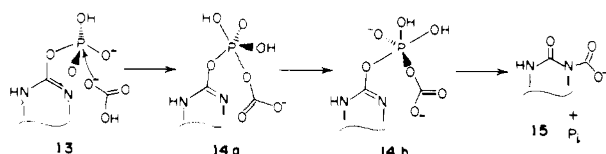
Finally, one can consider that carbamyl phosphate, in the absence of biotin, serves as an analogue of *O*-phosphobiotin and not of carboxyphosphate. Carboxyphosphate differs by



Scheme III



Scheme IV



having an extra negative charge at neutral pH. We feel that conclusive experimental evidence has not yet been obtained on this question.

Stereochemical Consequences of Carboxylation of *O*-Phosphobiotin in Enzymic Reactions. Conversion of *O*-phosphobiotin to carboxybiotin can occur by two stereochemically distinct pathways when the Westheimer rules for reaction intermediate stereochemistry at phosphorus[16] are followed. Usher pointed out that the possibility of pseudorotation of a pentacovalent intermediate permits attack at phosphorus to occur at the tetrahedral face that is opposite ("in line") or common ("adjacent") to the leaving group.[24] The latter result requires that the intermediate undergo a pseudorotation. The stereochemical consequences of these two modes are that the "in-line" mechanism causes an inversion of relative configuration at phosphorus and the "adjacent" mechanism leads to relative retention (equivalent to frontside displacement).

The two stereochemically distinct pathways for carboxylation of phosphobiotin (**13**) to form carboxybiotin (**15**) are given in Schemes III ("in-line" pathway) and IV ("adjacent" pathway). Mechanistic details have been minimized to emphasize stereochemistry.

The mechanism we have previously suggested[6] is an "adjacent" mechanism, as in Scheme IV, and its details were discussed. The "in-line" mechanism requires that bicarbonate attack phosphobiotin trans to the biotin ureido moiety. A concerted "in-line" attack and carboxylation is stereochemically impossible; therefore, carboxyphosphate must form, as shown in Scheme III. The "adjacent" mechanism in Scheme IV permits reaction to proceed without formation of carboxyphosphate, a potentially hyperreactive species.[25]

The conversion of ATP to ADP and P_i can be analyzed for the stereochemical fate of the displaced phosphate group.[26] The oxygen atom transferred from bicarbonate could replace the β-phosphate at ATP with relative retention, inversion, or randomization of configuration at the cleaved phosphate. The formation of phosphobiotin would likely occur with inversion. The conversion of phosphobiotin to carboxybiotin and phosphate can occur with retention (Scheme IV) or inversion (Scheme III). Overall, inversion-retention leads to inversion and inversion-inversion leads to retention. Reaction via direct attack of bicarbonate upon ATP to form carboxyphosphate[4] followed by carboxylation of biotin by that species leads to inversion at phosphorus in the simplest mechanism. Therefore, by this analysis, either stereochemical result would not rule out the involvement of *O*-phosphobiotin but may rule out direct displacement mechanisms. Structural analysis of an enzyme, however, may resolve the question. The mechanisms we have presented do place specific location requirements upon enzymatic binding sites for the various species. For example, NMR analysis of a (nonbiotin) enzyme, phosphoenol pyruvate carboxylase, revealed that an adjacent displacement by bicarbonate upon the phosphorus of phosphoenol pyruvate, with

implied pseudorotation (analogous to our Scheme IV), is the logical consequence of the enzyme's structural array.[27]

Experimental Section

Materials and Methods. General. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. ^{1}H NMR spectra were measured on a Varian T-60 instrument with tetramethylsilane (Me_4Si) as an internal standard in chloroform-*d* ($CDCl_3$) and methanol-d_4 (CD_3OD) solutions. In deuterium oxide (D_2O) solutions, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) was used as the internal standard. Deuterated solvents were purchased from Merck Sharp and Dohme of Canada, Ltd. ^{13}C NMR spectra were measured with a Varian CFT-20 instrument. Solutions in $CDCl_3$ were referenced to Me_4Si as an internal standard. Solutions in D_2O were referenced to Me_4Si using an insert. ^{31}P NMR decoupling experiments and 100-MHz spectra were determined on a Varian HA-100 instrument equipped with a Micro-Now decoupler.[12] ^{31}P NMR chemical shift positions are relative to external trimethyl phosphate.[12] Low-resolution mass spectra were measured on a Du Pont 21-490 mass spectrometer. High-resolution mass measurements were done on an A.E.I. MS3074. Solution acidities of less than 0.01 M acid were measured with a Radiometer pH meter and corrected for deuterium oxide in place of water by adding 0.40 to the meter value.[28] Acidities of 0.05 M or more ("pD" ≤1.6) were prepared with deuterium chloride and potassium chloride (to 0.5 M ionic strength). Solutions between "pD" 1.6 and 2.0 were made with 0.5 M dichloroacetate calibrated against standard deuterium chloride solutions. Buffers (0.5 M) and pD ranges used follow: 2.4–2.6, dihydrogen phosphate; 3.4–3.9, formate; 4.0–5.3, acetate. When 1 M formate was used in place of 0.5 M formate, no change in rate was observed.

