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On the Kinetics of Metal-Catalyzed Adenosine Triphosphate Dephosphorylation*

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ABSTRACT: Analysis of Tetas and Lowenstein's rate data (*Biochemistry 2*, 351 (1963)) on metal ion catalysts of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) dephosphorylation reveals that the role of the metal is not limited to the acceleration of water attack on phosphate linkages or of elimination of metaphosphate. Such mechanisms are enhanced by chelation of manganese(II) to ATP, but are actually inhibited for the manganese ADP chelate, although hydroxyl attack is accelerated in the latter case. For the copper(II)–ATP systems a completely new mechanism dominates the kinetics, one involving proton attack on the dimeric hydroxy chelate, $[CuATP(OH)]_2^{6-}$. Involvement of this dimer is confirmed by a study of the concentration dependence of the rate. On dilution, the pseudo-first-order rate constant decreases in parallel with the dimer concentration. A similar mechanism is probably operative in the Zn(II)–ATP system.

Divalent metal ions are required in most enzymatic reactions of ATP.[1] The interaction of metal ions with ATP in nonenzymatic systems has been studied in considerable detail. Stability constants have been measured (Sillén and Martell, 1964) and the sites of attachment of some metal ions to ATP have been determined (Cohn and Hughes, 1962; Brintzinger, 1963; Schneider *et al.*, 1964).

The influence of metal ions on the kinetics of nonenzymatic reactions of ATP, and also ADP, have been investigated by Lowenstein and coworkers (1958a,b, 1961, 1963). Of particular interest is their study on the hydrolysis (dephosphorylation) reaction (Tetas and Lowenstein, 1963). The results obtained are complex and were not interpreted quantitatively. The authors did, however, offer some suggestions on mechanisms. They proposed that chelation of ATP and ADP by metal ions serves to render the terminal phosphate susceptible to nucleophilic attack by water, or to elimination, and that specific differences among metal ions might result from changes in chelate structure. Schneider and Brintzinger (1964) made a more detailed study of copper(II) catalysis of ATP dephosphorylation, and proposed a specific chelate structure to account for the exceptional activity of Cu(II). The differences ob-

* From the Frick Chemical Laboratory, Princeton University, Princeton, New Jersey 08540. *Received August 8, 1967.* This investigation was supported by U. S. Public Health Service Grants GM-13498 to T. G. S. and GM-13667 to R. A. B.

[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate.

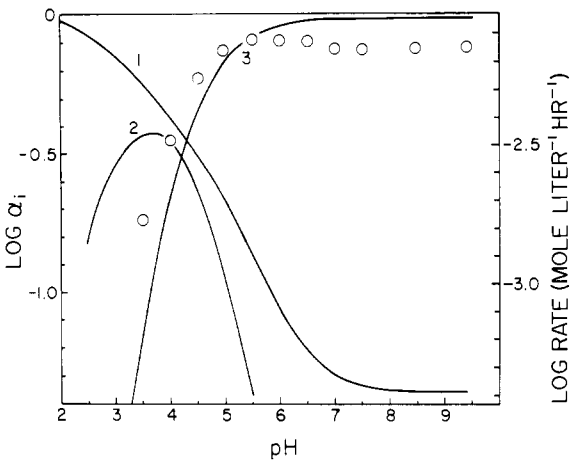

FIGURE 1: Concentration and rate–pH profiles for the Mn(II)–ATP systems at $C_{ATP} = C_{Mn} = 0.02$ M. α_i is the fraction of total ATP contained in the species i. Curve 1: i = unbound ATP; curve 2: i = $MnATPH^-$; curve 3: i = $MnATP^{2-}$. Rate data (○) from Tetas and Lowenstein (1963).

served by Tetas and Lowenstein, however, extend to the shapes of the rate–pH profiles and raise the possibility that the rate law, and not just the chelate structure, might vary from one metal ion to another. It was of interest, therefore, to determine the extent to which these variations could be attributed to differing mechanisms. In this report, available equilibrium data for these systems are used to interpret the rates. It is shown that Cu(II)-catalyzed ATP dephosphorylation proceeds *via* proton attack on the dimeric species $[CuATP(OH)]_2^{6-}$. A similar mechanism is suggested for the Zn(II)–ATP system as well.

