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COMPARISON OF THE METAL ION PROMOTED DEPHOSPHORYLATION OF ADENOSINE 5'-TRIPHOSPHATE AND URIDINE 5'-TRIPHOSPHATE

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Abstract - The dephosphorylation of ATP and UTP in dependence on pH proceeds for both nucleoside 5'-triphosphates (NTP) with the same rate indicating that the nucleic base moieties have no influence on this reaction. This is different in the presence of Cu^{2+} which promotes the scission of the terminal γ phosphate group with both NTPs, but with ATP the reaction is considerably more facilitated. Evidence is given that in the Cu^{2+}-ATP systems the base moiety is involved in the reaction, while UTP shows the properties of a simple organic triphosphate (R-TP), like methyltriphosphate, and for these the most reactive intermediate is a $Cu_2(R\text{-}TP)(OH)^-$ complex. This result contrasts with that for the Cu^{2+}-ATP system: here the reaction proceeds via a $[Cu_2(ATP)]_2(OH)_1^{-1/-2}$ or $_2$ dimer. The structures of both reactive intermediates are discussed and compared; reference to other NTP-metal ion systems is made, and some possible implications regarding enzymic systems are indicated.

1. INTRODUCTION

The transfers of phosphoryl and nucleotidyl groups are among the most important processes in biochemistry [1-3].[a] The transfer of a phosphoryl group to water (eq. 1) is the simplest of these processes *in vitro* and relatively easy

$$NTP + H_2O \longrightarrow NDP + PO_4 \qquad (1)$$

to study. As all enzymes catalyzing such transfer reactions require divalent metal ions [3,4], it is not surprising that the metal ion promoted dephosphorylation of nucleoside 5'-triphosphates (NTP) has long been recognized. About 25 years ago Liébecq [5] studied this reaction with ATP; he was followed by Tetas & Lowenstein [6], Schneider & Brintzinger [7], Miller & Westheimer [8] and others [9-11]. These early studies were strongly hampered [12] by the incomplete knowledge on the stability [13,14] and structure [14,15] of NTP complexes in solution [16] -- a problem which is still not completely overcome.

To learn something about the reactive intermediates in these dephosphorylation processes, we are taking the approach to compare the reactivity of several NTP systems [12,17-19]. Hence, a different reactivity must originate in the different structures of the nucleic base moieties, because otherwise the NTPs are identical. ATP is the most well-known representative of the purine-nucleotides,

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a) The numbers of references are given in square brackets and those of equilibria and reactions in parentheses.
Abbreviations: AMP and ATP, adenosine 5'-mono- and 5'-triphosphate; Bpy, 2,2'-bipyridyl; CTP, GTP, ITP, UTP, and TTP, cytidine, guanosine, inosine, uridine, and thymidine 5'-triphosphate, respectively; M^{2+}, bivalent metal ion; NDP and NTP, nucleoside 5'-di- and 5'-triphosphate; R-TP, triphosphate monoester, i.e. a triphosphate containing an organic moiety at one of the two terminal phosphate groups. The phosphate groups in NTP or R-TP are labelled as α, β and γ, where the latter refers to the terminal phosphate group. If nothing else is specified, the formula PO_4 represents all phosphate species which may be present in solution, i.e. H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}.

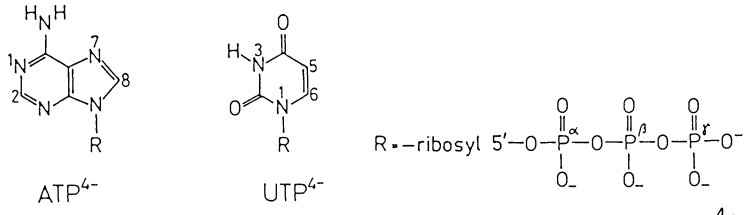

Fig. 1. Structures of adenosine 5'-triphosphate (ATP^{4-}) and uridine 5'-triphosphate (UTP^{4-}), together with the labelling system of the triphosphate chain.

while UTP is a pyrimidine-nucleotide (Fig. 1); the reactivity of systems containing these two nucleotides shall now be compared.[b] It should be emphasized in this connection that the stability of NTP-metal ion complexes is mainly determined by the triphosphate chain, i.e. the differences in stability between several NTP complexes of a given metal ion are relatively small [13] while their structures in solution may be quite different [15].