Transesterifications were studied by dissolving the ester in $CDCl_3$ with the alternative alcohol containing anhydrous HCl (see Results) and observing the ^{1}H NMR spectra.

Syntheses. *N,N'*-Dimethyl-*N*-methoxymethylurea (17). The urea was prepared by the procedure of Petersen.[29] 1,3-Dimethylurea (110 g, 1.25 mol) was dissolved in a 37% formaldehyde solution (135 g, 1.35 mol), adjusted to pH 8 with 2 N NaOH, and then stirred for 2 h at 40 °C. The solvent was removed in vacuo to give a viscous solution. To this were added 250 mL of methanol (MeOH) and 0.65 mL of concentrated HCl. The solution was stirred at 45 °C for 2 h and then neutralized with 40% NaOH. The excess MeOH was removed by simple distillation under reduced pressure (15 Torr). Distillation under high vacuum gave the product, collected from 84 to 90 °C (0.01 Torr) (bath temperature 112–124 °C). The yield was 62%. The product is a colorless, viscous liquid: IR (neat) 1634, 1538 cm^{-1} (C=O); ^{1}H NMR ($CDCl_3$) δ 2.81 (3 H, d, $^2J_{HH'}$ = 5.5 Hz, $NHCH_3$), 3.03 (3 H, s, CH_3), 3.37 (3 H, s, OCH_3), 4.70 (2 H, s, CH_2), 5.3 (1 H, bs, NH).

1-Methoxy-1,3-dioxo-2,4-dimethyl-1-phospha-2,4-diazacyclopentane (Cyclic Phosphonamide Methyl Ester) (5). Compound **5** was prepared from **17** by modification[17] of the procedure of Petersen and Reuther.[30] To **17** (5.33 g, 0.04 mol) dissolved in 20 mL of benzene was added an equimolar amount of concentrated HCl (4 mL). The flask was shaken for 5 min. The benzene was removed in vacuo and the remaining water was removed under high vacuum at 50 °C for 2 h. The resulting product proved to be a mixture of 1,3-dimethylurea and 1-chloromethyl-1,3-dimethylurea. The mixture was dissolved in 50 mL of freshly distilled trimethyl phosphite and heated at 90 °C for 1–2 h under a drying tube. The phosphite was then removed by simple distillation under reduced pressure (15 Torr) (bath temperature 50 °C, distillate temperature 25 °C). The remaining solution was heated at 150 °C (0.01 Torr) for 20 min and then allowed to cool to room temperature. Recrystallization from toluene removed some of the 1,3-dimethylurea side product. The viscous solution was then dissolved in a minimal amount of $CHCl_3$ and about 1.1 g of solution was chromatographed on a column of silica gel (Grace, 100–200 mesh, 17 g, column, 12 mm × 23 cm). Fractions of 25 mL each were collected. The eluants used were $CHCl_3$ for the first fraction and 4% CH_3OH/96% $CHCl_3$ for the remaining fractions. Upon removal of solvent, fractions 2 and 3 gave a clear, colorless oil. Combination and distillation of those fractions (bp 112–114 °C (0.7 Torr)) gave pure **5** in about 28% yield based on **17**. The sample is a clear, colorless oil: IR (neat) 1700 (C=O), 1290–1255 cm^{-1} (P=O); ^{1}H NMR ($CDCl_3$) δ 2.83 (3 H, d, $^3J_{PH}$ = 8 Hz, CH_3), 3.03 (3 H, d, $^4J_{PH}$ = 1 Hz, CH_3), 3.43 (2 H, d, $^2J_{PH}$ = 15 Hz, PCH_2), 3.85 (3 H, d, $^3J_{PH}$ = 12 Hz, $POCH_3$); ^{13}C NMR ($CDCl_3$) δ 25.07 (q, $^1J_{CH}$ = 41 Hz, CH_3), 32.16 (d of q, $^3J_{PC}$ = 12.7, $^1J_{CH}$ = 41 Hz, CH_3), 43.73 (d of t, $^1J_{PC}$ = 116, $^1J_{CH}$ = 41 Hz, PCH_2), 53.77 (d of q, $^2J_{PC}$ = 6.4, $^1J_{CH}$ = 41 Hz, $POCH_3$), 156.11 (d, $^2J_{PC}$ = 27.4 Hz, C=O); ^{31}P NMR ($CDCl_3$) δ −25.20; mol wt 178.0507 (calcd for $C_5H_{11}N_2O_3P$, 178.0507).

