

**Comparison of the Metal Ion Facilitated Hydrolysis for the 5'-Triphosphates of 1,N<sup>6</sup>-Ethenoadenosine ( $\epsilon$ -ATP), Adenosine (ATP), and Cytidine (CTP).<sup>1,2</sup>**  
**Dephosphorylation of  $\epsilon$ -ATP Proceeding with Zn<sup>2+</sup> and Cu<sup>2+</sup>**  
**via Structurally Different Species:**  
**Evidence for a Long-Sought, Monomeric, Back-Bound Complex with Cu<sup>2+</sup>/ $\epsilon$ -ATP**

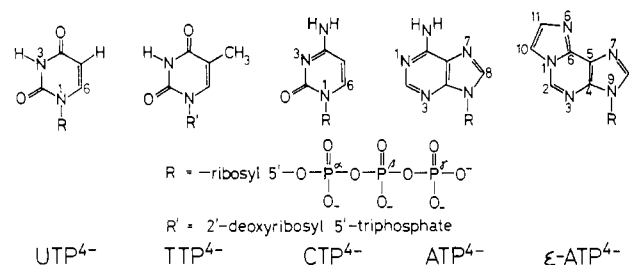
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The first-order rate constants (50 °C;  $I = 0.1$ , NaClO<sub>4</sub>) for the dephosphorylation of uncomplexed nucleoside 5'-triphosphates (= NTP =  $\epsilon$ -ATP, ATP, or CTP) in 1 mM solutions in the pH range 2–10 are virtually identical at the same pH, indicating that the nucleic base has no influence on the rate. In the presence of Cu<sup>2+</sup> or Zn<sup>2+</sup> (M<sup>2+</sup>:NTP = 1:1) this changes drastically, when the rates at the pH of the maximum promotion are compared: with Cu<sup>2+</sup>, ATP >  $\epsilon$ -ATP > CTP; with Zn<sup>2+</sup>,  $\epsilon$ -ATP > ATP > CTP. The effectiveness of the metal ions in the neutral pH range is Cu<sup>2+</sup> > Zn<sup>2+</sup> for ATP and CTP, but very surprisingly Zn<sup>2+</sup> > Cu<sup>2+</sup> for  $\epsilon$ -ATP. By combination of previous results for ATP and CTP with new results on  $\epsilon$ -ATP [obtained by using Job's method and by determining the relation between the initial rate of dephosphorylation ( $v_0 = d[\text{PO}_4]/dt$ ) and the concentration of M<sup>2+</sup>/ $\epsilon$ -ATP] the composition and the structure of the most reactive species may be compared: (i) For M<sup>2+</sup>/CTP and other triphosphates with a noncoordinating terminal organic residue the monomeric M<sub>2</sub>(NTP)(OH)<sup>-</sup> is the most reactive complex, while (ii) for M<sup>2+</sup>/ATP and Zn<sup>2+</sup>/ $\epsilon$ -ATP it is the dimeric species, [M<sub>2</sub>(NTP)]<sub>2</sub>(OH)<sup>-</sup>, which is a metal ion bridged stacked NTP dimer with a crucial M<sup>2+</sup>/base interaction. (iii) The most surprising result is obtained for Cu<sup>2+</sup>/ $\epsilon$ -ATP: the reactive complex, Cu<sub>2</sub>( $\epsilon$ -ATP)(OH)<sup>-</sup>, is monomeric with one Cu<sup>2+</sup> back-bound to the N-6,N-7 site; such a base/back-bound reactive NTP intermediate has long been sought. The observed different reactivities in the mentioned M<sup>2+</sup>/NTP systems may be explained by the formation of the different reactive species, but all three reactive intermediates have in common the coordination of two metal ions to the triphosphate residue: in cases ii and iii the  $\alpha,\beta$ -coordination of one metal ion is facilitated by its base interaction; the other metal ion (carrying the nucleophile OH<sup>-</sup>) coordinates to the terminal  $\gamma$ -phosphate group. The formation of the delicate reactive intermediates may easily be inhibited by mixed-ligand complex formation, e.g. with tryptophanate, and thus the reactivity of a system is diminished. Relations to enzymic transphosphorylations and possible problems in using  $\epsilon$ -ATP as a substrate are indicated: the incorporation of the 1,N<sup>6</sup>-etheno bridge into ATP alters its reactivity in the presence of metal ions considerably and unpredictably (with Cu<sup>2+</sup> inhibition and with Zn<sup>2+</sup> promotion occurs).

Phosphoryl and nucleotidyl transfers are widespread in nature and extremely important for all living organisms.<sup>3</sup> The enzymes involved in these reactions catalyze nucleophilic attack at phosphorus in phosphate residues of nucleotides,<sup>4</sup> and they all require divalent metal ions.<sup>4-7</sup> This requirement has stimulated considerable interest as to the possible roles of divalent metal ions in these reactions,<sup>4</sup> and the nonenzymic metal ion promoted dephosphorylation of nucleoside 5'-triphosphates (NTPs)<sup>8</sup> in aqueous solution to the corresponding diphosphates and orthophosphate has long been recognized.<sup>9-11</sup> Indeed, studies of the transfer of a phosphoryl group to a water molecule provide insight into

Chart I

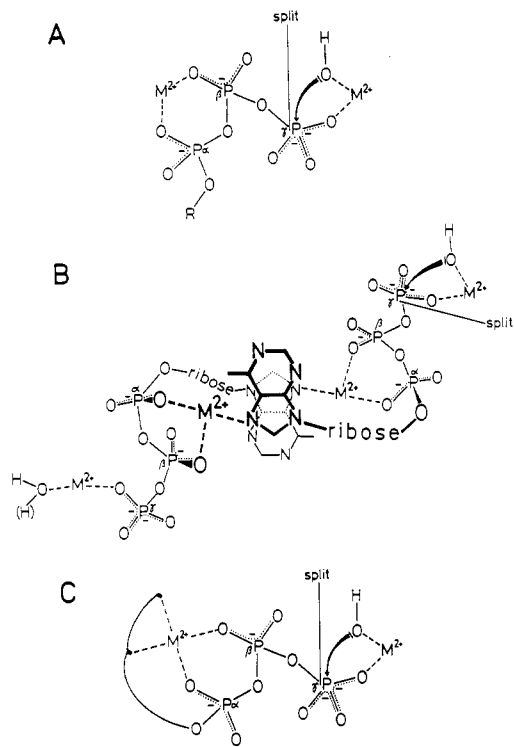


- (1) Part 10. Hydrolysis of Nucleoside Phosphates. Part 9: see ref 2.
- (2) Milburn, R. M.; Gautam-Basak, M.; Tribolet, R.; Sigel, H. *J. Am. Chem. Soc.* **1985**, *107*, 3315-3321.
- (3) Cohn, M. *Acc. Chem. Res.* **1982**, *15*, 326-332.
- (4) Cooperman, B. S. *Met. Ions Biol. Syst.* **1976**, *5*, 79-126.
- (5) Marzilli, L. G. *Prog. Inorg. Chem.* **1977**, *23*, 255-378.
- (6) Eichhorn, G. L. *Met. Ions Biol. Syst.* **1980**, *10*, 1-21.
- (7) Wu, F. Y.-H.; Wu, C.-W. *Met. Ions Biol. Syst.* **1983**, *15*, 157-192.
- (8) Abbreviations and definitions: Ado, adenosine;  $\epsilon$ -Ado,  $\epsilon$ -adenosine = 1,N<sup>6</sup>-ethenoadenosine; AMP and ATP, adenosine 5'-monophosphate and -triphosphate;  $\epsilon$ -AMP and  $\epsilon$ -ATP,  $\epsilon$ -adenosine 5'-monophosphate and -triphosphate; bpy, 2,2'-bipyridyl; CTP, GTP, ITP, UTP, and TTP, cytidine, guanosine, inosine, uridine, and thymidine 5'-triphosphate, respectively; M<sup>2+</sup>, bivalent metal ion; NTP, nucleoside 5'-triphosphate; Trp, L-tryptophan. The phosphate groups in NTP are labeled as  $\alpha$ ,  $\beta$ , and  $\gamma$ , where the latter refers to the terminal phosphate group (see Chart I). If nothing else is specified, the formula PO<sub>4</sub> represents all related species that may be present in solution, i.e. H<sub>3</sub>PO<sub>4</sub>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, HPO<sub>4</sub><sup>2-</sup>, and PO<sub>4</sub><sup>3-</sup>. The term "dephosphorylation" is used for the transfer of a phosphate group to a water molecule; the term "hydrolysis" is mainly used in connection with the formation of hydroxo complexes of metal ions. The terms monomeric or dimeric complexes mean that one or two NTP<sup>4-</sup> ions together with at least the corresponding equivalents of M<sup>2+</sup> are within the considered complex; hence, e.g., M<sub>2</sub>(NTP) is a monomeric (but dinuclear) nucleotide complex whereas [M(NTP)]<sub>2</sub><sup>4-</sup> is a dimeric one.
- (9) (a) Liébecq, C.; Jacquemotte-Louis, M. *Bull. Soc. Chim. Biol.* **1958**, *40*, 67-85, 759-765. (b) Liébecq, C. *Bull. Soc. Chim. Biol.* **1959**, *41*, 1181-1188.
- (10) Tetas, M.; Lowenstein, J. M. *Biochemistry* **1963**, *2*, 350-357.
- (11) Schneider, P. W.; Brintzinger, H. *Helv. Chim. Acta* **1964**, *47*, 1717-1733.

transphosphorylation mechanisms.<sup>12,13</sup> It is of interest to note in this connection that strong evidence exists<sup>14</sup> for a single-step direct phosphoryl transfer from ATP to water in the yeast inorganic pyrophosphatase catalysis of this reaction, i.e. the phosphoryl transfer does not involve formation of a phosphorylated enzyme intermediate.