2. COMPARISON OF THE DEPHOSPHORYLATION RATES IN ATP AND UTP SYSTEMS

In Figure 2 the first-order rate constants for the dephosphorylation in dependence on pH are plotted for several systems. It is evident that different properties regarding the scission of the terminal phosphate group (eq. 1) become apparent only in the presence of Cu^{2+}. This result must mean that in the metal

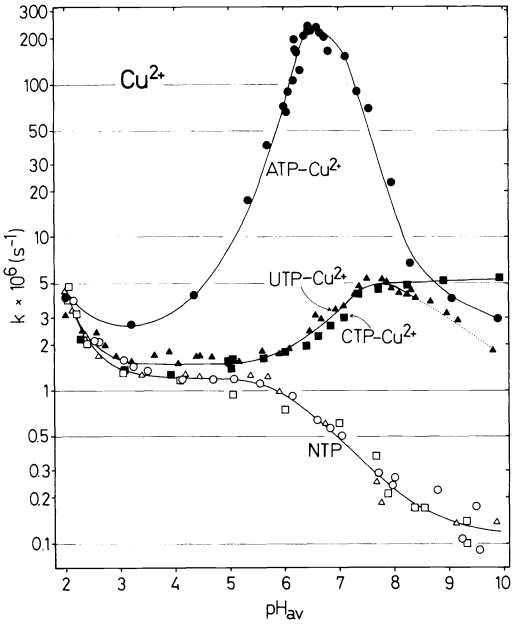

Fig. 2. Comparison of the Cu^{2+} promoted dephosphorylation of ATP (●) [21], UTP (▲) [19], and CTP (■) [22] (always in the ratio 1:1) in dependence on pH, characterized as the first-order rate constants k (s^{-1}). In addition is given: ATP (○), UTP (△), and CTP (□) alone. The concentration of all reagents was 10^{-3} M; I = 0.1, $NaClO_4$; 50°C. The dotted line portion indicates uncertainty due to precipitation.

b) The concentration of liberated PO_4 (eq. 1) was determined [12,17,19] with molybdate reagent using the procedure of Hirata & Appleman [20] as altered by Schneider & Brintzinger [7]. The reactivity was quantified by the initial rate of dephosphorylation, $v_o = d[PO_4]/dt$ (Ms^{-1}) [12], or by the first-order rate constant, k (s^{-1}) [17,21,22]; the latter procedure was used to enable us to compare our experimental results with earlier studies [7]. No buffers were employed, as this leads to the formation of mixed ligand complexes [23] and to an inhibition of the metal ion accelerated dephosphorylation [17].

ion free systems the base moieties are not involved in the hydrolysis process, while in the metal ion accelerated reaction they have a crucial influence.

This conclusion agrees with the observations made in the presence of 2,2'-bipyridyl (Bpy) [12,19]. The $Cu(Bpy)(NTP)^{2-}$ complexes dominate under 1:1:1 conditions over a wide pH range [12-14,19] and the coordination of Bpy releases the nucleic base moieties from the coordination spheres of the metal ions [24-28]. This means, in $Cu(Bpy)(ATP)^{2-}$ and $Cu(Bpy)(UTP)^{2-}$ only 2,2'-bipyridyl and the triphosphate chain are coordinated to the metal ion. Therefore, all $Cu(Bpy)(NTP)^{2-}$ complexes exhibit exactly the same dephosphorylation properties [12,19]: at pH 7 the rates of $Cu(Bpy)(NTP)^{2-}$ correspond to those of NTP alone, while at pH < 7 $Cu(Bpy)(NTP)^{2-}$ is more inert towards dephosphorylation and at pH > 7 it is somewhat more reactive ($k \simeq 1 \times 10^{-6}$ s^{-1}).

With these observations in mind it was attempted to learn something about the reactive intermediates in these Cu^{2+}-NTP systems; obviously at least in one case, the base moiety of the nucleotide must somehow be involved.

