Discrete Monomeric Metaphosphate Anion as an Intermediate in the Hydrolysis of μ-Monothiopyrophosphate

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Abstract: μ-Monothiopyrophosphate (MTP) is millions of times more reactive than pyrophosphate as a phosphoryl group donor to water. Addition of nucleophiles, including α-effect nucleophiles, at high concentrations to aqueous solutions of MTP does not increase the rate at which MTP is cleaved to thiophosphate. However, tris(hydroxymethyl)aminomethane (Tris) and ethylenediamine capture phosphoryl groups from MTP in competition with water. Phosphoryl capture by 3.0 M Tris at pH 9.9 partitions the phosphoryl group into *N*-phospho-Tris (53%) and *O*-phospho-Tris (47%). Fluoride and acetohydroxamate anion do not capture phosphoryl groups from MTP, despite being excellent nucleophiles for the phosphoryl group in highly reactive phosphoric esters such as acetyl phosphate and *N*-phosphopyridines. The results are interpreted in terms of a mechanism in which MTP undergoes cleavage into thiophosphate and discrete monomeric metaphosphate within a solvation sphere, from which metaphosphate cannot escape before being captured by a solvating nucleophile. Nucleophiles such as Tris can enter the solvation sphere by displacing water molecules in a preliminary equilibrium step; however, solvating Tris has little or no effect on the rate at which MTP undergoes cleavage into metaphosphate, and metaphosphate is captured by solvating water or Tris. Negatively charged nucleophiles such as fluoride and acetohydroxamate anion cannot solvate MTP, owing to electrostatic repulsion, and cannot capture metaphosphate. MTP differs from phosphoric esters, acyl phosphates, and phosphoramidates as a phosphoryl group donor in that its P–S bonds are weaker than P–O or P–N bonds. Phosphoric esters and anhydrides donate phosphoryl groups to nucleophiles within dissociative transition states that include the nucleophile (Herschlag, D.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 7579–7586). In the case of the trianion of MTP, the P–S bond is very weak and undergoes cleavage to metaphosphate and thiophosphate dianion without nucleophilic participation. Metaphosphate monoanion is so reactive with nucleophiles that it cannot escape from its solvation sphere; it is captured by water or another nucleophile that competes with water in solvating MTP.

μ-Monothiopyrophosphate (MTP[1]) is the most highly reactive phosphoryl group donor that has been subjected to a mechanistic analysis of its hydrolysis.[2] $HMTP^{3-}$ undergoes hydrolysis 17-million times faster than HPP_i^{3-}, and H_2MTP^{2-} undergoes hydrolysis 58-million times faster than $H_2PP_i^{2-}$.[2b] The large difference in rates is attributed to the dissociative nature of the hydrolysis mechanisms for MTP and PP_i.[2b] The transition state for phosphoryl group transfer from PP_i to water is characterized by a high degree of P–O bond cleavage, as it is for the reactions of phosphoric esters and anhydrides and for phosphoramidates.[3a-f] The weakness of the P–S bonds in MTP compared with the P–O bonds in PP_i accentuates the reactivity of MTP in dissociative processes and causes it to undergo hydrolysis millions of times faster than PP_i.[2b]

The dissociative nature of the transition state for phosphoryl group transfer from phosphoric esters, acyl phosphates, and phosphoramidates has been worked out through kinetic studies and product analysis of the reactions of nucleophiles and water with reactive phosphate compounds.[3] The hydrolysis of phosphomonoester monoanions was originally postulated to proceed by a stepwise mechanism through the intermediate formation of monomeric metaphosphate anion as an electrophilic intermediate.[4] This mechanism was supported by the thermodynamic activation parameters, by structure reactivity correlations, and by the absence of a solvent kinetic isotope effect in D_2O.[4e] An ingenious phase-transfer experiment further supported the existence of an electrophilic intermediate.[4f] Recent kinetic and stereochemical studies of the transfer of phosphoryl groups to nucleophiles in protic media failed to support the existence of monomeric metaphosphate as a diffusible intermediate.[3,5a,b] Linear free energy correlations showed that the transition state in water is characterized by a high degree of bond cleavage between the leaving group and the phosphoryl group in flight, by the presence of the acceptor nucleophile, and by the existence of weak bonding between the phosphoryl group and the acceptor. The stereochemical course of the solvolysis of chiral phosphomonoesters in alcoholic solvents has been found to proceed with inversion of configuration and no detectable racemization.[5a,b] The absence of either an inverse or a normal secondary kinetic isotope effect in the hydrolysis of glucose 6-[$^{18}O_3$]phosphate, in which the ^{18}O atoms occupy the peripheral positions of the phosphate group, has been interpreted to mean that the transition state is dissociative in nature and that there is significant bonding between the phosphorus atom and glycosyl-O-6 in the transition state.[6]

Nucleophilic participation in the transition state manifests itself in the increased rate at which reactive phosphate compounds phosphorylate nucleophiles other than water in aqueous solutions and in the stereochemical inversion of configuration at phosphorus

(1) Abbreviations: PP_i, pyrophosphate; HPP_i^{3-} and $H_2PP_i^{2-}$, the trianionic and dianionic forms of PP_i; MTP, μ-monothiopyrophosphate (the analog of PP_i with bridging sulfur in place of oxygen); $HMTP^{3-}$ and H_2MTP^{2-}, the trianionic and dianionic forms of MTP; pK_{nuc}, the pK_a of the conjugate acid of a nucleophile; pK_{lg}, the pK_a of the conjugate acid of a leaving group; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