1,1-Dimethyl-1,4-dioxo-3-methyl-1-phospha-3,5-diazahexane (Dimethyl Ester) (3). Compound **3** was prepared by several methods.

Method 1. During the preparation of **5**, compound **3** was obtained in approximately 25% yield based on **17**. By eliminating the heating at 150 °C in the preparation of **5**, cyclization was decreased and the yield of **3** increased.

Method 2. Compound **3** was also prepared by dissolving 1 g of **5** in 25 mL of dry methanol with a catalytic amount of sodium methoxide and stirring at room temperature for 1 h. The solvent was removed in vacuo to give a colorless oil. Upon the addition of dry, alcohol-free $CHCl_3$, the sodium methoxide was precipitated. Compound **5** was obtained quantitatively by filtering the solution through a glass-wool plug.

Method 3. Diacid **2** was dissolved in a minimal amount of dry methanol to which was added 2 equiv of diazomethane in ether. After removal of the solvent in vacuo, the oily product was purified by column chromatography. Approximately 1 g of sample was placed on a short column of silica gel (Grace, 100–200 mesh, 15 g, column, 19 mm × 10 cm) and eluted with 5% CH_3OH/95% $CHCl_3$. The pure, colorless oil obtained was compound **3**: IR (neat) 1644, 1540 (C=O), 1263 cm^{-1} (P=O); ^{1}H NMR ($CDCl_3$) δ 2.81 (3 H, d, $^3J_{HH'}$ = 5 Hz, $NHCH_3$), 2.98 (3 H, s, CH_3), 3.76 (2 H, d, $^2J_{PH}$ = 9.5 Hz, PCH_2), 3.81 (3 H, d, $^3J_{PH}$ = 11 Hz, $POCH_3$), 5.07 (1 H, b, NH); ^{13}C NMR ($CDCl_3$) δ 27.70 (q, $^1J_{CH}$ = 35 Hz, CH_3), 35.56 (q, $^1J_{CH}$ = 30 Hz, CH_3), 43.60 (d of t, $^1J_{PC}$ = 158, $^1J_{CH}$ = 33 Hz, PCH_2), 52.77 (d of q, $^2J_{PC}$ = 6.8, $^1J_{CH}$ = 36 Hz, $POCH_3$), 159.02 (d, $^3J_{PC}$ = 2.0 Hz, C=O); ^{31}P NMR ($CDCl_3$) δ −23.81 ppm; mol wt 210.0769 (calcd for $C_6H_{15}N_2O_4P$, 210.0769).

Lithium 1-Methoxy-1-oxy-1,4-dioxo-3-methyl-1-phospha-3,5-diazahexane (Methyl Ester Lithium Salt) (1). Compound **1** was prepared by treatment of **5** with 1 equiv of lithium hydroxide monohydrate in methanol and stirring at room temperature for 2 h. The sample is a white powder: IR (KBr) 1620, 1555 (C=O), 1210 cm^{-1} (P=O); ^{1}H NMR (D_2O) δ 2.67 (3 H, s, CH_3), 2.92 (3 H, s, CH_3), 3.52 (2 H, d, $^2J_{PH}$ = 9 Hz, PCH_2), 3.55 (3 H, d, $^3J_{PH}$ = 10 Hz); ^{31}P NMR (D_2O) δ −18.03. Anal. ($C_5H_{12}N_2O_4PLi$) C, H, N.