3. PROPERTIES OF THE Cu^{2+}-UTP SYSTEM

For comparison the rates of dephosphorylation of the Cu^{2+}-UTP system are plotted in Figure 3 together with the distribution of complex species in dependence on pH. Although the rates have been measured at 50°C and the distribution of complex species refers to 25°C, it is evident that the dephosphorylation rate parallels the concentration of the species $Cu(UTP\text{-}H)^{3-}$, but it must be noted that the curve assigned to $Cu(UTP\text{-}H)^{3-}$ actually represents the sum of the two isocharged species $Cu(UTP\text{-}H)^{3-}$ and $Cu(UTP)(OH)^{3-}$. Indeed, it is concluded [19] that the reactive intermediate derives from $Cu(UTP)(OH)^{3-}$ because:

(i) In the pH range up to about 8 the reactivity of Cu^{2+}-UTP corresponds closely to that of Cu^{2+}-CTP (see Fig. 2), and for the latter system it has been shown that the reactive species is connected with the formation of $Cu(CTP)(OH)^{3-}$ [12] (the cytidine moiety cannot ionize at N-3 [14]).

(ii) If the reactive species were derived from $Cu(UTP\text{-}H)^{3-}$, it would be difficult to see why $Cu(UTP\text{-}H)(OH)^{4-}$ should be so unreactive (compare the two parts of Fig. 3).

(iii) The release of a proton from N-3 leading to $Cu(UTP\text{-}H)^{3-}$ is connected with the formation of a macrochelate [14,18] and it is to be expected that an increase in temperature disfavors its formation, while the formation of hydroxo-complexes in triphosphate systems is favored [21].

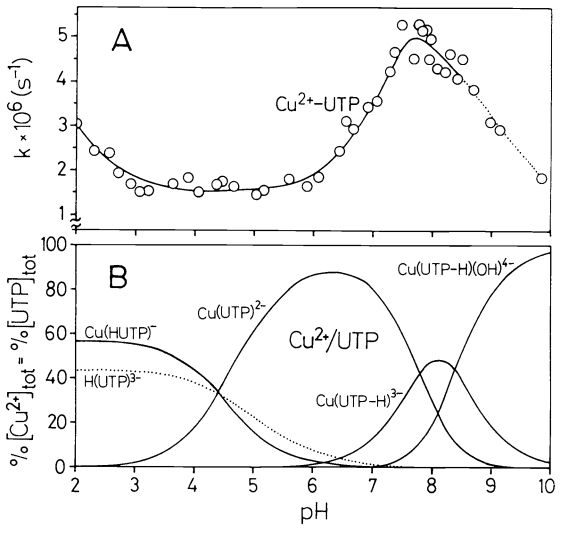

Fig. 3. *Upper part:* First-order rate constant, k (s^{-1}), for the dephosphorylation of UTP in the presence of Cu^{2+} (1:1) in dependence on pH. $[UTP]_{tot}$ = 10^{-3} M; I = 0.1, $NaClO_4$; 50°C ([19], see also Fig. 2). *Lower part:* Effect of pH on the concentration of the species present in an aqueous solution of $[UTP]_{tot} = [Cu^{2+}]_{tot} = 10^{-3}$ M at I = 0.1 and 25°C [13]. Results are given as the percentage of total UTP (or Cu^{2+}) present (see also [19]).

This tentative conclusion is confirmed by the properties of the more reactive 2:1 systems of Cu^{2+} and UTP or CTP; both nucleotide systems behave alike [19] and their dephosphorylation rates equal those of the Cu^{2+}-methyltriphosphate system [7], indicating that the base moieties are not involved and that these three triphosphates form a similar reactive intermediate.[c] Job's series show that the intermediate has a Cu^{2+}:triphosphate composition of 2:1 [19]; this fact together with the result of Figure 4 that the intermediate is of a monomeric nature and the observation that for the 2:1 system in the pH range 5 to 6 the reaction rate is proportional to $1/[H^+]$ (i.e. proportional to $[OH^-]$) suggests that the most reactive species has the composition $Cu_2(NTP)(OH)^-$, where NTP = UTP, CTP or methyltriphosphate [19]. Hence, the scheme given in Figure 5 can be proposed for the formation of the reactive intermediate in the metal ion promoted dephosphorylation of such organic triphosphates (R-TP). In this scheme it is implied that the coordination of a second metal ion forces one metal ion to the γ group and the other into the α,β position causing in this way a labilization of the γ group.