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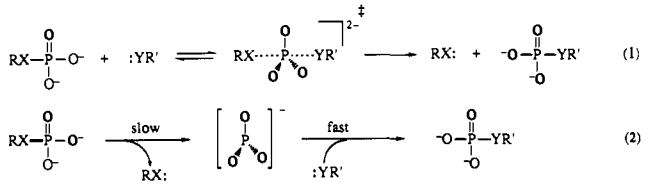
in protic solvents. Stereochemical studies in nonprotic solvents, and in the sterically hindered protic solvent *t*-butyl alcohol indicate that monomeric metaphosphate can be an intermediate under the special conditions of poor solvation.[5c,d]

The high hydrolytic reactivity of MTP, owing to the weakness of the P–S bonds, suggested to us that this molecule might undergo hydrolysis through a true dissociative mechanism and a discrete metaphosphate intermediate. Haake and Allen postulated that the solvolysis of dimethylguanidinium phosphate—which is also highly reactive but undergoes hydrolysis at about 1% the rate of MTP—may entail little or no nucleophilic participation by acceptors in the transition state.[7] They suggested that the solvolysis mechanism for dimethylguanidinium phosphate may be near an interface between the mechanism of eq 1, a nucleophilic displacement, and that of eq 2, a dissociative mechanism through a metaphosphate intermediate. According to their postulate, phosphate compounds that are less reactive than dimethylguanidinium phosphate react with nucleophilic participation in the transition state according to eq 1.



If dimethylguanidinium phosphate reacts near the mechanistic interface, the high reactivity and accentuated dissociative character of MTP-hydrolysis should place this reaction in the category of one proceeding by a mechanism analogous to that of eq 2. In this mechanism, the rate of RX: formation is insensitive to the nucleophilic reactivity of the acceptor R′Y:, because the rate is that at which metaphosphate is formed in the first step. In an aqueous solution, metaphosphate will be partitioned between water and an added nucleophile, but the concentration of an added nucleophile will not alter the rate at which RX: is formed.

We have investigated the effects of nucleophiles on the rate at which MTP is cleaved to thiophosphate in aqueous solutions at pH values in the range 9–10, where the most reactive form of MTP is $HMTP^{3-}$. The nucleophiles included amines, α-effect nucleophiles, and fluoride ion. The nucleophiles did not increase the rate at which MTP was cleaved to thiophosphate; however, Tris and ethylenediamine efficiently captured the phosphoryl group. These results implicated monomeric metaphosphate as a discrete intermediate in the hydrolysis of MTP. Portions of this work have appeared in a communication.[8]

Experimental Section

Materials. Li_4MTP was synthesized as described elsewhere.[9] HCl was distilled as a constant-boiling fraction at 110 °C to give a 6.1 M solution. Distilled water was freed of metal ions by passage over a mixed-bed ion-exchange resin, and organic impurities were removed. The water was freed of CO_2 by boiling for 10 min and then stored under N_2. KOH was standardized by titration with standard potassium hydrogen phthalate.

The hydrochlorides of hydrazine, hydroxylamine, methoxylamine, and semicarbazide were obtained from Aldrich (>98%) and recrystallized by published methods.[10] Stock solutions were prepared with addition of 1 equiv of KOH to the hydrochlorides, followed by adjustment of pH to the desired value by addition of HCl. Tris (99.9+%), ethylenediamine, and acetohydroxamate (Aldrich) were used without further purification. Stock solutions of acetohydroxamate were prepared with addition of 1 equiv of KOH, followed by addition of HCl to adjust the pH to the desired value. Stock solutions of ethylenediamine and Tris were adjusted to the desired value with HCl. NaCl and NaF (Mallinkrodt) were used as supplied.

Methods. The cleavage of MTP to thiophosphate at pH values between 9 and 10 was monitored spectrophotometrically at 226–232 nm. Initial rates were measured in the presence of hydroxylamine, methoxylamine, semicarbazide, and hydrazine and used to calculate first-order rate constants because of the instability of thiophosphate in the presence of high concentrations of these compounds. Reactions with hydroxylamine and methoxylamine were monitored at 227 nm, and first-order rate constants were calculated from initial velocities using the apparent extinction coefficient 3020 M^{-1} cm^{-1} at this wavelength. The apparent extinction coefficient is the difference between those for thiophosphate and MTP. The background extinctions at 227 nm in solutions containing semicarbazide and hydrazine were very high; therefore, the initial rates were measured at 232 nm and used with the apparent extinction coefficient 2745 M^{-1} cm^{-1} to calculate first-order rate constants. Thiophosphate was stable in the presence of Tris, ethylenediamine, NaF, and NaCl, so that the full course of reactions was monitored at 232 nm; therefore, the first-order rate constants were evaluated by fitting the reaction progress curves to the first-order rate law. The rate of thiophosphate formation in the presence of acetohydroxamate was determined by ^{31}P NMR, because of interference from background absorbance.