Experimental Procedure

Metal-catalyzed hydrolysis of ATP was carried out at 40° in a 5.0-ml reaction mixture containing 0.1 M KNO_3. $Na_2H_2[^{32}P]ATP$ labeled in the terminal phosphate position was prepared according to the procedure of Penefsky *et al.* (1960). All metal solutions were standardized by EDTA titration. Equimolar amounts of metal nitrate and $Na_2H_2[^{32}P]ATP$ (approximately 2×10^4 cpm/μmole) were added to the reaction mixture and an aliquot was removed for a zero time determination. The pH was then quickly adjusted to 5.5 (corresponding to the maximum in the rate profile of Tetas and Lowenstein, 1963) with 0.5 N NaOH. Constant pH was maintained during the course of the reaction with the aid of a Radiometer pH-Stat. At regular time intervals aliquots were removed into 0.35 ml of triethylamine–molybdate reagent (Sugino and Miyoshi, 1964) which quantitatively precipitates orthophosphate in the presence of organic phosphates. Cold carrier phosphate (0.5 μmole) was then added. After approximately 30 min, the precipitate was collected on a Millipore filter (type HA, 0.45 μ), washed with 5 ml of 1 N HCl, dried, and counted in a Nuclear-Chicago gas-flow counter. The pseudo-first-order rate constant was then determined from the initial portion of a plot of the logarithm of the fraction of ATP consumed against time.

Results

Analysis of pH Profiles. Our analysis of Tetas and Lowenstein's (1963) data proceeds by a comparison of the pH dependence of the rate of dephosphorylation in the Mn(II)–, Cu(II)–, and Zn(II)–ATP systems with the calculated pH profiles for the concentrations of the various ATP entities: unbound ATP; $MATP^{2-}$ (where M is the particular metal ion); $MATPH^-$; and, in the case of copper, $MATP(OH)^{3-}$, $[MATP(OH)]_2^{6-}$, and $MATP(OH)_2^{4-}$. If the rate is proportional to the concentration of one of these then the pH profiles of rate and concentration, plotted on a logarithmic scale, should be superimposable by translation along the ordinate. If more than one species is kinetically important then the rate profile should encompass all of them, but with different ordinate shifts, corresponding to the individual rate constants.

The concentrations of the various chelate species were calculated using the thermodynamic data of Taqui Khan and Martell (1966), who determined equilibrium constants for a number of metal–ATP systems in 0.1 M KNO_3 over a temperature range from 0.4 to 40°. Their enthalpy values were used to extrapolate the equilibrium constants to 80°, the temperature employed by Tetas and Lowenstein (1963). Concentrations were then calculated by computer with a modification of Ingri and Sillén's program KUSKA (1962).

The rate data were read directly from Figures 1 and 2 of Tetas and Lowenstein (1963) and converted into a logarithmic scale. A small correction was made for the reaction of unbound ATP by multiplying the rate, recorded in the same figures, for metal-free ATP by the calculated fraction of ATP remaining unbound, and subtracting this value from the metal–ATP rate.

There are a number of uncertainties in this analysis which should be noted at the outset. In the first place the quantity actually measured by Tetas and Lowenstein (1963) was reaction yield for an increment of time, not rate *per se*. The extent of reaction however for almost all of their experimental points was less than 30%, so that the measurements should be at least a reasonable approximation to initial rates. As for the calculation of concentrations applicable to Tetas and Lowenstein's conditions (1963), there are two difficulties. The extrapolation to 80° is based on the assumption that the enthalpies for the various equilibria remain constant to this temperature. This is by no means certain, but the errors introduced from this source are probably small and to some extent mutually cancelling. More serious is the question of reaction medium. The equilibrium constants were measured in 0.1 M KNO_3, in which weak binding of potassium to ATP occurs (O'Sullivan and Perrin, 1961), while the rate data were obtained in a variety of buffers, some of which bind the metal ions weakly (Sillén and Martell, 1964).

In spite of these difficulties, the results of the analysis seem to be unequivocal, as will be seen. The differences in pH profile for the concentration of the various species in each system are substantial, and the rate data are available over a wide pH range, so that the curve-fitting procedure is not sensitive to moderate errors. In each case the comparison clearly indicated one rate expression to the exclusion of others.