The reactivity observed in the Cu^{2+}:pyrimidine-NTP 1:1 systems at pH > 6 (Fig. 2) may be explained in two ways [19], using UTP as an example:

(i) The position of equilibria 2 and 3

$$2\ Cu(UTP)(OH)^{3-} \rightleftharpoons Cu_2(UTP)(OH)^- + UTP^{4-} + OH^- \quad (2)$$

$$Cu(UTP)(OH)^{3-} + Cu(UTP)^{2-} \rightleftharpoons Cu_2(UTP)(OH)^- + UTP^{4-} \quad (3)$$

may be such that a few percent $Cu_2(UTP)(OH)^-$ is formed. As the reactivity of $Cu_2(UTP)(OH)^-$ is much larger than that of $Cu(UTP)(OH)^{3-}$ the formation of small amounts of the 2:1 complex would be enough to explain the observed reactivity.

(ii) A partial release of the (α),β phosphate group(s) from the coordination sphere of Cu^{2+} in $Cu(UTP)(OH)^{3-}$ should also lead to a species with some reactivity.

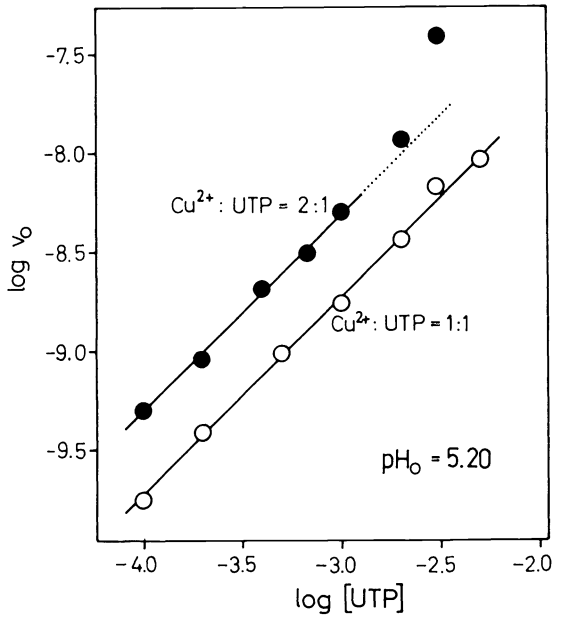

Fig. 4. Relationship between the initial dephosphorylation rate, v_o (Ms^{-1}), of UTP and the total concentrations of Cu^{2+} and UTP. Dependence of log v_o on log $[UTP]_{tot}$ = log $[Cu^{2+}]_{tot}$ (○) or log $[UTP]_{tot}$ = log $(1/2[Cu^{2+}]_{tot}$ (●); I = 0.1, $NaClO_4$; 50°C. The lines are drawn with the slope m = 1; the dotted portions indicate uncertainty due to precipitation. In the 1:1 system at pH_o = 7.80 also a slope of m = 1 is observed [19].

c) Indeed, stability (e.g. Cu^{2+}, Zn^{2+}) and ^1H-NMR shift data (Zn^{2+}, Cd^{2+}) of pyrimidine-nucleoside 5'-triphosphates show that there is no metal ion-base interaction in $M(NTP)^{2-}$ [15].