All rates were measured at 25 °C. Rates for the cleavage of MTP (63 or 76 μM) in the presence of a given amine were determined in a set of three kinetic runs, which differed only in the amine concentrations. An initial set was done for each amine with ionic strength being maintained at 0.5 with KCl. The pH was maintained with 20–30 mM K-CHES and was measured at the temperature of the experiment. Other sets of rates were measured with Tris and MTP with 0.1 M KCl or 5 mM K-CHES without maintenance of ionic strength; the reaction was insensitive to variations in ionic strength.[9] The concentrations of nucleophiles in each set of rate measurements were as follows: Tris, 0.1, 0.2, and 0.3 M; Tris, 0.74, 1.1, and 1.5 M; Tris, 2.0, 2.4, and 3.0 M; ethylenediamine, 0.073, 0.15, and 0.22 M (two sets); ethylenediamine, 0.46, 0.92, and 1.3 M. The concentrations of the α-effect nucleophiles hydroxylamine, methoxylamine, semicarbazide, and hydrazine were 0.1, 0.2, and 0.3 M. The effects of NaF and NaCl on the rate of MTP-cleavage were determined at 0.25 M halide.

^{31}P NMR Experiments. All experiments were carried out at 25 °C in 10-mm NMR tubes in a Bruker AM500 NMR spectrometer equipped with an Aspect 3000 computer, the lock signal being provided by D_2O in a coaxial insert tube. All chemical shifts were measured relative to an external standard of 85% H_3PO_4. The NMR samples contained 5 mM K-CHES and 1.9 mM Li_4MTP, except as noted below. The final pH values were as follows: 1.7 M Tris, pH 9.78; 3.0 M Tris, pH 9.93; 1.3 M ethylenediamine, pH 8.80; 0.65 M NaF, pH 9.51. The fluoride reaction contained 1.1 mM Li_4MTP. The acetohydroxamate reaction contained 1.2 M acetohydroxamate, 18 mM K-CAPS buffer, and 1.1 mM Li_4MTP at pH 10.3. The reactions were monitored by obtaining spectra at various times until a reaction was >97% completed. Each timed spectrum was obtained from 300 to 1000 scans, and the final spectrum was obtained from 1000 to 10000 accumulations.

Results

Effects of Nucleophiles on the Rate of MTP-Cleavage. The rate constants collected in Table I show that nucleophiles do not increase the rate at which MTP is cleaved to thiophosphate in aqueous solutions. Consider first Tris base and the α-effect nucleophiles hydrazine, hydroxylamine, and methoxylamine. These compounds have no effect on the rate at which MTP is cleaved to thiophosphate. Semicarbazide has a small apparent effect on the rate constant that is independent of the semicarbazide concentration. This effect appears to be due to a secondary reaction of semicarbazide with the product thiophosphate; we know from independent experiments that semicarbazide reacts with thiophosphate under the reaction conditions to form products with increased absorbance at 232 nm, the wavelength at which the cleavage of MTP was monitored.

Consider sodium fluoride as an added nucleophile. Fluoride ion is reactive toward activated phosphate compounds to form phosphorofluoridate. For example, fluoride ion reacts with acetyl phosphate and *N*-phosphopyridines in aqueous solutions to increase the rate of cleavage of these compounds and produce phosphorofluoridate.[3h,11] From Table I, our first conclusion is that sodium fluoride does not increase the cleavage rate. Indeed, the rate is somewhat slowed by sodium fluoride, presumably by complex formation between MTP and sodium fluoride. We know from earlier work on the coordination of MTP with monovalent and

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Table I. Observed First-Order Rate Constants for the Cleavage of MTP to Thiophosphate in the Presence of Nucleophiles[a]

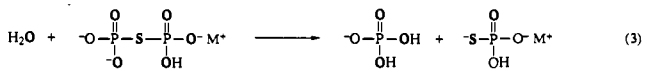
nucleophile[b]	conc[c] (M)	pH	k_{obs}[d] (min^{-1})	k_{hyd}[e] (min^{-1})
$(HOCH_2)_3CNH_2$	0.10–0.30	9.87	0.055 ± 0.014	0.059 ± 0.021
	0.10–0.30	9.15	0.187 ± 0.008	0.25 ± 0.05
	0.74–1.5[f]	9.71	0.069 ± 0.009	0.082 ± 0.007
	2.0–3.0[g]	9.90	0.055 ± 0.010	0.055 ± 0.006
H_2NNH_2	0.1–0.3	9.40	0.131 ± 0.005[h]	0.155 ± 0.013
$HONH_2$	0.1–0.3	9.33	0.176 ± 0.007[h]	0.178 ± 0.013
CH_3ONH_2	0.1–0.3	9.43	0.116 ± 0.019[h]	0.146 ± 0.012
$H_2NCONHNH_2$	0.1–0.3	9.48	0.158 ± 0.004[h]	0.132 ± 0.008
$H_3CCONHO^-$[i]	1.2	10.3	0.020	0.025
NaF	0.25[g]	9.48	0.10[j]	0.132 ± 0.008
NaCl	0.25[g]	9.44	0.10[j]	0.143 ± 0.012
$^+H_3NCH_2CH_2NH_2$	0.073–0.22	9.27	0.109 ± 0.015	0.20 ± 0.03
	0.076–0.23	9.01	0.13 ± 0.02	0.33 ± 0.08
	0.46–1.3	8.80	0.118 ± 0.011	0.46 ± 0.04
H_2O		9.47	0.129 ± 0.005	0.135 ± 0.008
H_2O		10.70	0.0146 ± 0.0008	0.012 ± 0.001