Studies involving substitution-labile divalent metal ions,<sup>2,12,13</sup> like Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, or Cd<sup>2+</sup>, showed for all kinds of triphosphates with a single terminal organic residue that the most reactive species in the dephosphorylation process is a complex containing two metal ions per triphosphate.<sup>12,13,15,16</sup> However, the metal ion promoted dephosphorylation of ATP and other purine nucleoside 5'-triphosphates is much more pronounced<sup>12,16</sup> than that of UTP, TTP, and CTP (Chart I). The larger reactivity of the purine NTPs, e.g. ATP, originates in the additional M<sup>2+</sup>/N-7 interaction,<sup>17-19</sup> which facilitates the formation of the

- (12) Sigel, H.; Hofstetter, F.; Martin, R. B.; Milburn, R. M.; Scheller-Krattiger, V.; Scheller, K. H. *J. Am. Chem. Soc.* **1984**, *106*, 7935-7946.
- (13) Sigel, H.; Hofstetter, F. *Eur. J. Biochem.* **1983**, *132*, 569-577.
- (14) Gonzalez, M. A.; Webb, M. R.; Welsh, K. M.; Cooperman, B. S. *Biochemistry* **1984**, *23*, 797-801.
- (15) Miller, D. L.; Westheimer, F. H. *J. Am. Chem. Soc.* **1966**, *88*, 1514-1517.
- (16) Sigel, H.; Amsler, P. E. *J. Am. Chem. Soc.* **1976**, *98*, 7390-7400.
- (17) Scheller, K. H.; Hofstetter, F.; Mitchell, P. R.; Prijs, B.; Sigel, H. *J. Am. Chem. Soc.* **1981**, *103*, 247-260.



**Figure 1.** Comparison of three different reactive species formed during the metal ion promoted dephosphorylation of nucleoside 5'-triphosphates. A: Likely structure of the reactive species formed with organic triphosphates undergoing only a metal ion-phosphate coordination, like pyrimidine-NTPs or methyl triphosphate (R = organic residue).<sup>12,13</sup> B: Proposed structure for the reactive  $[M_2(ATP)]_2(OH)^-$  dimer, which occurs in low concentration during ATP dephosphorylation.<sup>12</sup> The intramolecular attack of OH<sup>-</sup> is indicated on the right side, while the left side is ready to transfer also into the reactive state by deprotonation of the coordinated water molecule or to undergo an intermolecular water attack. C: Simplified structure of the long-sought,<sup>4,11,23</sup> due to back-binding especially (in comparison with A) reactive complex of a triphosphate having correctly positioned additional metal ion binding sites. The participation of a second metal ion carrying a OH<sup>-</sup> nucleophile is proposed here on the basis of previous experience (see structures A and B, and ref 12).

reactive species. With pyrimidine NTPs metal ions coordinate to the phosphate chain only; there is no M<sup>2+</sup>/base interaction<sup>17,18</sup> as long as the proton at N-3 is not ionized.<sup>17,20</sup> Consequently, in the metal ion promoted dephosphorylation, all pyrimidine NTPs show the same properties as methyltriphosphate:<sup>13</sup> the reaction proceeds via a monomeric<sup>8</sup> triphosphate complex containing two metal ions; the most likely structure of this reactive species is shown in Figure 1A.

ATP and other purine NTPs exhibit a pronounced tendency for self-stacking.<sup>17,18</sup> This together with the M<sup>2+</sup>/N-7 interaction leads to the formation of dimeric  $[M_2(ATP)]_2$  species,<sup>8,21</sup> which may be dephosphorylated by an intramolecular attack of coordinated OH<sup>-</sup> or (less effective) under certain conditions via an intermolecular water attack.<sup>12</sup> A schematic representation of this reactive dimer is given in Figure 1B.

Figure 1C indicates a third reactive species that is monomeric and in which the  $\alpha,\beta$ -coordination of one metal ion at the phosphate chain is facilitated by the presence of additional binding sites in a sterically favorable position. The search for this species had been initiated in 1956 by the hypothesis of Szent-Györgyi<sup>22</sup> that macrochelate formation in nucleotide complexes is of im-

**Table I.** Comparison of the Coordinating Properties of ATP and  $\epsilon$ -ATP toward Zn<sup>2+</sup> or Cu<sup>2+</sup>, Together with Related Data ( $I = 0.1$ ; 25 °C)<sup>a</sup>

M <sup>2+</sup>	const	ATP	$\epsilon$ -ATP
...	$pK^H_{H_2(NTP)}$	$4.01 \pm 0.01^{b,c}$	$4.45 \pm 0.02^c$
...	$pK^H_{H(NTP)}$	$6.49 \pm 0.01^{b,d}$	$6.50 \pm 0.01^d$
Zn <sup>2+</sup>	$\log K^{Zn}_{Zn(HNTP)}$	$2.67^e$	$3.26 \pm 0.19$
	$\log K^{Zn}_{Zn(NTP)}$	$5.16 \pm 0.08$	$5.44 \pm 0.13$
	$pK^H_{Zn(HNTP)}$	4.0	4.3
	$pK^H_{Zn(NTP)(H_2O)}$	$8.87 \pm 0.04^f$	$\sim 9.0$
Cu <sup>2+</sup>	$\log K^{Cu}_{Cu(HNTP)}$	$3.57 \pm 0.08^b$	$7.3 \pm 1^g$
	$\log K^{Cu}_{Cu(NTP)}$	$6.32 \pm 0.04^b$	$9 \pm 1^g$
	$pK^H_{Cu(HNTP)}$	$3.74^b$	$4.8 \pm 0.1$
	$pK^H_{Cu(NTP)(H_2O)}$	$8.17^h$	$>8.5^i$

<sup>a</sup>If nothing else is mentioned, the data are abstracted from Table I in ref 25b (Zn<sup>2+</sup>) and Table I in ref 25c (Cu<sup>2+</sup>). The range of error given with the constants is *three times* the standard error of the mean value or the sum of the systematic errors, whichever is larger. <sup>b</sup>From ref 28. <sup>c</sup>The considered proton is at N-1 in H<sub>2</sub>(ATP)<sup>2-</sup> and at N-6 in H<sub>2</sub>( $\epsilon$ -ATP)<sup>2-</sup>; see Chart I. <sup>d</sup>The proton in H(NTP)<sup>3-</sup> is at the terminal  $\gamma$ -phosphate group (Chart I).<sup>29</sup> <sup>e</sup>From ref 31. <sup>f</sup>From ref 20. <sup>g</sup>See text in ref 25c. There is in addition evidence<sup>25c</sup> for dimer formation according to  $Cu(H\epsilon\text{-ATP})^- + Cu(\epsilon\text{-ATP})^{2-} \rightleftharpoons Cu_2H(\epsilon\text{-ATP})_2^{3-}$  ( $\log K_D = 3.6 \pm 0.3$ ). <sup>h</sup>Estimate<sup>32</sup> obtained at 25 °C from 10<sup>-3</sup> M 1:1 reactant solutions at pH  $\leq 8.0$ ; the corresponding estimation at 50 °C gave  $pK^H_{Cu(ATP)(H_2O)} = 7.82$ . Hydroxo-complex formation of Cu(ATP)<sup>2-</sup> is connected with a release of N-7 from Cu<sup>2+</sup>.<sup>12</sup> The given value describes well hydroxo-complex formation for a Cu<sup>2+</sup>/ATP 1:1 system up to pH 9.5,<sup>33</sup> despite the formation of dimeric species.<sup>33</sup> <sup>i</sup>Spectrophotometric measurements (see Figure 1 in ref 25c) gave no evidence for hydroxo-complex formation in the pH range up to 8, hence  $pK^H_{Cu(\epsilon\text{-ATP})(H_2O)} > 8.5$ .

portance for biological systems. Consequently, this third reactive complex has long been sought in model studies<sup>4,23</sup> and in NTP dephosphorylations;<sup>11</sup> in model studies no significant rate enhancement was observed,<sup>4</sup> and for NTP systems other reactive complexes have been identified.<sup>12,13</sup> However, in our studies of the ATP derivative 1,*N*<sup>6</sup>-ethenoadenosine 5'-triphosphate ( $\epsilon$ -ATP; Chart I), we discovered that the Cu<sup>2+</sup>-promoted dephosphorylation proceeds via a species as indicated in Figure 1C, while the Zn<sup>2+</sup>-facilitated reaction again involves a complex similar to the one shown in Figure 1B.