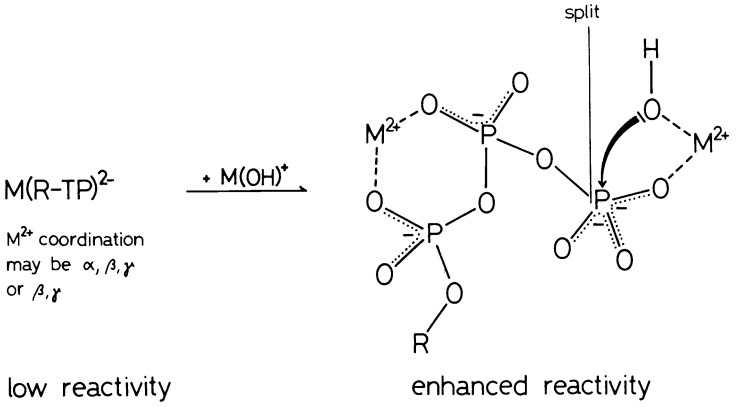

Fig. 5. Formation of the reactive complex during the metal ion promoted dephosphorylation of organic triphosphates (R-TP) undergoing only a metal ion-phosphate coordination, like UTP, CTP or methyltriphosphate, and its tentative and simplified structure (depictured from [19]).

4. PROPERTIES OF THE Cu^{2+}-ATP SYSTEM

Aside from the fact that the Cu^{2+}-ATP system is much more reactive, its kinetic properties [12] are partly similar to those of the Cu^{2+}-UTP system and partly quite different. The *similarities* are that (i) the 2:1 Cu^{2+}-ATP system in the pH range 3 - 6 is more reactive than the 1:1 system, and indeed Job's series show that at pH 4.5 the reactive intermediate has the Cu^{2+}:ATP stoichiometry of 2:1, and (ii) in the pH range 4 to 5 the reactivity in the 2:1 system is approximately proportional to the concentration of OH^-, indicating again the participation of $Cu(OH)^+$. The *differences* are that (i) maximal reactivity is reached already at pH 6.5 (Fig. 2), i.e. in a pH range where $Cu(ATP)^{2-}$ is the dominating species; the formation of $Cu(ATP)(OH)^{3-}$ inhibits the reaction. (ii) The most important difference however is that in plots of log v_o versus log [ATP] straight lines with a slope of two are obtained for Cu^{2+}-ATP 1:1 and 2:1 systems, indicating that the reaction proceeds via a dimer [12].

The summary of these kinetic observations leads to the conclusion that the most reactive intermediate in the dephosphorylation of the Cu^{2+}-ATP system is a dimeric species with the composition $[Cu_2(ATP)]_2(OH)_1^{-1/-2}$ or $_2$. The observed reactivity in the Cu^{2+}-ATP 1:1 systems may be explained by equilibria analogous to those given for Cu^{2+}-UTP (eq. 2,3) but taking into account also dimerization or by attributing a certain reactivity also to $[Cu(ATP)]_2(OH)_0^{-4/-5}$ or $_1$.

It is evident that any hypothesis about the structure of the reactive dimeric intermediate must involve the adenine base moiety, because ATP and UTP differ only by their bases and in the Cu^{2+}-UTP system the base is not of importance (see Section 3 and Fig. 5). Indeed, it was previously deduced from several indirect hints and observations [12] that in the dimer the two adenine bases are stacked and that metal ions bridge the two ATPs by coordinating to the phosphate chain of one and to the N-7 of the other ATP. This conclusion is now further supported by ^{1}H-NMR shift experiments [15] which demonstrate self-association of ATP via base stacking, i.e. upfield shifts of the resonance signals of H-2 and H-8 are observed. The extent of stacking may be promoted by neutralizing part of the negative charge of the phosphate chain by coordinating Mg^{2+}. However, metal ions like Zn^{2+} or Cd^{2+} are significantly more effective: they promote the formation of dimers further by forming an *inter*molecular metal ion bridge between two stacked ATPs. This bridge involves the phosphate chain of one ATP^{4-} and N-7 of the adenine moiety of the other. The shifts of H-8 for complete stacking (δ_∞) are listed in the Table for several ATP systems: the downfield shifts observed for H-8 of $[Zn(ATP)^{2-}]_\infty$ and $[Cd(ATP)^{2-}]_\infty$ in comparison with $[Mg(ATP)^{2-}]_\infty$ agree with this interpretation. In addition, at very low concentrations, where only monomeric $M(ATP)^{2-}$ complexes are formed, N-7 still participates in complex formation, e.g., with Cu^{2+}, Zn^{2+} or Cd^{2+}: now macrochelates are formed by the coordination of a metal ion to the phosphate chain and to N-7 of the *same* ATP^{4-} [15].