[a] Rate constants were measured spectrophotometrically as described in the Experimental Section at 25 °C in 10–30 mM K-CHES buffer at 63–76 μM Li_4MTP and I = 0.5 maintained with KCl unless otherwise noted. [b] The pK_a values for the conjugate acids of the nucleophiles are as follows:[8] $(HOCH_2)_3CNH_3^+$, 8.10; $^+H_3NNH_2$, 8.07; $^+H_3NCH_2CH_2NH_2$, 7.52; $^+H_3NCH_2CH_2NH_3^+$, 9.98; $HONH_3^+$, 5.97; $CH_3ONH_3^+$, 4.60; $H_2NCONHNH_3^+$, 3.65. [c] Total concentrations uncorrected for ionic forms. [d] The average rate constant from three runs which differed only in the nucleophile concentration within the indicated range. The rate constants for a given set of runs showed no trend with nucleophile concentration, and the estimate of error shown refers to the range of values observed. [e] The values of k_{hyd} are calculated from the pH-rate profile of Halkides and Frey,[2b] which was measured at 25 °C and I = 0.1 with KCl. [f] 0.1 M KCl. [g] 5 mM K^+ from K-CHES buffer. [h] Initial velocities were used to calculate these rate constants. [i] Single kinetic run by ^{31}P NMR. The reaction mixture contained 1.1 mM MTP, 21 mM LiCl, 10 mM CAPS buffer, and 1.2 M KCl. [j] Single spectrophotometric run.

divalent cations that K^+ is coordinated to $HMTP^{3-}$ under the conditions of Table I, and coordination of other monovalent cations in place of potassium alters the cleavage rate.[12] Sodium, lithium, and tetramethylammonium ions are inhibitory relative to potassium, whereas cesium ions increase the cleavage rate. The modest inhibitory effect of sodium fluoride on the rate of MTP-cleavage is explained by the inhibitory effect of sodium ions. Sodium chloride also inhibits the rate to about the same degree as sodium fluoride, presumably because of the effect of sodium ions.

Consider ethylenediamine, which exists largely in the form of the monoprotonated monocation under the conditions of Table I. This compound significantly inhibits the rate at which MTP is cleaved to thiophosphate, and the inhibition is slightly increased at the higher concentrations of ethylenediamine. Again, complex formation is reasonable, given that the predominant form of ethylenediamine under the reaction conditions is the monocationic form, which can displace potassium ion from coordination with $HMTP^{3-}$. We postulate that ethylenediamine monocation displaces potassium from MTP and slightly slows the cleavage to thiophosphate.

The effects of monocations on the rate at which MTP undergoes cleavage are presumably related to their relative effects in stabilizing thiophosphate as the leaving group in eq 3. The larger



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monovalent alkali metal ions are presumably less solvated than the smaller ones so that their positive charges are less shielded from the leaving group and provide greater stabilization. The alkyl ammonium ions also are presumably solvated through hydrogen bonding to water, which tends to dissipate the positive charge and reduce its effectiveness in stabilizing the leaving group.

Analysis of Reaction Products by ^{31}P NMR. The fact that added nucleophiles do not increase the rate at which thiophosphate is produced from MTP means that the nucleophiles do not participate in the transition state for P–S bond cleavage. Certain of the nucleophiles can, nevertheless, undergo phosphorylation if the mechanism is stepwise and requires the formation of metaphosphate as a discrete intermediate (eg. eq 2). Tris and ethylenediamine both undergo phosphorylation by MTP in aqueous solutions under the conditions of Table I, as shown by the ^{31}P NMR analysis of reaction products in shown Table II. Fluoride

Table II. Phosphorylation of Nucleophiles by MTP as Analyzed by ^{31}P NMR[a]

nucleophile	conc[b] (M)	pH	$ROPO_3^{2-}$ (%)	XPO_3^{2-}[c] (%)	HPO_4^{3-} (%)
$(HOCH_2)_3CNH_2$	1.7	9.8	15	17	69
	3.0	9.9	23	29	48
$^+H_3NCH_2CH_2NH_2$	1.3	8.8		43	57
F^-	0.65	9.5		0	100
$H_3CCONHO^-$	1.2	10.3	0		100

[a] The phosphorylation products were analyzed by ^{31}P NMR (202.46 MHz) after more than 97% of MTP had been consumed. In the reactions of Tris and ethylenediamine, the reaction mixtures consisted of 1.9 mM MTP and 21 mM LiCl in 10 mM K-CHES buffer. In the reaction of acetohydroxamate, the reaction mixture consisted of 1.1 mM MTP, 21 mM LiCl, and 1.2 M KCl in 10 mM K-CAPS buffer. In the reaction of fluoride, the solution contained 1.1 mM MTP in 10 mM K-CHES buffer containing 0.25 M NaF. [b] Total concentration of all species uncorrected for protonation states. [c] X = N or F.

and acetohydroxamate, unlike Tris and ethylenediamine, are not phosphorylated by MTP.