Sodium 1,3-Dioxo-2,4-dimethyl-1-phospha-2,4-diazacyclopentane (Cyclic Phosphonamide Sodium Salt) (4). Compound **4** was prepared by the following method: 450 mg (2.53 mmol) of **4** and 1.0 g (6.67 mmol) of NaI were dissolved in 25 mL of dry acetone ($MgSO_4$) and refluxed for 3 h. Compound **4** separated as a white solid. It was filtered, washed with acetone, and dried. The product was dissolved in methanol to remove traces of impurities, reprecipitated by the addition of ether, filtered, and washed with ether. The compound is a white powder: IR (KBr) 1680 (C=O), 1300 cm^{-1} (P=O); ^{1}H NMR (D_2O) δ 2.74 (3 H, d, $^3J_{PH}$ = 7 Hz, $PNCH_3$), 2.90 (3 H, d, $^4J_{PH}$ = 1 Hz, CH_3), 3.32 (2 H, d, $^2J_{PH}$ = 14 Hz, PCH_2); ^{31}P NMR (D_2O) δ −18.55.

1,1-Dihydroxy-1,4-dioxo-3-methyl-1-phospha-3,5-diazahexane (Diacid) (2). Compound **2** was prepared by two methods.

Method 1. The crude reaction mixture of compound **5**, after removal of phosphite, was dissolved in 10% HCl and allowed to stand overnight. After removal of water in vacuo, the crude mixture of **2** and 1,3-dimethylurea was dissolved in a minimal amount of boiling water. Acetone was then added until the hot solution turned cloudy. The solution was allowed to cool to room temperature and then placed in a refrigerator overnight to crystallize. The crystals were collected by suction filtration and recrystallized from water/acetone, giving pure diacid.

Method 2. Compound **1** was dissolved in 1.0 N HCl and hydrolyzed to the diacid in ca. 1 h. The diacid was then isolated using a Dowex ion exchange column prepared by eluting with 1.0 N HCl. The product was crystallized from water/acetone, as above, in good yield. The pure compound is a white solid: mp 160–161 °C; IR (KBr) 1560 (C=O), 1240 cm^{-1} (P=O); ^{1}H NMR (D_2O) δ 2.72 (3 H, s, CH_3), 2.92 (3 H, s, CH_3), 3.72 (2 H, d, $^2J_{PH}$ = 10 Hz, PCH_2); ^{13}C NMR (D_2O) δ 28.46 (q, $^1J_{CH}$ = 37 Hz, CH_3), 36.76 (q, $^1J_{CH}$ = 36 Hz, CH_3), 46.80 (d of t, $^1J_{PC}$ = 152, $^1J_{CH}$ = 35 Hz, PCH_2), 161.71 (d, $^3J_{PC}$ = 1.5 Hz, C=O); ^{31}P NMR (D_2O) δ −19.34. Anal. ($C_4H_{11}N_2O_4P$) C, H, N, P.

1-Ethoxy-1,3-dioxo-2,4-dimethyl-1-phospha-2,4-diazacyclopen-

tane (Cyclic **Phosphonamide Ethyl Ester**) (**18**). The preparation of compound **18** was similar to that of **4** except that triethyl phosphite was used in place of trimethyl phosphite. After heating at 150 °C, a crude mixture of **18**, **7**, and 1,3-dimethylurea was distilled (bp 128–130 °C (0.7 Torr)) and then separated by column chromatography as in the preparation of **5**. Fraction 3 gave compound **18**. Distillation (bp 128–130 °C (0.7 Torr)) gave pure **18**. The pure sample is a clear, colorless oil: IR (neat) 1700 (C=O), 1275 cm^{-1} (P=O); ^1H NMR ($CDCl_3$) δ 1.38 (3 H, t, $^3J_{HH'}$ = 7 Hz, $POCH_2CH_3$), 2.80 (3 H, d, $^3J_{PH}$ = 7.5 Hz, $PNCH_3$), 2.98 (3 H, d, $^4J_{PH}$ = 1 Hz, CH_3), 3.45 (2 H, d of d, $^3J_{PH}$ = 15, $^2J_{HH'}$ = 1 Hz, PCH_2), 4.23 (2 H, d of q, $^3J_{PH}$ = 10, $^3J_{HH'}$ = 7 Hz, $POCH_2$); ^{13}C NMR ($CDCl_3$) δ 16.58 (d of t, $^3J_{PC}$ = 5.6, $^1J_{CH}$ = 38 Hz, $POCH_2CH_3$), 25.19 (q, $^1J_{CH}$ = 39 Hz, CH_3), 32.36 (d of q, $^2J_{CH}$ = 12.7, $^1J_{CH}$ = 40 Hz, $PNCH_3$), 44.80 (d of t, $^1J_{PC}$ = 117, $^1J_{CH}$ = 40 Hz, PCH_2), 63.67 (d of q, $^2J_{PC}$ = 6.7, $^1J_{CH}$ = 39 Hz, $POCH_2$), 156.20 (d, $^2J_{PC}$ = 27.6 Hz, C=O); mol wt 192.0661 (calcd for $C_6H_{13}N_2O_3P$, 192.0664).