TABLE. Evidence for the importance of the M^{2+}/N-7 interaction for the self-association of the $M(ATP)^{2-}$ complexes of Zn^{2+} and Cd^{2+}, as judged from the comparison of the chemical shifts (ppm) of H-8 in stacked (δ_∞) ATP^{4-} with the corresponding shifts of $M(ATP)^{2-}$ complexes (in D_2O, 27°C, I ≃ 2)[a,b]

System	δ_∞ of H-8	downfield shift, $\Delta\delta_\infty$[c]
adenosine[a]	8.07 ± 0.04	
ATP^{4-}	7.92 ± 0.06	
$Mg(ATP)^{2-}$	8.02 ± 0.04	
$Zn(ATP)^{2-}$	8.36 ± 0.06	0.34 ± 0.10
$Cd(ATP)^{2-}$	8.49 ± 0.08	0.47 ± 0.12

a) The corresponding shift of adenosine is given for comparison (27°C; I = 0.1, $NaNO_3$).
b) The data are abstracted from Tables III and IV in [15].
c) Shift difference $\Delta\delta_\infty$ for H-8 between $Zn(ATP)^{2-}$ or $Cd(ATP)^{2-}$ and $Mg(ATP)^{2-}$.

5. COMPARISON OF THE REACTIVE INTERMEDIATES IN THE Cu^{2+} SYSTEMS WITH ATP OR UTP

A tentative and simplified structure of the reactive $[Cu_2(ATP)]_2(OH)_{1\text{ or }2}^{-1/-2}$ intermediate in the Cu^{2+} promoted dephosphorylation of ATP is shown in Figure 6. The main point is that the shift of M^{2+} along the phosphate backbone into an α,β coordination of one ATP is facilitated by coordination of this metal ion to N-7 of the other ATP, thus leading to a γ phosphate group ready for an intramolecular attack by OH^- from the γ coordinated $Cu(OH)^+$ unit. This N-7 facilitated shift of the metal ion in the dimer into an α,β position is thought to be the main reason for the larger dephosphorylation rate of Cu^{2+}-ATP compared with Cu^{2+}-UTP (Fig. 2); in the latter system the α,β coordination is only enforced by coordination of a further metal ion. However, both intermediates shown in Figures 5 and 6 have in common that the hydrolysis of the terminal γ group proceeds via an M(α,β)-M(γ) coordinated unit.

In the reactive intermediate shown in Figure 6 one ATP seems to be needed at first only for structural purposes, i.e. to facilitate the formation of the reactive state of the other. This view is supported by the observation that

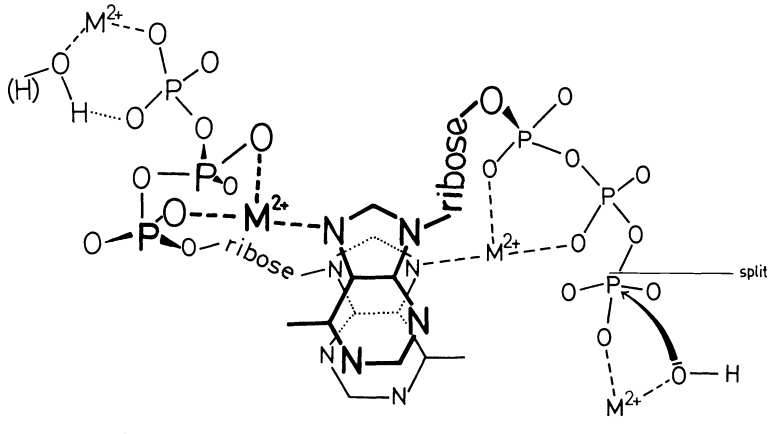

Fig. 6. Tentative and simplified structure of the reactive dimer, which occurs in low concentrations during the metal ion promoted dephosphorylation of ATP. The intramolecular attack of OH^- is indicated on the right side, while the left side is ready to transform into the reactive state. This structure is an altered version of the one proposed earlier in Figure 14 of [12] by taking into account the more recent experiences.

at pH 6.7 the reactivity of the Cu^{2+}-ATP 1:1 and 2:1 systems may be further enhanced by the addition of AMP, while with phosphate or adenosine the reaction is inhibited [18,29]. It appears that AMP, having the N-7 *and* one phosphate group, is able to take over the role of the 'structuring' ATP, thus leading to mixed AMP/ATP stacks and forcing more ATP into the reactive form. This observation with AMP is thus further support for the basic validity of the view expressed in Figure 6.