In the experiments with Tris, the products consisted of thiophosphate (δ_P = 34.8 ppm), *N*-phospho-Tris (δ_P = 7.0 ppm) and *O*-phospho-Tris (δ_P = 5.1 ppm), and phosphate (δ_P = 3.5 ppm). The signal for *N*-phospho-Tris was identified by the absence of proton coupling and its disappearance over time in dilute acid, which is consistent with the known hydrolytic lability of phosphoramidates in weakly acidic solutions. The signal for *O*-phospho-Tris was identified by its stability in acidic solutions and the presence of splittings due to proton coupling (triplet, J_H = 5.8 Hz). *O*-Phospho-Tris could not have arisen through isomerization of *N*-phospho-Tris because their relative signal intensities remained constant throughout the course of the reaction. Phosphorylation of Tris was 2.5 times more probable than hydrolysis, on the basis of the product analysis and relative concentrations of Tris and water.

In the experiments with ethylenediamine monocation, the products were thiophosphate (δ_P = 35 ppm), phosphate (δ_P = 3.0 ppm), and *N*-phosphoethylenediamine (δ_P = 8.9 ppm). Phosphorylation of ethylenediamine was 18 times more probable than hydrolysis under the conditions of Table II, on the basis of the relative concentrations of ethylenediamine and water.

Fluoride and acetohydroxamate anion were not phosphorylated by MTP. The only phosphate products in the fluoride reaction were thiophosphate and phosphate. Fluorophosphate was found in separate experiments to be stable under the conditions of Table

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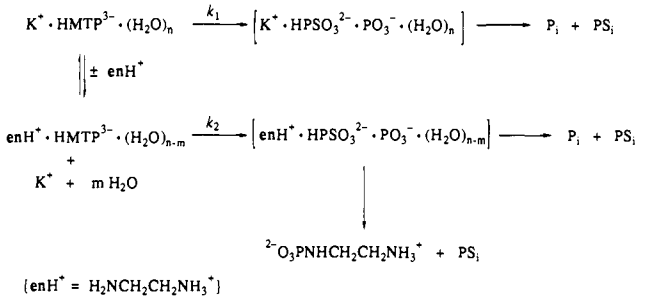
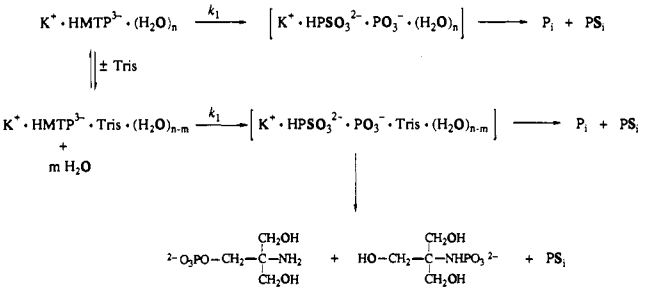
Scheme I



Scheme II

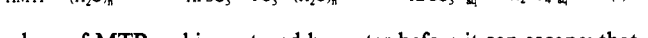


II, so it could not have been formed as an intermediate. Acetohydroxamate anion also was not phosphorylated. The products in the acetohydroxamate reaction were slightly more complex, in that they consisted of phosphate, thiophosphate, and diphosphoryl disulfide (δ_P = 16.3 ppm), which arose from the oxidation of thiophosphate. No phosphorylated acetohydroxamate was detected.

Discussion

A Dissociative Mechanism for the Hydrolysis of MTP. The hydrolytic mechanism for MTP at pH values between 9 and 10 must account for five experimental facts set forth in this paper: (1) Added nucleophiles do not participate in the transition state for the cleavage of the P–S bonds in MTP. (2) Tris and ethylenediamine compete with water for phosphorylation by MTP. (3) Fluoride ion and acetohydroxamate anion are not phosphorylated by MTP. (4) MTP phosphorylates Tris and ethylenediamine more efficiently than water in aqueous solutions. (5) MTP phosphorylates both the amino and hydroxyl groups of Tris at comparable rates, despite the higher nucleophilic reactivity of the amino group.

The facts can be explained by a stepwise mechanism in which a P–S bond of MTP undergoes cleavage in the rate-limiting step without nucleophilic participation to form a metaphosphate anion as a discrete intermediate, and water or another nucleophile immediately captures metaphosphate in a second step. The mechanism should be related to but different from eq 2, which cannot explain how ethylenediamine inhibits the cleavage of MTP but is more efficient than water in capturing metaphosphate nor why fluoride and acetohydroxamate anion do not capture metaphosphate from MTP. The simplest mechanism for the hydrolysis of MTP that can be adapted to explain all of the facts is that in eq 4, in which metaphosphate is generated within the solvation



(4)

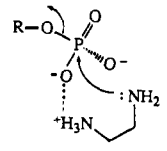
sphere of MTP and is captured by water before it can escape; that is, metaphosphate is a discrete but not a diffusible intermediate. The mechanism is consistent with the pH-rate profile and the activation parameters for the hydrolysis of MTP. The most reactive form of MTP at pH values between 9 and 10 is the trianion, and the entropy of activation is +0.2 eu, which is consistent with a unimolecular transformation of the ground state into the transition state.[2b]