MANGANESE–ATP. This is a fairly simple system involving the unprotonated and protonated chelate species and unbound ATP. For the total concentrations used by Tetas and Lowenstein (1963), 0.02 M Mn(II) and 0.02 M ATP, their distribution is shown in Figure 1, where the logarithm of the fraction of ATP present at equilibrium in each form is plotted against pH. Superimposed on this distribution are the dephosphorylation rate data of Tetas and Lowenstein (1963), recast as described above. Clearly, these data follow closely the concentration of the normal chelate, $MnATP^{2-}$ (curve 3). The small deviations observed are within the uncertainties of the analysis as discussed above. Thus it appears that the rate data for this system support a rate law first order in $MnATP^{2-}$ concentration, and, by implication, the basic mechanisms proposed by Tetas and Lowenstein (1963), *i.e.*, attack by water on coordinated ATP, or elimination of metaphosphate.

COPPER–ATP. The chemistry of this system is considerably more complex, involving, in addition to the normal and protonated chelate, a monohydroxy chelate, $CuATP(OH)^{3-}$, its dimer, $[CuATP(OH)]_2^{6-}$, and a dihydroxy chelate, $CuATP(OH)_2^{4-}$. The rate–pH profile is quite different from that of Mn(II)–ATP, exhibiting a sharp maximum at pH 5.5. This has been reported by Schneider and Brintzinger (1964) as well. If the mechanism of dephosphorylation involves the normal chelate species, then formation of the hydroxylated compounds might account for the drop in rate above pH 5.5. The distribution of the various forms is shown in Figure 2, as is Tetas and Lowenstein's rate data. It is evident that the latter do not follow the concentration of $CuATP^{2-}$ (curve 3). Although this species passes through a pH maximum, it does so at a somewhat lower pH than does the rate data, and more important, for the descending branch on the acid side a difference of some 2 pH units is involved. Moreover, the rate profile does not coincide, even approximately, with the concentration profile of any of the species. The involvement of more than one species in the rate law is excluded since in this case the rate–pH profile would be broadened to include all of the relevant concentration profiles; in fact the rate profile is narrower than any of the concentration profiles except for $[CuATP(OH)]_2^{6-}$.

These data then do not support a dephosphorylation mechanism involving simply water attack on any of the species. Alternatively, attack by a proton or hydroxyl ion may be considered. These possibilities can be tested by plotting instead of the concentration–pH profiles, the pH profiles of $(X)(H^+)$ or $(X)(OH^-)$, where X is the particular species under consideration. A rate law involving $(X)(H^+)$ cannot be distinguished kinetically from one involving (XH), nor can one involving $(X)(OH^-)$ be distinguished from one involving (XOH). Consequently new curve-fitting possibilities are introduced only by the combinations $(CuATPH^-)(H^+)$ and $[CuATP(OH)_2^{4-}](OH^-)$, which produce monotonic curves at either end of the pH scale and are therefore of no interest, and by $([CuATP(OH)]_2^{6-})(H^+)$ and $([CuATP(OH)]_2^{6-})(OH^-)$. Because $[CuATP(OH)]_2^{6-}$ contains two coppers and two hydroxides, multiplication of its concentration by (H^+) or (OH^-) does not lead to a profile superimposable with the concentration profile of any other species. Multiplication by (OH^-) shifts the profile to higher pH while multiplication by (H^+) shifts it to lower pH, the required direction. In fact a plot of log $([CuATP(OH)]_2^{6-})(H^+)$ (curve 4) gives an excellent fit to the observed rate–pH profile, as shown in Figure 2. There are moderate deviations at the base of the profile, suggesting the emergence of other kinetic paths. But there can be no doubt that the main path can be quite adequately accounted for by a rate law first order in both dimer and hydrogen ion concentration, and by no other reasonable rate law.