$\epsilon$ -ATP has caught our attention because it is widely applied in enzymic reactions as a fluorescent probe, substituting ATP as a substrate,<sup>24</sup> although its metal ion coordinating properties differ significantly from those of ATP, at least for the divalent 3d transition-metal ions.<sup>25</sup> Due to the scarcity of  $\epsilon$ -ATP the present study is restricted to systems involving Zn<sup>2+</sup> and Cu<sup>2+</sup>. Zn<sup>2+</sup> was selected for the dephosphorylation experiments because it is a divalent metal ion occurring in many enzymes,<sup>26</sup> including RNA polymerase,<sup>6,7,27</sup> and because its coordinating properties toward ATP and  $\epsilon$ -ATP appear at first sight as relatively similar (Table I);<sup>25,28-33</sup> hence, it seemed interesting to test if significant dif-

- (18) Scheller, K. H.; Sigel, H. *J. Am. Chem. Soc.* **1983**, *105*, 5891-5900.  
 (19) Sigel, H.; Scheller, K. H.; Milburn, R. M. *Inorg. Chem.* **1984**, *23*, 1933-1938.  
 (20) Sigel, H. *J. Am. Chem. Soc.* **1975**, *97*, 3209-3214.  
 (21) Regarding properties of ITP and GTP systems, see footnote 90 in ref 12.  
 (22) Szent-Györgyi, A. In *Enzymes: Units of Biological Structure and Function*, Gaebler, O. H., Ed.; Academic: New York, 1956; p 393.

- (23) Cooperman, B. S. *Biochemistry* **1969**, *8*, 5005-5010.  
 (24) (a) Stryer, L. *Annu. Rev. Biochem.* **1978**, *47*, 819-846. (b) Secrist, J. A., III; Barrio, J. R.; Leonard, N. J.; Weber, G. *Biochemistry* **1972**, *11*, 3499-3506. (c) Loscalzo, J.; Reed, G. H. *Biochemistry* **1976**, *15*, 5407-5413. (d) Roberts, D.; Kellett, G. L. *Biochem. J.* **1980**, *189*, 561-567. (e) Yanagida, T. *J. Mol. Biol.* **1981**, *146*, 539-560. (f) Jacobson, M. A.; Colman, R. F. *Biochemistry* **1982**, *21*, 2177-2186. (g) Weston, M. F.; Kotake, S.; Davis, L. C. *Arch. Biochem. Biophys.* **1983**, *225*, 809-817. (h) Rosenfeld, S. S.; Taylor, E. W. *J. Biol. Chem.* **1984**, *259*, 11920-11929. (i) Miki, M.; Wahl, P. *Biochim. Biophys. Acta* **1985**, *828*, 188-195.  
 (25) (a) Scheller, K. H.; Sigel, H. *Eur. J. Biochem.* **1986**, *157*, 147-153. (b) Sigel, H.; Scheller, K. H.; Scheller-Krattiger, V.; Priejs, B. *J. Am. Chem. Soc.* **1986**, *108*, 4171. (c) Kaden, T. A.; Scheller, K. H.; Sigel, H. *Inorg. Chem.* **1986**, *25*, 1313.  
 (26) Sigel, H., Ed. *Met. Ions Biol. Syst.* **1983**, Vol. 15.  
 (27) Scrutton, M. C.; Wu, C. W.; Goldthwait, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 2497-2501.  
 (28) Tribolet, R.; Malini-Balakrishnan, R.; Sigel, H. *J. Chem. Soc., Dalton Trans.* **1985**, 2291-2303.  
 (29) Martin, R. B.; Mariam, Y. H. *Met. Ions Biol. Syst.* **1979**, *8*, 57-124.  
 (30) Scheller, K. H.; Sigel, H. *J. Am. Chem. Soc.* **1983**, *105*, 3005-3014.  
 (31) Khan, M. M. T.; Martell, A. E. *J. Am. Chem. Soc.* **1966**, *88*, 668-671.

ferences in reactivity would arise.  $\text{Cu}^{2+}$  was used for several reasons: (i) there are indications that  $\text{Cu}(\text{ATP})^{2-}$  might be a natural active form of  $\text{Cu}^{2+}$ ;<sup>34</sup> (ii) the  $\text{Cu}(\epsilon\text{-ATP})^{2-}$  complex is much more stable than  $\text{Cu}(\text{ATP})^{2-}$  (Table I); (iii) that this increased stability could give rise to different properties in reactions was already indicated by  $\epsilon\text{-AMP}$ ,<sup>12</sup> addition of this AMP-derivative to a  $\text{Cu}^{2+}/\text{ATP}$  system inhibits dephosphorylation strongly, while addition of AMP itself further facilitates the reaction.

### Experimental Section

**Materials and Apparatus.** The disodium salt of 1,*N*<sup>6</sup>-ethenoadenosine 5'-triphosphate was purchased from Sigma Chemical Co., St. Louis, MO. The salt initially contained 3.5% free orthophosphate. Due to the scarcity of  $\epsilon\text{-ATP}$  we also employed in the dephosphorylation experiments some substance that had first been used in <sup>1</sup>H NMR shift studies;<sup>25a</sup> this  $\epsilon\text{-ATP}$  could then contain up to 9% free orthophosphate. However, the free phosphate content was taken into account, and such less pure substance was preferably used in experiments where free phosphate does not matter, e.g. in dephosphorylation experiments of  $\epsilon\text{-ATP}$  alone.

All the other chemicals and the apparatus were the same as given in ref 12.

**Determination of Dephosphorylation Rates.** All dephosphorylation experiments were carried out at 50 °C and  $I = 0.1 \text{ M}$  ( $\text{NaClO}_4$ ). As buffers inhibit the metal ion accelerated dephosphorylation of NTPs,<sup>35</sup> the pH was adjusted with  $\text{NaOH}$  or  $\text{HClO}_4$  with use of a glass stick (the change in volume was negligible).<sup>16,32,35</sup> The concentration of liberated phosphate from NTP was determined with molybdate reagent in samples taken at suitable intervals.<sup>32</sup>  $[\text{NTP}]_t$  at time  $t$  is given by  $[\text{NTP}]_t = [\text{NTP}]_0 - [\text{PO}_4]_t$ , where  $[\text{NTP}]_0$  is the initial concentration of NTP, and  $[\text{NTP}]_t$  and  $[\text{PO}_4]_t$  are concentrations at time  $t$ . The free phosphate initially present was taken into account.

The (pseudo) first-order rate constant,  $k$  ( $\text{s}^{-1}$ ), which is generally used for comparisons,<sup>11,12</sup> was determined from the slope of the straight-line portion of a  $\log [\text{NTP}]_t/t$  plot. The corresponding  $\text{pH}_{\text{av}}$  was obtained by averaging the pH values measured for those samples that gave points on the straight-line portion. Examples are shown in the figures of ref 32, 35, and 36.

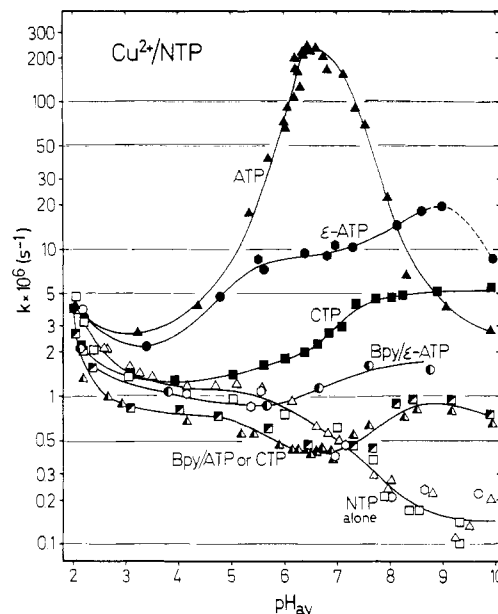
For mechanistic considerations, where an exact relationship between rate and pH is essential,<sup>16</sup> the initial rate of dephosphorylation,  $v_0 = d[\text{PO}_4]/dt$  ( $\text{M s}^{-1}$ ), was determined from the slope of the tangent of the  $[\text{PO}_4]_t/t$  curve at the time  $t = 0$ . The corresponding initial pH of the reaction solution, i.e.  $\text{pH}_0$ , was determined analogously.<sup>32</sup> To obtain  $v_0$  for a given system at a particular  $\text{pH}_0$ , two experiments were carried out in this range of pH (one slightly above and the other below the desired value), and these results were then interpolated to the desired  $\text{pH}_0$ . An example of this procedure is shown in Figure 1 of ref 16.

The two quantification methods for the dephosphorylation rates may be transferred into each other by the relation  $v_0 = d[\text{PO}_4]/dt = k[\text{NTP}]_t$ , and by also taking into account that  $\text{pH}_0$  is usually by about 0.2 log unit larger than  $\text{pH}_{\text{av}}$ .

### Results and Discussion

The rate of dephosphorylation of  $\epsilon\text{-ATP}$  alone is presented in Figure 2: the corresponding first-order rate constants are plotted as a function of pH. Some of these data, as well as some referring to the influence of  $\text{Cu}^{2+}$ , have been obtained in preliminary experiments by Hofstetter.<sup>37</sup>

Figure 2 shows that the entire pH-rate profile for  $\epsilon\text{-ATP}$  from pH 2 to 10 corresponds within experimental error to that of ATP and CTP. In fact, all NTPs studied so far<sup>12</sup> show this same profile; consequently,  $\epsilon\text{-ATP}$  behaves in this respect like any other NTP. This result is important, because it proves, in accordance with earlier observations,<sup>12</sup> that alterations at the nucleic base moieties are not reflected in the dephosphorylation rate as long as metal ions are absent. Clearly, this also means that any different properties observed for NTPs in the presence of metal ions must be a reflection of their different base moieties.