The effects of nucleophiles other than water on the solvolysis of MTP strongly support the mechanism of eq 4 as the basic mechanism. Scheme I is an extension of this mechanism that accounts for the inhibition of MTP-cleavage by ethylenediamine monocation and the phosphorylation of ethylenediamine by MTP in competition with water. The steps designated by the rate constants k_1 and k_2 are rate-limiting, the other steps being either at equilibrium or controlled by rate constants that are much larger than k_1 or k_2. MTP binds monovalent cations, which are included in Scheme I, and the rate of its hydrolysis varies with different cations.[12] The tetramethylammonium salt of MTP undergoes hydrolysis at one-half to one-fourth the rate of the potassium salt, depending on conditions, and so it is not surprising that ethylenediamine monocation inhibits the cleavage. The upper line of Scheme I is the basic mechanism of eq 4, in which the solvated complex of potassium–MTP undergoes transformation into an intermediate complex of potassium ion, thiophosphate, monomeric metaphosphate, and water of solvation in the rate-limiting step governed by k_1. Metaphosphate is captured by a water molecule within the solvation sphere. In another process, shown in the lower part of Scheme I, ethylenediamine monocation enters into an exchange equilibrium with the potassium ion of the potassium–MTP complex. In addition to the replacement of potassium with ethylenediamine, this process may change the number of solvating water molecules, as indicated in the scheme by the change from n to $n - m$ waters of solvation, where m is a small positive or negative integer. The complex of ethylenediamine monocation and MTP undergoes a rate-limiting cleavage into an intermediate complex, consisting of ethylenediamine monocation, thiophosphate, metaphosphate, and solvent. The rate constant governing this process is k_2, which is smaller than k_1, owing to the replacement of potassium ion with ethylenediamine monocation. In this complex, either water or the unprotonated amino group of ethylenediamine monocation can capture metaphosphate in the second step.

A different variation of the hydrolysis mechanism is shown in Scheme II, which accounts for the absence of an effect on the rate of MTP-cleavage by Tris, while allowing for the partitioning of metaphosphate between water and Tris. As in Scheme I, the upper line is the basic mechanism of the hydrolysis of the potassium–MTP complex governed by the rate constant, k_1, for the cleavage of the P–S bond. Tris is largely in its neutral form between pH 9 and 10 (pK_a = 8.1), and the neutral form is postulated to interact with potassium–MTP, *without* displacing potassium ion, by undergoing an exchange with water of solvation; that is, Tris at high concentrations competes with water in solvating the potassium–MTP complex. The solvated potassium–MTP complex undergoes cleavage to the metaphosphate complex at the same rate, governed by k_1, in the presence or absence of Tris, because solvating molecules do not participate in the cleavage of the P–S bond in MTP and the counterion is potassium ion. However, when Tris is in the solvation sphere, it competes with water in capturing metaphosphate. Moreover, the capture of metaphosphate is nearly a random process, in which the highly nucleophilic amino group of Tris has little advantage over a hydroxyl group of Tris or a water molecule, and metaphosphate is partitioned into phosphate, *O*-phospho-Tris, and *N*-phospho-Tris.

The fact that Tris is phosphorylated almost indiscriminately on either oxygen or nitrogen does not prove that a discrete metaphosphate anion exists as an intermediate. Phosphorylation with apparent lack of discrimination within a solvation complex of MTP with Tris might be a coincidence arising from the greater steric hindrance around the amino group relative to the hydroxyl groups of Tris. However, if steric hindrance were the explanation for nearly random phosphorylation of Tris and there were nucleophilic participation in the transition state, we should expect ethylenediamine—which is sterically unhindered around the amino group—to react much faster with MTP than water. The modestly slower reaction of ethylenediamine is explained by the displacement of potassium ion from MTP by ethylenediamine monocation, but this effect would be overridden in the overall cleavage of MTP if there were nucleophilic participation by the unprotonated amino group. For example, the second-order rate constant for the phosphorylation of ethylamine by *N*-phosphopyridine is about 15 000 times the second-order rate constant for the reaction of

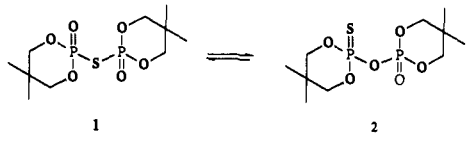
water.[3d] Moreover, diamine monocations *enhance* the rate at which phosphomonoesters are cleaved.[3a,d] This effect may arise through the formation of ionic complexes between diamine monocations and phosphomonoesters, followed by nucleophilic attack by the amino group on the phosphate group of the ester, as illustrated below.



The absence of any rate enhancement in the cleavage of MTP by ethylenediamine monocation, under conditions of complexation, suggests that nucleophilic attack is unimportant in the transition state.

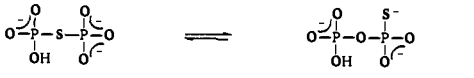
Fluoride reacts with reactive phosphate compounds in aqueous solutions to form phosphorofluoridate in competition with hydrolysis.[3h,11] However, fluoride does not react with MTP. The most obvious interpretation of this fact, in light of the mechanisms of MTP-cleavage in eq 4 and Schemes I and II, is that fluoride, being negatively charged, does not form a complex with MTP. According to these mechanisms, monomeric metaphosphate is generated from various cation–$HMTP^{3-}$ complexes and is captured by any nucleophilic species within the solvation sphere of this complex. An anionic nucleophile presumably could not occupy the solvation sphere or form an association complex with MTP because of electrostatic repulsion, and so it cannot react with metaphosphate. For the same reason, acetohydroxamate anion also cannot capture metaphosphate from MTP.