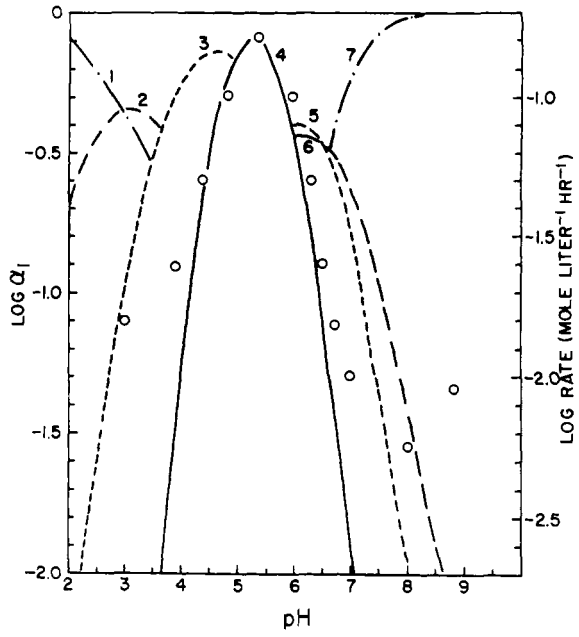

FIGURE 2: Concentration and rate–pH profiles for the Cu(II)–ATP system at $C_{ATP} = C_{Cu} = 0.02$ M. α_i is defined in Figure 1. Curve 1: i = unbound ATP; curve 2: i = $CuATPH^-$; curve 3: i = $CuATP^{2-}$; curve 5: i = $(CuATPOH)_2^{6-}$; curve 6: i = $CuATPOH^{3-}$; curve 7: i = $CuATP(OH)_2^{4-}$. Curve 4 is a plot of log $\alpha[CuATP(OH)]_2^{6-}$ + log (H^+); the ordinate for this curve is the left-hand scale reading minus 5.9. The interior descending branches of the other curves are omitted for clarity. Rate date (O) from Tetas and Lowenstein (1963).

ZINC–ATP. The rate profile resembles that of copper–ATP in that it has a maximum at pH 5.5 and falls off rapidly on both sides, although the rates themselves are

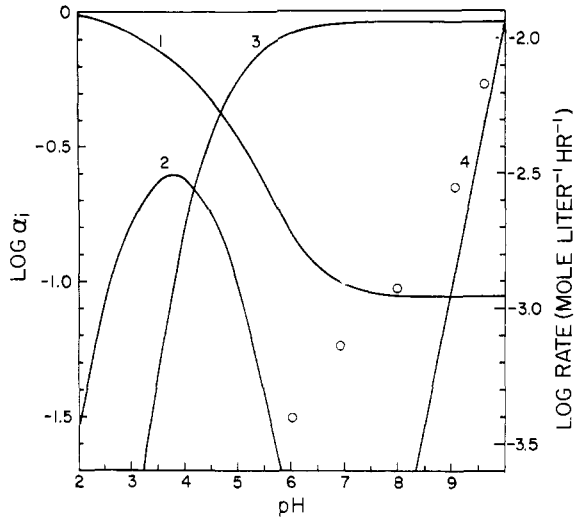

FIGURE 3: Concentration and rate–pH profiles for the Mn(II)–ADP system at $C_{ADP} = C_{Mn} = 0.02$ M. α_i is defined in Figure 1. Curve 1: i = unbound ADP; curve 2: i = MnADPH; curve 3: i = $MnADP^-$. Curve 4 is a plot of log α_{MnADP^-} + log (OH^-); the ordinate is the left-hand scale minus 4.0. Rate data (○) from Tetas and Lowenstein (1963).

a factor of five or so lower. On the other hand, the available equilibrium constants are quite similar to those of manganese–ATP, with no report of the formation of hydroxylated species. Yet it is difficult to understand the drop in rate above pH 5.5 without the formation of such species, no matter which mechanism is considered. It is suggestive that with ADP zinc does form a hydroxylated chelate, as well as a dimer (Taqui Khan and Martell, 1962). Perhaps such species also form in the zinc–ATP system, but at concentrations sufficiently low that they remain undetected in the equilibrium studies. In that event the rate law could be similar to that found for copper–ATP, involving the product of concentrations of a hydroxylated dimer and hydrogen ion. The profile for this product would lie at pH values where the fraction of total ATP bound in the hypothetical dimer would be quite low. We offer positive evidence for this possibility below.

ADP SYSTEMS. Although our main concern in this work is with the mechanisms of ATP dephosphorylation, it is instructive to compare the rate data for ADP dephosphorylation also published by Tetas and Lowenstein (1963). Rate profiles for the copper, zinc, and manganese systems are given in Figures 5 and 6 of their paper. Direct comparison with concentration profiles is less practicable here because enthalpies for the metal ion ADP equilibria are unavailable. However we note that the zinc- and copper–ADP rate profiles are similar to the corresponding ATP profiles in that again they show a maximum at about pH 5. Since hydroxylated chelates and dimers are formed in these ADP systems (Taqui Khan and Martell, 1962), the inference is that they are again involved in the rate law.