**Figure 2.** Comparison of the  $\text{Cu}^{2+}$ -promoted dephosphorylation of  $\epsilon\text{-ATP}$  ( $\bullet$ ;  $\bullet$ <sup>37</sup>), ATP ( $\blacktriangle$ ),<sup>32</sup> and CTP ( $\blacksquare$ )<sup>36</sup> (always in the ratio 1:1) as a function of pH, characterized as the first-order rate constants  $k$  ( $\text{s}^{-1}$ ). In addition are given values for  $\epsilon\text{-ATP}$  ( $\circ$ ;  $\circ$ <sup>37</sup>), ATP ( $\triangle$ ),<sup>12,32</sup> and CTP ( $\square$ )<sup>36</sup> alone and  $\epsilon\text{-ATP}$  ( $\bullet$ ),<sup>37</sup> ATP ( $\triangle$ ),<sup>32</sup> and CTP ( $\blacksquare$ )<sup>36</sup> in the presence of  $\text{Cu}^{2+}$  and 2,2'-bipyridyl (1:1:1). The concentration of each reactant (when present) was always  $10^{-3} \text{ M}$ ;  $I = 0.1$ ,  $\text{NaClO}_4$ ; 50 °C. The broken line portion indicates uncertainty due to precipitation.

**1. Comparison of the Influence of  $\text{Cu}^{2+}$  on the Dephosphorylation Rate of  $\epsilon\text{-ATP}$ , ATP, and CTP.** ATP was selected for this comparison, because it is the parent compound of  $\epsilon\text{-ATP}$  (Chart I), and CTP was used as the simplest representative of the pyrimidine NTPs, which all have no base/metal ion interaction<sup>17</sup> and consequently show identical dephosphorylation properties.<sup>13</sup> Differences between pyrimidine NTPs occur only at  $\text{pH} > 8$  upon deprotonation of N-3 (Chart I).<sup>13</sup>

From Figure 2 it is evident that the dephosphorylation rate in the  $\text{Cu}^{2+}/\text{ATP}$  1:1 system is more pronounced than in the corresponding system with  $\epsilon\text{-ATP}$ . However, it is also clear that  $\text{Cu}^{2+}/\epsilon\text{-ATP}$  is considerably more reactive than the  $\text{Cu}^{2+}/\text{CTP}$  1:1 system. As the properties of the latter system are characteristic for an organic triphosphate undergoing only a phosphate-metal ion coordination (Figure 1A), this comparison indicates that the  $\epsilon\text{-adenine}$  moiety influences the rate and, hence, participates in metal ion coordination; this conclusion is in agreement with recent spectrophotometric measurements.<sup>25c</sup>

In  $\text{Cu}^{2+}/\text{ATP}$  the metal ion is interacting at the base moiety with N-7 (Chart I),<sup>12</sup> while in the case of  $\epsilon\text{-ATP}$  a phenanthroline-like coordination to the N-6/N-7 site occurs.<sup>25c,30</sup> Hence, these different coordinating properties of the two base residues must be responsible for the observed differences in reactivity, and one aim will now be to see if this is also reflected in different reaction pathways (see section 7).

That the  $\text{Cu}^{2+}/\text{base}$  interaction is crucial for the dephosphorylation reaction is confirmed by the experiments carried out in the presence of equivalent amounts of 2,2'-bipyridyl. This second ligand enforces the release of the base moiety from the coordination sphere of  $\text{Cu}^{2+}$  in  $\text{Cu}(\text{bpy})(\text{NTP})^{2-}$  complexes; as under the experimental conditions the formation degree of this species with ATP<sup>32</sup> or CTP<sup>36</sup> is close to 100%, these two ternary systems behave quite alike.<sup>12</sup> The inhibition of the dephosphorylation rate in the  $\text{Cu}^{2+}/\text{bpy}/\epsilon\text{-ATP}$  system is somewhat less pronounced, which is understandable, as the formation degree (calculated with the equilibrium constants of ref 25b) is only about 90% at a pH of around 7.

**2. Influence of  $\text{Zn}^{2+}$  on the Dephosphorylation Rate of  $\epsilon\text{-ATP}$ , ATP, and CTP and Comparison with the Corresponding  $\text{Cu}^{2+}$  Systems.** In Figure 3 the first-order rate constants,  $k$  ( $\text{s}^{-1}$ ), for the dephosphorylation of these NTPs in the presence of  $\text{Zn}^{2+}$  are

(32) Buisson, D. H.; Sigel, H. *Biochim. Biophys. Acta* **1974**, *343*, 45–63.

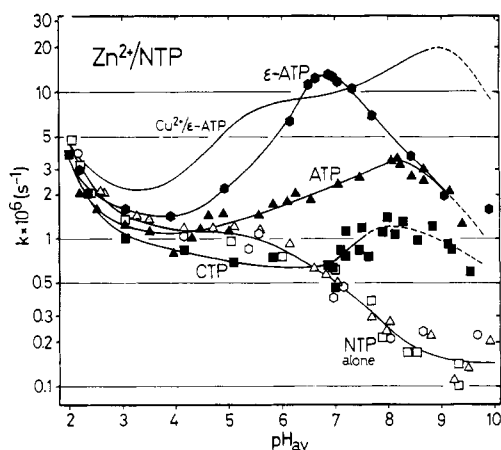
(33) Werner, E. R.; Rhode, B. M. *Inorg. Chim. Acta* **1983**, *80*, 39–46.

(34) Tallineau, C.; Barriere, M.; Boulard, M.; Boulard-Heitzmann, P.; Pontcharraud, R.; Reiss, D.; Guillard, O. *Biochim. Biophys. Acta* **1984**, *775*, 51–56.

(35) Amsler, P. E.; Sigel, H. *Eur. J. Biochem.* **1976**, *63*, 569–581.

(36) Sigel, H.; Buisson, D. H.; Priejs, B. *Bioinorg. Chem.* **1975**, *5*, 1–20.

(37) (a) Hofstetter, F. Ph.D. Thesis, University of Basel, 1980. (b) Part of the original experiments have been reevaluated by us.



**Figure 3.** Comparison of the  $\text{Zn}^{2+}$ -promoted dephosphorylation of  $\epsilon$ -ATP ( $\bullet$ ), ATP ( $\blacktriangle$ ),<sup>35</sup> and CTP ( $\blacksquare$ )<sup>35</sup> (always in the ratio 1:1) as a function of pH, characterized as the first-order rate constants  $k$  ( $\text{s}^{-1}$ ). In addition are given (see Figure 2) values for  $\epsilon$ -ATP ( $\circ$ ,  $\circ$ ), ATP ( $\triangle$ ), and CTP ( $\square$ ) alone. For further comparison the line representing the reactivity of the  $\text{Cu}^{2+}/\epsilon$ -ATP 1:1 system is inserted from Figure 2. The concentration of each reactant (when present) was always  $10^{-3}$  M;  $I = 0.1$ ,  $\text{NaClO}_4$ ;  $50^\circ\text{C}$ . The broken line portions indicate uncertainty due to precipitation.

plotted in dependence on pH. Comparisons with Figure 2 reveal that, in the metal ion facilitated dephosphorylation of ATP and CTP,  $\text{Zn}^{2+}$  is considerably less efficient than  $\text{Cu}^{2+}$ ; this is in agreement with general experience:<sup>12,13</sup> e.g., for ATP the effectiveness decreases within the series  $\text{Cu}^{2+} > \text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Mn}^{2+} > \text{Mg}^{2+}$ .<sup>12</sup>

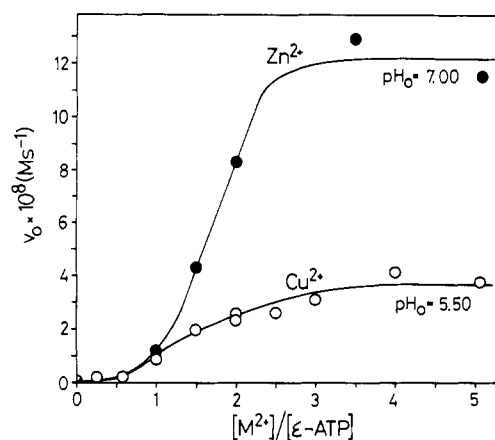
For the first time for a nucleoside 5'-triphosphate a different order is observed: in 1 mM solutions and in the pH region around 7,  $\text{Zn}^{2+}$  is clearly a more efficient promoter for the dephosphorylation of  $\epsilon$ -ATP than  $\text{Cu}^{2+}$  (Figure 3). This observation is the first hint that different mechanisms might be operating for the dephosphorylation of  $\epsilon$ -ATP in the presence of these two metal ions.

Another important aspect is that for the  $\text{Zn}^{2+}/\text{NTP}$  1:1 systems the reactivity decreases in the pH range 3–10 in the order  $\epsilon$ -ATP > ATP > CTP (Figure 3). This contrasts with the  $\text{Cu}^{2+}/\text{NTP}$  1:1 systems, which show the order ATP >  $\epsilon$ -ATP > CTP (Figure 2). This reversed order for  $\epsilon$ -ATP and ATP indicates different effects of  $\text{Zn}^{2+}$  and  $\text{Cu}^{2+}$  on the reaction. However, the CTP system is the least reactive one with both metal ions, showing again that in the systems with ATP and  $\epsilon$ -ATP the base/metal ion interaction is important.