The dissociative mechanisms in eq 4 and Schemes I and II refer to the forward reactions, which are shown as irreversible processes. The reverse reactions should take place, but they are thermodynamically unfavorable and would be very difficult to observe. In the true reversal, thiophosphate would be phosphorylated on sulfur to form MTP and, by the principle of microscopic reversibility, it would proceed by the same mechanism through monomeric metaphosphate. In practice, thiophosphate would be phosphorylated on oxygen in preference to sulfur, to form the stronger P–O bond, and MTP would not be formed in significant amounts. The MTP tetraester **1** undergoes a unimolecular thermal isomerization to the more stable unsymmetrical ester **2** according to eq 5.[13] The equilibrium constant is large, so that the thermal



(5)

1 **2**



(6)

isomerization is a good method for synthesizing the unsymmetrical ester. The equilibrium constant for the isomerization of MTP to the unsymmetrical isomer according to eq 6 can be expected to be much larger owing to the greater stability of the negative charge on sulfur relative to oxygen.[14] The formation of the P–O bond in unsymmetrical monothiopyrophosphate does not necessarily proceed by the same mechanism as the formation of the P–S bond in MTP, and it probably does not for the reasons outlined in the following section.

Mechanistic Differences in the Hydrolysis of MTP Compared with Phosphomonoesters and Phosphoramidates. The hydrolysis of reactive phosphomonoesters, acyl phosphates, and phosphoramidates differs from the hydrolysis of MTP most fundamentally in that the kinetics for their cleavage reveals the existence of nucleophilic participation in the transition states. The clearest experimental manifestation of this fact is that the observed first-order rate constants for the cleavage of these compounds increase upon the addition of nucleophiles that are more reactive than water. Thus, the rate of the decomposition of *p*-nitrophenyl phosphate to *p*-nitrophenol in aqueous solutions increases when nucleophiles such as hydroxylamine are added. The observed first-order rate constant can be expressed as $k_{obs} = k_o + k_{nuc}$[nucleophile], in which k_o is the first-order rate constant for the hydrolysis and k_{nuc} is the second-order rate constant for the reaction of the added nucleophile with *p*-nitrophenyl phosphate to form the phosphorylated nucleophile. The values of k_{nuc} are significant in the reactions of phosphomonoesters, acyl phosphates, and phosphoramidates; however, they are insignificant in the reaction of MTP. For example, the value of k_o for the hydrolysis of *p*-nitrophenyl phosphate is 1×10^{-6} min^{-1}, and the second-order rate constant, k_{nuc}, for the reaction of hydroxylamine with *p*-nitrophenyl phosphate is 2×10^{-5} M^{-1} min^{-1} at 25 °C.[3a] Therefore, the rate at which *p*-nitrophenyl phosphate is cleaved to *p*-nitrophenol in a solution containing 0.3 M hydroxylamine is 6 times the rate in the absence of hydroxylamine. In contrast, 0.3 M hydroxylamine has no detectable effect on the rate at which MTP is cleaved to thiophosphate (Table I). If we consider the ratios $(k_{nuc}[0.3]/k_o)$, which refer to the presence of 0.3 M added nucleophile, in the reactions of *N*-phosphoisoquinoline, phosphoramidate, *N*-phospho-4-morpholinopyridine, *N*-phospho-3-methoxypyridine, and *p*-nitrophenyl phosphate, the ratios range from 1.7 to over 300 for various α-effect nucleophiles.[3a,c,d,15] The comparable limiting ratios for MTP range from <0.19 to <0.03. The simplest interpretation of these relationships is that nucleophiles participate in cleaving the P–O and P–N bonds in reactive phosphomonoesters and phosphoramidates. The absence of any kinetic effect of added nucleophiles in the cleavage of the P–S bonds in MTP shows that there is no nucleophilic participation in the transition state for MTP-cleavage.

The second-order rate constants for the phosphorylation of nucleophiles by phosphomonoesters and phosphoramidates increase with increasing basicity of the nucleophile, with some exceptions discussed below. This sensitivity to nucleophilic reactivity corresponds to β_{nuc} values of 0.1 to more than 0.2 in several systems. Moreover, linear free energy correlations show that, in phosphoryl group transfers between substituted pyridines, the reactivities of the attacking nucleophiles are correlated with the reactivities of the leaving groups by interaction coefficients, $p_{xy} = \partial\beta_{lg}/\partial\beta_{nuc}$, of about 0.013.[3d,f] The absence of nucleophilic participation in the transition state for reactions of MTP precludes the observation of such relations in phosphorylation of nucleophiles by this compound.

The fact that MTP phosphorylates Tris to form both *O*-phospho-Tris and *N*-phospho-Tris in comparable amounts shows that the phosphorylating species is highly electrophilic and reacts indiscriminately with nucleophiles. Monomeric metaphosphate is such an electrophile, as illustrated in Schemes I and II. Indiscriminate reaction of monomeric metaphosphate with nucleophiles such as Tris would correspond to a β_{nuc} value of zero in a structure reactivity correlation of log *k* versus pK_a of the nucleophile; however, nucleophiles do not participate in the transition state, so that we have no values of k_{nuc} to plot versus pK_a.