There is however a marked difference in the rate profiles for manganese–ATP and –ADP dephosphorylation. In the former case a rise at pH 4–5 culminates in a plateau beyond pH 5.5. On the other hand, the dephosphorylation rate for manganese–ADP is depressed below the value found for metal-free ADP up to pH 7 and then rises without limit as the pH is further increased. This behavior is quite inconsistent with a rate law first order in $MnADP^-$ alone. Figure 3 compares the rate data with the concentration profiles in the manganese–ADP system. To calculate these concentrations we assumed that the enthalpies for the ADP equilibria (Taqui Khan and Martell, 1962) are the same as those for the corresponding manganese–ATP equilibria (Taqui Khan and Martell, 1966). This introduces another uncertainty into the comparison, but it cannot affect the obvious conclusion that the rates do not follow the concentration of $MnADP^-$ (curve 3). Rather, they are seen to approach asymptotically curve 4, which corresponds to the product $(MnADP^-)(OH^-)$. These data suggest that the main path for manganese–ADP dephosphorylation is through the hydroxyl attack on the chelate, with water attack playing only a minor role.

ATP Dephosphorylation Kinetics and Metal Ion Concentration. In order to test the hypothesis that dephosphorylation in the copper–ATP system proceeds *via* the species $[CuATP(OH)]_2^{6-}$ in the rate-determining step, we have carried out kinetic studies over a wide range of copper concentrations. The concentration of ATP was set equal to that of copper and the pH was held constant at 5.5, corresponding to the maximum in the rate profile of Tetas and Lowenstein (1963). The medium was that used by Taqui Khan and Martell (1966), and the temperature was 40°, the highest temperature for which they measured equilibrium constants. The fraction of ATP present in $[CuATP(OH)]_2^{6-}$ decreases as the solution is diluted, and the effective rate constant should therefore decrease in the same direction if the hypothesis is valid.

The rate may be tentatively expressed by

$$-\frac{dC_{ATP}}{dt} = k'(X) \qquad (1)$$

where C_{ATP} is the concentration of total ATP, and X is the metal–ATP species involved in the rate-determining step. The rate constant (k') includes any pH dependence. Since $(X) = \alpha_x C_{ATP}$, where α_x is the fraction of ATP bound in X, eq 1 may be written as

$$-\frac{dC_{ATP}}{C_{ATP}} = k'\alpha_x dt$$

which, on integration, gives

$$\log \frac{C_{ATP}}{C_{ATP}^0} = \frac{k'\alpha_x}{2.303} t$$

if α_x does not change with time, which is approximately the case in the initial part of the reaction. C_{ATP}^0 is the

initial value of C_{ATP}. The initial slope of the plot of the logarithm of the fraction of ATP unhydrolyzed against time, k'', then gives the pseudo-first-order rate constant, $2.303k'' = k'\alpha_x$. A plot of log 2.303k'' against log C_{Cu} should be superimposable on a plot of log α_x against log C_{Cu} for the appropriate X, with an ordinate shift corresponding to log k'. The result of such a comparison is shown in Figure 4, which gives the distribution of the concentrations of $CuATP^{2-}$, $[CuATP(OH)]_2^{6-}$ and of unbound ATP as a function of the total ATP or metal concentration, at pH 5.5. The species $CuATPH^-$, $CuATP(OH)^{3-}$, and $CuATP(OH)_2^{4-}$ are omitted; their curves essentially parallel that of $CuATP^{2-}$, but at lower values. The experimental points clearly follow the concentration of the dimer, over the concentration range C_{Cu} = 0.0453–0.0024 M. Thus the involvement of the dimer in the rate-determining step can be taken as established.