1,1-Diethyl-1,4-dioxo-3-methyl-1-phospha-3,5-diazahexane (Diethyl Ester) (7). Compound **7** was prepared in a manner similar to that of **3** using triethyl phosphite instead of trimethyl phosphite. The compound was obtained in approximately 25% yield from column chromatography of a sample using preparation method 1. The pure sample is a colorless oil: IR (neat) 1640, 1540, (C=O), 1218 cm^{-1} (P=O); ^1H NMR ($CDCl_3$) δ 1.33 (3 H, t, $^3J_{HH'}$ = 7 Hz, $POCH_2CH_3$), 2.78 (3 H, d, $^3J_{HH'}$ = 4.5 Hz, $NHCH_3$), 3.00 (3 H, s, CH_3), 3.69 (2 H, d, $^2J_{PH}$ = 9 Hz, PCH_2), 4.17 (2 H, quintet, $^3J_{HH'}$ = 7, $^3J_{PH}$ = 7 Hz, $POCH_2CH_3$), 5.27 (1 H, b, NH); ^{13}C NMR ($CDCl_3$) δ 16.47 (d of t, $^3J_{PC}$ = 5.8, $^1J_{CH}$ = 34 Hz, $POCH_2CH_3$), 27.76 (q, $^1J_{CH}$ = 35 Hz, CH_3), 35.97 (q, $^1J_{CH}$ = 34 Hz, CH_3), 45.07 (d of t, $^1J_{PC}$ = 160, $^1J_{CH}$ = 33 Hz, PCH_2), 62.52 (d of q, $^2J_{PC}$ = 6.8, $^1J_{CH}$ = 34 Hz, $POCH_2$), 159.27 (s, C=O); mol wt 238.1070 (calcd for $C_8H_{19}N_2O_4P$, 238.1082).

Kinetic Procedure. The hydrolysis of the methyl phosphonates (**1**, **3**, and **5**) was followed by measuring the integrated absorbances of the methanol singlet at 3.5 ppm in the ^1H NMR spectrum. That methanol was released was confirmed by the addition of methanol to the NMR tube after completion of the reaction and obtaining a coincident spectral peak. The compounds all hydrolyze to diacid **2**. The hydrolysis of cyclic phosphonamide sodium salt **4** was followed by measuring the integrated absorbances of the disappearing PCH_2 doublet at 3.32 ppm. All hydrolysis experiments were done at 35 °C, the temperature of the spectrometer probe.

In mixed-solvent experiments, we used acetone-d_6/DCl, 50:50 v/v. A typical procedure follows: 0.18 mL of a 0.2 M solution of substrate in acetone-d_6 was placed in an NMR tube maintained at 35 °C; 0.18 mL of 38% deuterium chloride in D_2O (maintained at 35 °C) was added to the NMR tube. The tube was shaken and placed in the NMR probe. Integrated absorbances were measured periodically.

The hydrolysis experiments using buffers in solutions that would be acidified by the product (pK_a = 1.3) followed the same general procedure except that 0.25 mL (0.10 M) of compound in D_2O was mixed with 0.25 mL of buffer (1.0 M) in D_2O.

The observed first-order hydrolysis rate constants were obtained by plotting percent substrate (from integrated resonance signals) remaining (logarithmic scale) against time. Each rate constant was determined from the slope of an estimated best fit straight line through the experimental points, usually for 3 half-lives. Each second-order rate constant for hydrolysis was determined by plotting the observed first-order rate constants against pD or −log [DCl] (Figure 1). These points fit a line of unit slope. The value at 1.0 M acid concentration gives the numerical value of the second-order rate constant.

References and Notes

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