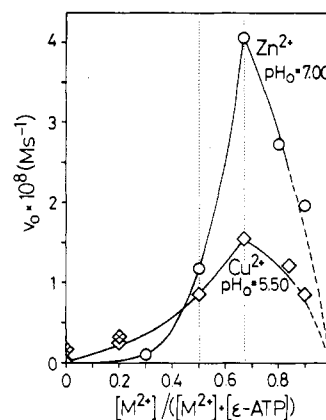
**3. Influence of Excess of  $\text{M}^{2+}$  on the Dephosphorylation Rate of  $\epsilon$ -ATP.** Previous experience is that the most reactive NTP species contains more than one metal ion (Figure 1A,B). With this result in mind the influence of increasing amounts of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  on the dephosphorylation rate of  $\epsilon$ -ATP was measured. These experiments had to be carried out at a constant pH, and therefore the initial rate of the dephosphorylation reaction,  $v_0 = d[\text{PO}_4]/dt$  ( $\text{M s}^{-1}$ ), was determined together with the corresponding initial  $\text{pH}_0$  (see Experimental Section).

Figure 4 summarizes the effect of increasing amounts of  $\text{Cu}^{2+}$  or  $\text{Zn}^{2+}$  on a 1 mM  $\epsilon$ -ATP solution at  $\text{pH}_0$  5.50 (cf.<sup>38</sup>) or 7.00, respectively. Obviously, the reactivity in the 1:1 systems of  $\text{Cu}^{2+}/\epsilon$ -ATP and  $\text{Zn}^{2+}/\epsilon$ -ATP may be significantly enhanced by the addition of further metal ions; especially in the  $\text{Zn}^{2+}$  system the effect is very pronounced. In both systems at  $[\text{M}^{2+}]:[\epsilon\text{-ATP}] > 3$ , limiting rate values are reached.

Job's method<sup>39</sup> was used to identify the composition of the reactive complex in the  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}/\epsilon$ -ATP systems. The initial



**Figure 4.** Dependence of the initial rate  $v_0$  for the  $\text{Cu}^{2+}$ - ( $\circ$ ;  $\text{pH}_0$  5.50)<sup>38</sup> and  $\text{Zn}^{2+}$ - ( $\bullet$ ;  $\text{pH}_0$  7.00) promoted dephosphorylation of  $\epsilon$ -ATP on the ratio  $[\text{M}^{2+}]:[\epsilon\text{-ATP}]$ .  $[\epsilon\text{-ATP}]_{\text{tot}} = 10^{-3}$  M;  $I = 0.1$ ,  $\text{NaClO}_4$ ;  $50^\circ\text{C}$ .



**Figure 5.** Job's series<sup>39</sup> for the  $\text{Cu}^{2+}/\epsilon$ -ATP system ( $\diamond$ ) at  $\text{pH}_0$  5.50 (cf. ref 38) and for the  $\text{Zn}^{2+}/\epsilon$ -ATP system ( $\circ$ ) at  $\text{pH}_0$  7.00.  $[\text{M}^{2+}]_{\text{tot}} + [\epsilon\text{-ATP}]_{\text{tot}} = \text{constant} = 2 \times 10^{-3}$  M;  $I = 0.1$ ,  $\text{NaClO}_4$ ;  $50^\circ\text{C}$ . The measured points (cross-hatched diamond) were corrected for the dephosphorylation of uncomplexed  $\epsilon$ -ATP by assuming complete 1:1 complex formation. The broken line portions indicate uncertainty due to precipitation. The vertical dotted lines give the positions of the ratios  $\text{M}^{2+}:\epsilon\text{-ATP} = 1:1$  or  $2:1$  (see text in section 3).

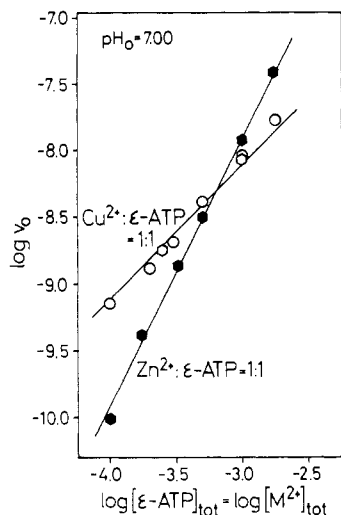
rates were measured and plotted versus the ratios  $[\text{M}^{2+}]:([\text{M}^{2+}] + [\epsilon\text{-ATP}])$ , keeping  $[\text{M}^{2+}] + [\text{ATP}]$  constant (Figure 5). A maximum rate at values of 0.33, 0.5, or 0.67 would indicate a composition of the reactive species of  $\text{M}^{2+}:\epsilon\text{-ATP} = 1:2$ ,  $1:1$ , or  $2:1$ , respectively. It is evident for both systems that the most reactive complex contains two metal ions per  $\epsilon$ -ATP in accordance with previous experience<sup>12,13,16</sup> in other triphosphate systems. Therefore it appears safe to assume that the  $\text{M}^{2+}:\epsilon\text{-ATP} = 2:1$  ratio is valid over the whole pH range from 4 to 9 and not just at the pH values used in the experiments for Figure 5.

**4. Relation between the Rate of Dephosphorylation and the Concentration of  $\text{M}^{2+}/\epsilon$ -ATP.** To learn more about the mechanism of the reaction, i.e. the dependence of the rate  $v_0$  on the total concentration of  $\text{M}^{2+}/\epsilon$ -ATP, further experiments were carried out and  $\log v_0$  was plotted vs.  $\log [\epsilon\text{-ATP}] (= \log [\text{M}^{2+}])$  as shown in Figure 6. For both systems at  $\text{pH}_0$  7.00 the data fit on straight lines, but for  $\text{Cu}^{2+}/\epsilon$ -ATP a slope of 1 is obtained while for  $\text{Zn}^{2+}/\epsilon$ -ATP a slope of 2 results, indicating<sup>16</sup> that in the  $\text{Cu}^{2+}$  system the reaction proceeds via a monomeric complex while in the  $\text{Zn}^{2+}$  system a dimeric species must be involved. In fact, a high-order concentration dependency in case of  $\text{Zn}^{2+}$  is already indicated by the pronounced "curvature" observed for  $\text{Zn}^{2+}/\epsilon$ -ATP at the left side of the peak in Figure 5.

The results summarized in the upper parts of Figure 7 show that at pH 7  $\text{Cu}(\epsilon\text{-ATP})^{2-}$  is formed to more than 95%. Taking now also into account the above-mentioned result of Figure 6, it is evident that this monomeric  $\text{Cu}(\epsilon\text{-ATP})^{2-}$  complex must be involved in the formation of the most reactive species. Our results

(38) The experiments with  $\text{Cu}^{2+}$  were carried out at  $\text{pH}_0$  5.50 to prevent immediate precipitation of copper hydroxide under those conditions where  $\text{Cu}^{2+}$  is in excess. At this pH  $\text{Cu}(\epsilon\text{-ATP})^{2-}$  still exists to about 50% as seen in Figure 7A (see also section 4).

(39) Job, P. C. R. *Hebd. Seances Acad. Sci.* 1933, 196, 181–183.

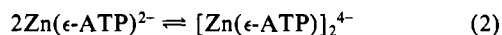


**Figure 6.** Relationship between the initial dephosphorylation rate  $v_0$  ( $\text{M s}^{-1}$ ) of  $\epsilon$ -ATP and the total concentrations of  $\text{Cu}^{2+}$  ( $\circ$ ;  $\circ^{37}$ ) or  $\text{Zn}^{2+}$  ( $\bullet$ ) and  $\epsilon$ -ATP at  $\text{pH}_0 = 7.00$  showing dependence of  $\log v_0$  on  $\log [\epsilon\text{-ATP}]_{\text{tot}} = \log [M^{2+}]_{\text{tot}}$ ;  $I = 0.1$ ,  $\text{NaClO}_4$ ;  $50^\circ\text{C}$ . The solid lines are drawn with the slopes  $m = 1$  ( $\text{Cu}^{2+}$ ) and  $m = 2$  ( $\text{Zn}^{2+}$ ). A least-squares calculation for the  $\text{Cu}^{2+}/\epsilon\text{-ATP}$  system gives  $m = 1.12 \pm 0.05$  (for the first five points only:  $m = 1.08 \pm 0.14$ ) and for the  $\text{Zn}^{2+}/\epsilon\text{-ATP}$  system one obtains  $m = 2.10 \pm 0.16$  (without the first point;  $m = 1.96 \pm 0.05$ ); the error range corresponds to twice the standard deviation.

allow no final conclusion about the species responsible for the reactivity at  $\text{pH} < 6.5$ : again  $\text{Cu}(\epsilon\text{-ATP})^{2-}$  could be involved, as it may be formed from  $\text{Cu}_2\text{H}(\epsilon\text{-ATP})_2^{3-}$  via equilibrium 1, but  $\text{Cu}_2\text{H}(\epsilon\text{-ATP})_2^{3-}$  itself or a derivative thereof could also own some dephosphorylation reactivity.