The literature contains other examples of the phosphorylation of nucleophiles proceeding with small and even negative values of β_{nuc}. The values of β_{nuc} in the phosphorylation of nitrogen nucleophiles in aqueous solutions by *N*-phosphoisoquinoline, phosphoramidate, *N*-phospho-4-morpholinopyridine, *N*-phospho-3-methoxypyridine, and *p*-nitrophenyl phosphate range from 0.13 to 0.22, depending on the type of nucleophile.[3a,c,d,15] These values refer to k_{nuc} in the equation $k_{obs} = k_o + k_{nuc}$[nucleophile], and they show that nucleophiles participate in the transition states for phosphorylation by these phosphomonoesters

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and phosphoramidates. In a few cases, however, the value of β_{nuc} is near zero or even negative. For the phosphorylation of substituted pyridines by 2,4-dinitrophenyl phosphate, β_{nuc} is approximately zero;[3b] for the phosphorylation of quinuclidines by *N*-phospho-4-morpholinopyridine, $\beta_{nuc} = -0.01$;[16] for the phosphorylation of quinuclidines by 2,4-dinitrophenyl phosphate, $\beta_{nuc} = -0.10$;[16] for the phosphorylation of primary amines by *p*-nitrophenyl phosphate, $\beta_{nuc} = -0.05$.[16] Jencks and co-workers explain the negative values of β_{nuc} on the basis that nucleophiles are solvated in aqueous solution and their reactions with phosphate compounds require their desolvation or partial desolvation.[16] The effect of increasing pK_a on the desolvation should be to decrease the rate, the opposite of its effect on nucleophilic reactivity, because solvent should be increasingly strongly held by nucleophiles of increasing basicity. Therefore, the second-order rate constants for the reactions as nucleophiles can be sensitive to desolvation in cases in which desolvation limits the rate at which the nucleophile undergoes phosphorylation and the negative values of β_{nuc} represent the effects of pK_a on the desolvation of the nucleophiles. In the case of MTP, β_{nuc} cannot be measured because of the absence of a second-order term for the nucleophile in the rate law; that is, there is no nucleophilic participation in the transition state.

In concluding that discrete monomeric metaphosphate is not an intermediate in solvolysis reactions in aqueous solutions, Herschlag and Jencks have estimated that discrete metaphosphate could be generated on a reaction trajectory between nitrogen and oxygen bases if the $pK_{nuc} = -20$ or the $pK_{lg} = -13$. These estimates do not refer to the transfer of PO_3^- between oxygen and sulfur, as in the hydrolysis of MTP. The P-S bond differs from the P-O bond in that it does not participate in resonance delocalization and π-bonding.[14] This difference is attributed by Reed and Schleyer to the relative absence of p_π-σ^* negative hyperconjugation in the P-S bond compared with the P-O bond.[17] The P-S bond is also weaker than the P-O bond by about 30 kcal mol^{-1}.[18] These bonding differences apparently allow the P-S bond in MTP to undergo cleavage to PO_3^- without nucleophilic participation, despite the fact that the pK_a of the leaving group, $HPSO_3^{2-}$, is 5.4.

The mechanism of eq 4 is the preassociation mechanism advanced by Jencks as a means by which monomeric metaphosphate might be an intermediate in phosphoryl group transfer reactions that proceed with nucleophilic participation in the transition state.[19] Structure reactivity correlations exclude this mechanism for phosphoryl group transfer between nitrogen bases and between nitrogen and oxygen bases in water.[5c,d] The preassociation stepwise mechanism remains possible for phosphoryl group transfer reactions in aprotic solvents or *t*-butyl alcohol, which proceed with racemization of chiral phosphate.[5c,d] Positional isotope exchange in [β-$^{18}O_4$]ADP or [α,β-^{18}O]ADP reisolated from solvolysis reactions in acetonitrile can also be explained by the preassociation stepwise mechanism, although the transient participation of acetonitrile to stabilize metaphosphate cannot be excluded.[20] The hydrolysis of MTP seems to follow the preassociation stepwise mechanism in water, in which monomeric metaphosphate appears to be the transient intermediate that is generated in the preassociation complex.

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General Acid Catalysis of the Reduction of *p*-Benzoquinone by an NADH Analog. Evidence for Concerted Hydride and Hydron Transfer

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Abstract: Rate constants for general acid catalysis of the reduction of *p*-benzoquinone by an NADH analog, [9-$^1H_2$]- and [9-$^2H_2$]-10-methylacridan, were determined in water and deuterium oxide at 25 °C. Catalysis by substituted acetic acids follows a Brønsted correlation with slope $\alpha = 0.85$. There is a 60-fold negative deviation for hydronium ion catalysis and a 6-fold positive deviation for catalysis by cacodylic acid. For RCOOH catalysts, solvent isotope effects k^L_{AH}/k^L_{AD} range from 1.2 to 3.0 for [9-L_2]-10-methylacridans (L = ^1H, ^2H). Substrate deuterium isotope effects $k^H_{AL}/k^D_{AL} = 1.5 \pm 0.1$ in water or deuterium oxide are small and essentially independent of the catalyst pK_a. Several reaction mechanisms are discussed, including reactions involving the semiquinone radical and radical anion generated by one-electron transfers. It is concluded that these results are most consistent with a concerted one-step hydride transfer assisted by proton transfer from the catalyst to form 4-hydroxycyclohexa-2,5-dienone that enolizes rapidly to hydroquinone.

Introduction

We are interested the mechanisms of coupling of hydride transfers from NADH analogs with proton transfers to the carbonyl group (eq 1) for several reasons. First, acids and bases in the active sites of enzymes often play a crucial role in catalyzing these types of reactions.[1,2] For example, in the case of lactate dehydrogenase, an active site histidine has been implicated as an

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