As one might expect, the observations in mixed Cu^{2+}/ATP/AMP systems contrast with the effect that AMP has on a Cu^{2+}-UTP 1:1 system: in the latter case the formation of the $Cu(UTP)(AMP)^{4-}$ complex [19] inhibits the reaction.

6. OTHER M^{2+}-NTP SYSTEMS

The rate of dephosphorylation in 1:1 systems of Cu^{2+} and ITP or GTP [12] is between that of Cu^{2+}-ATP and of Cu^{2+}-UTP or Cu^{2+}-CTP (Fig. 2). The lower reactivity of these Cu^{2+} purine-NTP systems may be ascribed to their lower stacking tendency [15] and the altered metal ion affinity of N-7 [15,16].

Thymidine 5'-triphosphate (TTP) is structurally very similar to UTP; in agreement herewith no differences in the dephosphorylation properties between the Cu^{2+} systems with UTP or TTP have been observed [19]. Some of these NTP systems have also been studied in the presence of Mn^{2+}, Ni^{2+}, or Zn^{2+} [12,17,19].

The Zn^{2+} and Ni^{2+} systems of UTP [19] and ATP [12,17] show (as far as the studies have been carried out) the same properties regarding the stoichiometry of the reactive intermediates as described now for the corresponding Cu^{2+} systems. However, the promotion of the hydrolysis reaction by these metal ions is less pronounced than with Cu^{2+}; especially Ni^{2+} is a relatively poor promoter. Possible reasons for the differences in efficiency between several metal ions in promoting the dephosphorylation of nucleoside 5'-triphosphates have been discussed elsewhere [19].

7. GENERAL CONCLUSIONS

The tentative structures given in Figures 5 and 6 for the reactive intermediates indicate that the coordination unit M(α,β)-M(γ) facilitates a scission between the β and γ group of a triphosphate. As many of the enzyme-nucleotide systems operating in nature contain two or more metal ions [1,2,30], reactive intermediates similar to those discussed here could well play a role: the mentioned M(α,β)-M(γ) coordination should facilitate a transfer either of a nucleoside diphosphate or of a phosphate group. Other coordination types can as well be envisaged, e.g., a M(α)-M(β,γ) coordination promoting a nucleoside monophosphate or diphosphate transfer.

One of the two metal ions used for activation (Figures 5 and 6) could well be replaced by an ionic interaction, e.g., with an arginine residue of the enzyme, or by a hydrogen bond between a suitable enzymic group and an oxygen of the phosphate moiety. The result regarding promotion and selectivity of the transfer reaction would be the same.

It is well-known that enzymes modify the structures of substrate molecules: e.g., Mn^{2+} binds to all three phosphate groups of thymidine 5'-triphosphate in the binary complex, but only to the terminal γ phosphate group in the ternary complex which involves also DNA polymerase I of *Escherichia coli* [31].

The crucial part in creating the reactive intermediate may be played by ligating groups of the enzyme[d] forcing the metal ion into a certain position along the phosphate backbone, *provided* the nucleoside 5'-triphosphate is anchored somewhere. This anchoring may occur between polar groups of the enzyme and the triphosphate chain, and/or via the nucleic base moiety: e.g., a purine

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d) Imidazole moieties of histidyl residues would be especially appropriate as ligating groups from the enzyme, because mixed ligand complexes containing imidazole and ligands with O donors, like phosphate groups, are selectively favored in their formation via an increased stability [28,32-35].

moiety could stack with an indole residue of an enzyme positioning in this way the triphosphate chain toward an enzyme-bound metal ion such that activation of the desired bond results. Stacking interactions between the indole residue of tryptophanate and ATP in ternary complexes are known [27,28,36] as are the corresponding hydrophobic interactions [37,38] with the isopropyl residue of leucinate [39].

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