Comparisons of the lower and middle parts of Figure 7, which apply to temperatures of  $25$  and  $50^\circ\text{C}$ , respectively, show that the reactivity decreases with the advent of  $\text{Zn}(\epsilon\text{-ATP})(\text{OH})^{3-}$  (see section 8); this is especially evident if one takes into account that hydroxo-complex formation in these systems is favored with increasing temperature (footnote *h* in Table I and ref 32). However, at the  $\text{pH}$  where the dephosphorylation rate reaches its maximum,  $\text{Zn}(\epsilon\text{-ATP})^{2-}$  is clearly the dominating species; this together with the mentioned slope of 2 shown in Figure 6 may be rationalized with the monomer-dimer equilibrium 2. If this equilibrium is

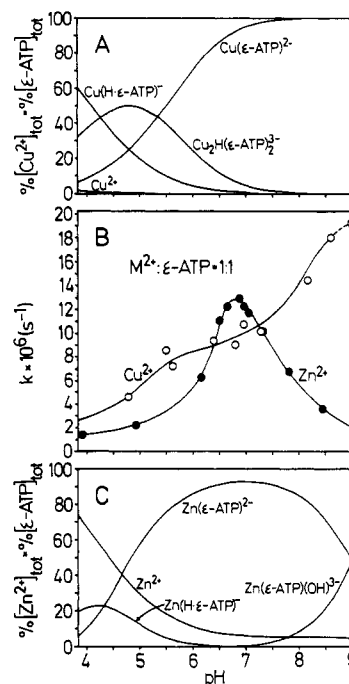


on the left, the concentration of the dimer is small and proportional to the square of the total concentration because  $K_D = [\text{dimer}]/([\text{monomer}]^2)$ ; hence,  $[\text{Zn}(\epsilon\text{-ATP})_2]^{4-}$  is involved in the formation of the most reactive species in the  $\text{Zn}^{2+}/\epsilon\text{-ATP}$  system. Evidence for the formation of such dimers has already been obtained earlier<sup>25a</sup> from  $^1\text{H}$  NMR shift experiments.

Finally, it should be noted that for all  $\text{M}^{2+}/\text{NTP}$  systems studied so far,<sup>12,13,16</sup> the slopes of the straight lines (be it 1 or 2) obtained by plotting  $\log v_0$  vs.  $\log [\text{NTP}]$  were always identical for 1:1 and 2:1 ratios between  $\text{M}^{2+}$  and a given NTP. The same may now also be surmised for the two  $\text{M}^{2+}/\epsilon\text{-ATP}$  systems.

**5. Evidence for the Participation of a  $\text{M}(\text{OH})^+$  Unit in the Reactive Complexes.** There is one additional aspect to be considered in connection with Figure 5: unbound  $\text{Cu}^{2+}$  begins to hydrolyze in a  $2\text{ mM}$  solution at  $\text{pH}$  about 5; hence, it is to be expected that, in a species containing two  $\text{Cu}^{2+}$  and one  $\epsilon\text{-ATP}^{4-}$ ,  $\text{Cu}^{2+}$  will also be partly hydrolyzed in this  $\text{pH}$  region, because one  $\epsilon\text{-ATP}^{4-}$  is not able to saturate the coordination spheres of two  $\text{Cu}^{2+}$  ions. The corresponding reasoning holds for  $\text{Zn}^{2+}$  in the  $\text{pH}$  region around 7. Hence, this indicates that a  $\text{M}(\text{OH})^+$  unit is involved in the most reactive intermediates.

A comparison of the middle and lower parts of Figure 7 at  $\text{pH}$  5.5 points into the same direction:  $\text{Zn}(\epsilon\text{-ATP})^{2-}$  is already formed to more than 76%, while the dephosphorylation rate is still far



**Figure 7.** Comparison of the variation of (B) the  $\text{Cu}^{2+}$  ( $\circ$ ) or  $\text{Zn}^{2+}$  ( $\bullet$ ) promoted dephosphorylation of  $\epsilon\text{-ATP}$  (each  $10^{-3}\text{ M}$ ) in aqueous solution at  $50^\circ\text{C}$  as a function of  $\text{pH}$  ( $I = 0.1$ ,  $\text{NaClO}_4$ ; cf. Figures 2 and 3), with the effect of  $\text{pH}$  at  $25^\circ\text{C}$  ( $I = 0.1$ ,  $\text{NaNO}_3$ ) on the concentration of the species present in an aqueous solution of  $\text{Cu}^{2+}$  (A) or  $\text{Zn}^{2+}$  (C) and  $\epsilon\text{-ATP}$  (each  $10^{-3}\text{ M}$ ). These results were computed with the constants listed in Table I, and they are given as the percentage of the total  $\text{M}^{2+}$  present (=total  $\epsilon\text{-ATP}$ ).

from its maximum. This indicates that the  $\text{pH}$  of a reaction solution is playing an additional role; in other words, this is also an indication for the formation of a  $\text{M}(\text{OH})^+$  unit within the reactive species (see sections 6 and 7), because with increasing  $\text{pH}$  such a unit will be favored. However, an excess of  $\text{OH}^-$  destroys the reactive complex as is evident from the formation of  $\text{Zn}(\epsilon\text{-ATP})(\text{OH})^{3-}$  (sections 4 and 8).

Clearly, the available experiments with the  $\text{M}^{2+}/\epsilon\text{-ATP}$  systems give only indirect indications for the intramolecular participation of an  $\text{M}(\text{OH})^+$  unit in the  $\epsilon\text{-ATP}$  systems, but these are in line with the direct evidence given in ref 12 for the reactive monomeric (Figure 1A) and dimeric (Figure 1B) intermediates in several  $\text{M}^{2+}/\text{NTP}$  systems. Indeed, a correctly positioned  $\text{OH}^-$  in a  $\text{M}(\text{OH})^+$  unit can be a potent nucleophile<sup>40-42</sup> despite its reduced basicity.<sup>41,42</sup>

However, water attack is also possible: a plot of  $\log k$  vs.  $\log [\text{H}^+]$  for the 1:1 system of  $\text{Zn}^{2+}/\epsilon\text{-ATP}$  (see the middle part of Figure 7) gives a straight line in the  $\text{pH}$  range of about 5.9–6.9 with a slope of approximately  $-0.6$ . As this is a slope larger than  $-1$  (which would indicate that  $v_0 \propto 1/[\text{H}^+]$ , i.e.  $v_0 \propto [\text{OH}^-]$ ), this evaluation evidences that *not* just  $\text{OH}^-$  attack [via  $\text{M}(\text{OH})^+$ ] is occurring. Indeed, for  $\text{M}^{2+}/\text{ATP}$  systems it has previously been shown<sup>12</sup> that *intermolecular* water attack does also occur under certain conditions.

**6. Composition and Tentative Structure of the Reactive Intermediate in the  $\text{Zn}^{2+}$ -Facilitated Dephosphorylation of  $\epsilon\text{-ATP}$ .** A summary of the facts regarding the composition of the reactive intermediate gives the following points: (i) the  $\text{Zn}^{2+}:\epsilon\text{-ATP}$  ratio is 2:1 (section 3); (ii) the intermediate is a dimer based on  $\text{Zn}(\epsilon\text{-ATP})^{2-}$  (section 4); (iii) in accord with previous experience,<sup>12</sup> the attack occurs preferably via  $\text{OH}^-$  in an intramolecular fashion through a  $\text{M}(\text{OH})^+$  unit (section 5). Hence, the most reactive

(40) Martin, R. B. *J. Inorg. Nucl. Chem.* **1976**, *38*, 511–513.

(41) Buckingham, D. A.; Keene, F. R.; Sargeson, A. M. *J. Am. Chem. Soc.* **1974**, *96*, 4981–4983.

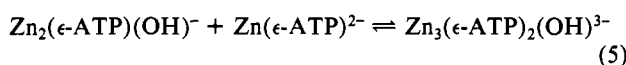
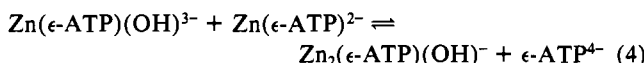
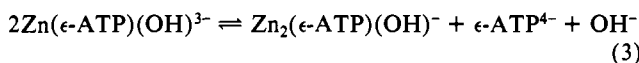
(42) Brown, R. S.; Huguet, J.; Curtis, N. J. *Met. Ions Biol. Syst.* **1983**, *15*, 55–59.

intermediate has the composition  $[\text{Zn}_2(\epsilon\text{-ATP})_2(\text{OH})^-]$ , although an *intermolecular* attack of water on  $[\text{Zn}_2(\epsilon\text{-ATP})_2]$  may also occur to some extent (section 5).

Taking into account the crucial metal ion/base interaction (section 2), as well as the recent evidence from an <sup>1</sup>H NMR shift study<sup>25a</sup> that  $\text{Zn}^{2+}$  promotes the self-association of  $\epsilon\text{-ATP}$  by bridging neighboring  $\epsilon\text{-ATP}$  species, a structure analogous to the one shown in Figure 1B for ATP and other purine NTP/ $\text{M}^{2+}$  systems may be proposed. In other words, the purine moieties in Figure 1B have to be replaced by the  $\epsilon$ -adenine residues with  $\text{Zn}^{2+}$  coordinated to the N-6,N-7 site,<sup>25,30</sup> to describe the situation for  $\text{Zn}^{2+}/\epsilon\text{-ATP}$ .

The coordination of one metal ion to a NTP commonly leads to an ( $\alpha$ )  $\beta,\gamma$ -coordination.<sup>29,43</sup> As suggested earlier,<sup>12,13,16</sup> coordination of a second metal ion forces one metal ion to the  $\gamma$ -phosphate group (which is the most basic site) and the other into the  $\alpha,\beta$ -position, causing a labilization of the  $\gamma$ -group. In the present case, the shift of one  $\text{Zn}^{2+}$  into the  $\alpha,\beta$ -position is facilitated by its simultaneous coordination to the N-6,N-7 site of the neighboring  $\epsilon\text{-ATP}$ .

In general,<sup>12,13,16</sup> 2:1 mixtures of  $\text{M}^{2+}/\text{NTP}$  systems are more reactive than 1:1 mixtures; this is also confirmed by the Job's series shown in Figure 5 for  $\text{Zn}/\epsilon\text{-ATP}$ . However, on the basis of Figure 1B the reactivity in the  $\text{Zn}^{2+}/\epsilon\text{-ATP}$  1:1 system (see Figure 3) may well be explained by assuming that the position of equilibria 3–5, or of any related equilibria, is such that a few percent of

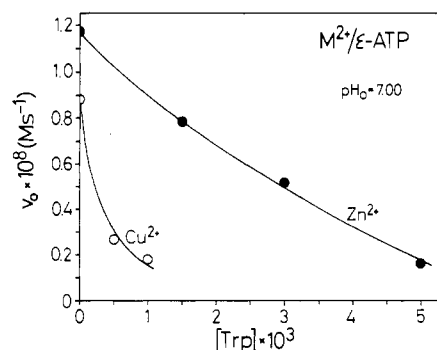


$\text{Zn}_3(\epsilon\text{-ATP})_2(\text{OH})^{3-}$  are formed. In this species two metal ions stabilize the dimer via bridging and the third coordinates to one of the two  $\gamma$ -phosphate groups to facilitate the nucleophilic attack.

As outlined, the mechanistic properties of the  $\text{Zn}^{2+}/\epsilon\text{-ATP}$  system correspond to those of  $\text{Zn}^{2+}/\text{ATP}$ . However, Figure 3 shows that the dephosphorylation rate in the 1:1 systems is significantly larger for  $\text{Zn}^{2+}/\epsilon\text{-ATP}$  than for  $\text{Zn}^{2+}/\text{ATP}$ : e.g., at pH 7, the  $\epsilon\text{-ATP}$  system is more reactive by a factor of about 5. This experimental fact must be connected with the exchange of the adenine by the  $\epsilon$ -adenine moiety (Chart I).<sup>44</sup> It appears likely that the larger coordination tendency of the N-6,N-7 site in  $\epsilon\text{-ATP}^{4-}$  ( $\log K^{\text{Zn}}_{\text{Zn}(\epsilon\text{-Ado})} = 1.5$ ),<sup>30</sup> compared to that of N-7 in  $\text{ATP}^{4-}$  ( $\log K^{\text{Zn}}_{\text{Zn}(\text{Ado})} = -0.3$ ),<sup>30</sup> is responsible: this could facilitate bridging of the two NTPs (Figure 1B) and especially promote further the shift of a  $\text{Zn}^{2+}$  ion into the reactive  $\alpha,\beta$ -position.

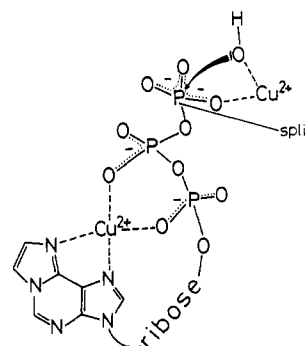
**7. Composition and Tentative Structure of the Reactive Intermediate in the  $\text{Cu}^{2+}$ -Facilitated Dephosphorylation of  $\epsilon\text{-ATP}$ .** The following facts regarding the composition of the most reactive species must be considered: (i) the  $\text{Cu}^{2+}:\epsilon\text{-ATP}$  ratio is 2:1 (section 3); (ii) the intermediate is of a monomeric nature (section 4); (iii) there are indications, in accordance with previous results,<sup>12</sup> that the nucleophilic attack occurs intramolecularly via a  $\text{Cu}(\text{OH})^+$  unit (section 5). Hence, the most reactive intermediate has the composition  $\text{Cu}_2(\epsilon\text{-ATP})(\text{OH})^-$ .

This composition corresponds to that deduced for the reactive intermediate in  $\text{M}^{2+}/\text{pyrimidine NTP}$  systems.<sup>13</sup> However, the  $\text{Cu}^{2+}/\epsilon\text{-ATP}$  1:1 system at pH 7 is more reactive by a factor of about 3 than the corresponding CTP system (Figure 2). The only



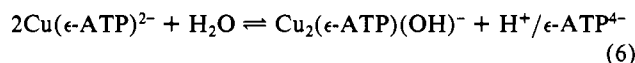
**Figure 8.** Inhibition of the  $\text{Cu}^{2+}$ - (O) and  $\text{Zn}^{2+}$ - (●) promoted dephosphorylation of  $\epsilon\text{-ATP}$  by the addition of L-tryptophan (Trp) at  $\text{pH}_0$  7.00.  $[\text{M}^{2+}]_{\text{tot}} = [\epsilon\text{-ATP}]_{\text{tot}} = 10^{-3}$  M;  $I = 0.1$ ,  $\text{NaClO}_4$ ;  $50^\circ\text{C}$ .

**Chart II**



difference between  $\text{Cu}^{2+}/\epsilon\text{-ATP}$  and  $\text{Cu}^{2+}/\text{CTP}$  being the absence of a  $\text{M}^{2+}/\text{base}$  interaction in the latter nucleotide,<sup>12,17</sup> the increased reactivity of  $\text{Cu}^{2+}/\epsilon\text{-ATP}$  has to be attributed to the high affinity of the N-6,N-7 site for  $\text{Cu}^{2+}$  ( $\log K^{\text{Cu}}_{\text{Cu}(\epsilon\text{-Ado})} = 2.8$ ).<sup>30</sup> Taking this into account, the likely structure for the most reactive species formed during the  $\text{Cu}^{2+}$ -promoted dephosphorylation of  $\epsilon\text{-ATP}$  is shown in Chart II. The results of sections 1, 3, and 4 give for the first time reliable evidence for the formation of such a back-bound reactive NTP species.

For the observed reactivity in the  $\text{Cu}^{2+}/\epsilon\text{-ATP}$  1:1 system (Figure 2) several explanations may be offered (no further suggestions are made for the pH range below 6.5; see section 4): (i) The position of equilibrium 6 may be such that appreciable



amounts of the reactive  $\text{Cu}_2(\epsilon\text{-ATP})(\text{OH})^-$  species are formed. (ii)  $\text{Cu}(\epsilon\text{-ATP})^{2-}$  could well be directly suitable for dephosphorylation via water attack: it seems possible that the back-binding to the N-6,N-7 site may enforce in an intramolecular equilibrium the formation of some  $\text{Cu}(\epsilon\text{-ATP})^{2-}$  with only a  $\gamma$ -phosphate coordination (instead of the usual  $\beta,\gamma$ -binding) thus favoring an intermolecular water attack at the  $\gamma$ -group. (iii) A further possibility, connected with point ii, is the formation of some  $\text{Cu}(\epsilon\text{-ATP})(\text{OH})^{3-}$  in which the metal ion is only coordinated to N-6,N-7 and the  $\gamma$ -phosphate group, allowing thus an *intramolecular* attack at the  $\gamma$ -phosphorus of an also bound  $\text{OH}^-$ ; clearly, the normally formed  $\text{Cu}(\epsilon\text{-ATP})(\text{OH})^{3-}$  species is not very reactive as indicated in Figure 2 at  $\text{pH} > 9$  (see also section 8).

**8. Inhibition of the Metal Ion Promoted Dephosphorylation by Mixed-Ligand Complex Formation.** Be it the dimer (analogous to Figure 1B) responsible for the dephosphorylation of  $\text{Zn}^{2+}/\epsilon\text{-ATP}$  or the monomer of the  $\text{Cu}^{2+}/\epsilon\text{-ATP}$  system (Chart II), the formation of both these reactive complexes is obviously a delicate matter. Consequently, any ligand with a larger coordination tendency toward  $\text{Zn}^{2+}$  or  $\text{Cu}^{2+}$  than the one of the N-7,N-6 site of  $\epsilon\text{-ATP}^{4-}$  is expected to inhibit the reactivity. Indeed, the results assembled in Figure 8 show that the addition of tryptophan leading to formation of ternary  $\text{M}(\epsilon\text{-ATP})(\text{Trp})^{3-}$  complexes<sup>25b</sup> inhibits the dephosphorylation of  $\epsilon\text{-ATP}$  in the  $\text{Cu}^{2+}$  and in the  $\text{Zn}^{2+}$

(43) (a) Connolly, B. A.; Eckstein, F. *J. Biol. Chem.* **1981**, *256*, 9450–9456. (b) Goody, R. S.; Hofman, W.; Konrad, M. *FEBS Lett.* **1981**, *129*, 169–172.

(44) One might assume that the slightly larger self-stacking tendency of  $\epsilon\text{-ATP}^{4-}$  ( $K_{\text{self}} = 1.9 \pm 0.2 \text{ M}^{-1}$ ;<sup>25a</sup> for  $\text{ATP}^{4-}$ ,  $K_{\text{self}} = 1.3 \pm 0.2 \text{ M}^{-1}$ )<sup>17</sup> is playing a role, but  $\text{Zn}^{2+}$  promotes the self-association of  $\text{ATP}^{4-}$  considerably better than the self-association of  $\epsilon\text{-ATP}$ ;<sup>25a</sup> hence, this can hardly be the cause for the formation of a higher concentration of the reactive dimer.

**Table II.** Comparison of the Initial Rate of Dephosphorylation,  $v_0$  ( $M s^{-1}$ ), of  $\epsilon$ -ATP, ATP, and CTP (NTP) in  $10^{-4}$ ,  $10^{-3}$ , and  $10^{-2}$  M Solutions in the Presence of  $Cu^{2+}$  or  $Zn^{2+}$  (1:1) ( $I = 0.1$ ,  $NaClO_4$ ; 50 °C) at  $pH_0$  7.00<sup>a</sup>

syst	concn of reactants (syst)					
	$10^{-4}$ M		$10^{-3}$ M		$10^{-2}$ M	
	$v_0$	PF <sup>b</sup>	$v_0$	PF <sup>b</sup>	$v_0$	PF <sup>b</sup>
NTP	0.005 <sup>c</sup>		0.05		0.5	
$Cu^{2+}/\epsilon$ -ATP	0.09	18	0.9	18	9	18
$Zn^{2+}/\epsilon$ -ATP	0.012	2.4	1.2	24	120	240
$Cu^{2+}/ATP$	0.18	36	18	360	1800	3600
$Zn^{2+}/ATP$	0.0023	0.46	0.23	4.6	23	46
$Cu^{2+}/CTP$	0.03	6	0.3	6	3	6
$Zn^{2+}/CTP$	0.007	1.4	0.07	1.4	0.7	1.4

<sup>a</sup>The initial rates are calculated (or directly taken) from the information assembled in Figures 2, 3, and 6 (see also Figure 9 in ref 16) and the known relations between  $v_0$  and the reactant concentrations (for comparison see also Tables I and II in ref 2). All initial rates are given as  $v_0 \times 10^8$ . <sup>b</sup>The values given in *italics* are the promotion factors (PF) based on the dephosphorylation rate of NTP alone. For example: PF = 0.09/0.005 = 18. <sup>c</sup>Calculated under the assumption of  $v_0 \propto [NTP]$ ; for ATP and UTP this has been proven in the concentration range  $10^{-3}$ – $10^{-2}$  M at  $pH_0$  5.50 and 7.50 (Tables I and II in ref 2). For ATP we have confirmed this assumption now also in the concentration range  $10^{-4}$ – $10^{-3}$  M at  $pH_0$  7.00; the experiments with  $[ATP] = 10^{-4}$  were carried out in the presence of 10% EDTA (i.e.,  $10^{-5}$  M) to prevent interference of traces of metal ions.

systems, confirming thus indirectly the deduced structures of the reactive complexes. Of course, the formation of the more simple reactive intermediate shown in Figure 1A may also be inhibited with tryptophan,<sup>13</sup> if large enough quantities are used.

As indicated, the result with tryptophan is expected, and it is in line with the observations made with 2,2'-bipyridyl in the  $Cu^{2+}/\epsilon$ -ATP system (Figure 2 and section 1), where the formation<sup>25b</sup> of  $Cu(bpy)(\epsilon\text{-ATP})^{2-}$  also inhibits the reaction drastically. The fact that large parts of all  $M(bpy)(NTP)^{2-}$  complexes exist in form of the isomer with an intramolecular stack between the base residues and the pyridyl rings<sup>19,25b,28</sup> is of minor significance in this connection, what is important is that in any case the  $Cu^{2+}$ /base interaction<sup>12</sup> is inhibited.

Moreover, from the middle and bottom parts of Figure 7 it is evident that even the formation of the simple ternary  $Zn(\epsilon\text{-ATP})(OH)^{3-}$  complex inhibits the dephosphorylation in the  $Zn^{2+}/\epsilon$ -ATP system (Section 4). This observation is understandable if one assumes for  $Zn(\epsilon\text{-ATP})^{2-}$ , as has been proven for several  $M(ATP)^{2-}$  systems ( $M^{2+} = Cu^{2+}, Zn^{2+}, Cd^{2+}$ ),<sup>12</sup> that hydroxo-complex formation is connected with a release of the base moiety from the coordination sphere of the metal ion. Indeed, at  $pH > 8.7$   $Zn^{2+}/\epsilon$ -ATP and  $Zn^{2+}/ATP$  show the same reactivity, which is also approaching that of  $Zn^{2+}/CTP$  at a  $pH$  about 9.5 (Figure 3).

The decrease in reactivity in the  $Cu^{2+}/\epsilon$ -ATP system at  $pH > 9$  (Figure 2) is most probably also due to the formation of  $Cu(\epsilon\text{-ATP})(OH)^{3-}$ ; it is understandable that in this case hydroxo-complex formation occurs only at a rather high  $pH$  because in  $Cu(\epsilon\text{-ATP})^{2-}$  the equatorial part of the  $Cu^{2+}$  coordination sphere is saturated and therefore the substitution of N-6 and/or N-7 needs a relatively large  $OH^-$  concentration. Indeed, in the  $pH$  range of about 10 the reactivities in the  $Cu^{2+}$  systems of ATP,  $\epsilon$ -ATP or CTP are getting comparable (see Figure 2), an observation in accordance with our interpretation regarding the release of the base moiety in  $Cu(\epsilon\text{-ATP})(OH)^{3-}$ .

### Conclusions

A comparison of the mechanistic details summarized in sections 6 and 7 for the  $Zn^{2+}$ - and  $Cu^{2+}/\epsilon$ -ATP systems with the results obtained previously<sup>12</sup> for the corresponding ATP systems leads to the following questions: (i) Why is the pathway for the de-

phosphorylation of ATP and  $\epsilon$ -ATP the same with  $Zn^{2+}$  as promoter? (ii) Why does the dephosphorylation reaction in the  $Cu^{2+}/ATP$  system proceed via dimers, i.e. via  $[Cu_2(ATP)]_2$  (Figure 1B) and  $[Cu(ATP)]_2(OH)^{5-}$  (see ref 12), and in the  $Cu^{2+}/\epsilon$ -ATP system via monomers (Chart II)?

This latter difference in the pathways of the dephosphorylation must be connected with the N-6,N-7 site in  $\epsilon$ -ATP as this is the only difference to ATP (Chart I). Hence, the reason could be the increased coordination tendency of the N-6,N-7 site in  $\epsilon\text{-ATP}^{4-}$  ( $\log K_{Cu}^{Cu(\epsilon\text{-Ado})} = 2.8$ )<sup>30</sup> compared with that of N-7 in  $ATP^{4-}$  ( $\log K_{Cu}^{Cu(Ado)} = 0.8$ )<sup>30</sup>; however, this suggestion is not convincing as a corresponding change in the coordinating affinities for  $Zn^{2+}$  did not alter the mechanism (Section 6). Therefore, it seems more probable that a geometric factor and the degree of saturation of the coordination sphere are responsible for the mechanistic differences: With  $ATP^{4-}$  only three equatorial binding sites of the tetragonal  $Cu^{2+}$  ion are occupied ( $\beta,\gamma$ -phosphate and N-7), while with  $\epsilon\text{-ATP}^{4-}$  the equatorial positions are saturated ( $\beta,\gamma$ -phosphate, N-6 and N-7) thus probably enforcing the different pathway.  $Zn^{2+}$  with the possibility for a hexadentate, octahedral coordination sphere is more adaptable with regard to the steric conditions required for dimer formation (Figure 1B).

Evidently great care should be exercised if  $\epsilon$ -ATP is employed as substrate instead of ATP in enzymic reactions in the presence of metal ions. The incorporation of the 1,N<sup>6</sup>-etheno bridge into ATP is giving rise to different coordinating properties<sup>25</sup> and is also leading to different reactivities: the observation that at concentrations  $> 0.7$  mM (see Figure 6) the  $Zn^{2+}/\epsilon$ -ATP 1:1 system is *more reactive* than the corresponding  $Cu^{2+}$  system is quite unexpected. The reason is the different proportionality between the initial rate,  $v_0$ , and the total reactant concentration: with  $Cu^{2+}$  the reaction proceeds via a monomer (Chart II;  $v_0 \propto [Cu(\epsilon\text{-ATP})^{2-}]$ ), but with  $Zn^{2+}$  via a dimer (Figure 1B;  $v_0 \propto [Zn(\epsilon\text{-ATP})^{2-}]^2$ ). This example nicely illustrates the difficulties and "pitfalls" (see ref 2) in making general statements about the relative promotional effects of different metal ions in NTP dephosphorylations, because different dependencies between rate and concentrations may exist. To illustrate this point, the data of Table II have been assembled: obviously, depending on the considered concentrations of the reactants, different conclusions about promotional effects may be reached.

Another point that warrants emphasis is the feature common for the three reactive intermediates described for NTP dephosphorylation (Figure 1 and Chart II): *two* metal ions bound to a triphosphate chain lead to especially favorable conditions for an intramolecular nucleophilic attack of  $OH^-$ . It is to be expected that not only coordinated  $OH^-$  remains a nucleophile of high quality but also that this occurs with other groups, e.g. alcoholic and related residues, to which transphosphorylations occur in nature. Clearly, the role of the "structuring" NTP in the reactive dimer (Figure 1B) or of the  $\epsilon$ -adenine moiety in the reactive monomer (Chart II) will be taken over in nature by enzymes.<sup>12</sup> It is interesting to note that a growing number of pertinent enzymic systems are becoming known,<sup>6,7,45</sup> which contain two or more metal ions.

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**Registry No.**  $\epsilon$ -ATP, 37482-17-0; Zn, 7440-66-6; Cu, 7440-50-8.

- (45) (a) Li, T. M.; Mildvan, A. S.; Switzer, R. L. *J. Biol. Chem.* **1978**, *253*, 3918–3923. (b) Otvos, J. D.; Armitage, I. M.; Chlebowski, J. F.; Coleman, J. E. *J. Biol. Chem.* **1979**, *254*, 4707–4713. (c) Granot, J.; Mildvan, A. S.; Bramson, H. N.; Kaiser, E. T. *Biochemistry* **1980**, *19*, 3537–3543. (d) Cooperman, B. S.; Panackal, A.; Springs, B.; Hamm, D. J. *Biochemistry* **1981**, *20*, 6051–6060.