We have not tested here for the involvement of hydrogen ion, which is implied by the analysis of Tetas and Lowenstein's (1963) rate profiles presented above. However it is encouraging to note that the second-order rate constant (k), calculated on the assumption that

$$-\frac{dC_{ATP}}{dt} = k[(CuATP(OH)_2^{6-}](H^+)$$

is quite consistent for the two sets of data. From Figure 4 and at pH 5.5 we calculate $k_{40°} = 1.6 \times 10^4$ mole l.$^{-1}$ min^{-1} while from Figure 2 we estimate $k_{80°} = 26 \times 10^4$ mole l.$^{-1}$ min^{-1}. This gives $k_{80°}/k_{40°} = 16$ the ratio expected on the simple rule of thumb that a 10° rise gives a doubling of the rate constant.

We also carried out similar experiments for zinc–ATP, and the results are likewise plotted in Figure 4. From simple dissociation of $ZnATP^{2-}$, the fraction of the ATP bound to zinc decreases by less than 0.1 log unit over the range covered, while the drop in measured rate constant is over 1.2 log units. It seems clear that a polynuclear species must be involved in the rate-determining step for zinc–ATP as well as for copper–ATP.

Finally, as a control we measured the rate of dephosphorylation for manganese–ATP under the same conditions. The measured rate constants at two concentrations (0.0480 and 0.0048 M) were essentially identical: 1.3×10^{-5} min^{-1}. A 25% decrease (0.1 log unit) is predicted, based on the fraction of ATP bound at the two concentrations, but this difference is probably within experimental error for the very low rates involved in this system.

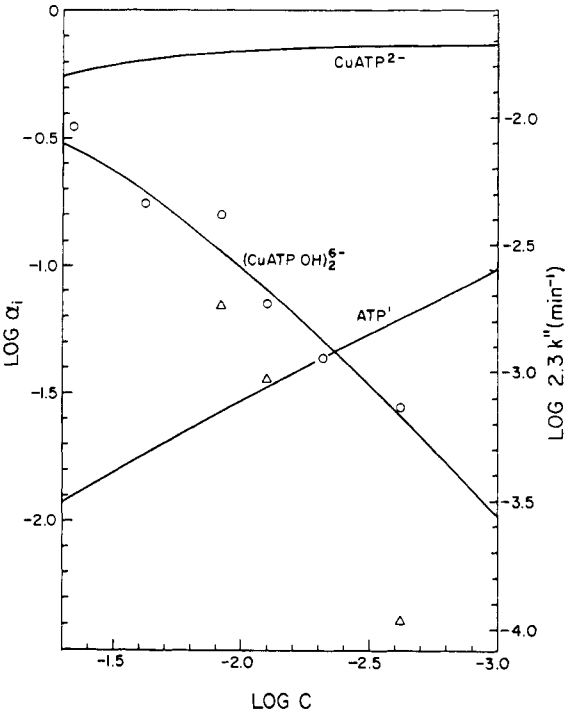

FIGURE 4: Variation of concentrations and rates with dilution at pH 5.5. C is the molar concentration of total ATP (or Cu(II) or Zn(II)). α_i is defined in Figure 1. The species i are indicated on the curves; ATP' represents the concentration of ATP unbound to copper. 2.3k'', pseudo-first-order rate constant for Cu(II)–ATP (○) and Zn(II)–ATP(△).

Discussion

A general conclusion reached from studies of the hydrolysis of a variety of phosphate esters (Bruice and Benkovic, 1966) is that rates are enhanced by protonation of anionic forms, which facilitates either nucleophilic attack by water or elimination of a metaphosphate intermediate. It seems reasonable to assume, as did Tetas and Lowenstein (1963), that association of metal ions to ATP and ADP should have the same effect as protonation. The present analysis suggests that the situation is more complex.

In the Mn(II)–ATP system a rate law first order in $MnATP^{2-}$ is evidently adequate to explain the data. Whether the mechanism involves attack by water or elimination of metaphosphate is undetermined. Neither mechanism can be important in the case of Mn(II)–ADP hydrolysis, however, since the rate for this system is lower than that for metal-free ADP up to pH 7. In other words, chelation by Mn(II) actually confers some stability on the diphosphate group of ADP with respect to either water attack or to metaphosphate elimination. Only when hydroxyl attack becomes important does the rate increase substantially. The lability of ATP when chelated to Mn(II) must then be associated with the extra phosphate group. Although the nuclear magnetic resonance evidence indicates that $MnATP^{2-}$ has all three phosphates coordinated (Cohn and Hughes, 1962), it could presumably switch to an α,β chelate in the transition state, leaving the terminal phosphate vulnerable to water attack or elimination.

The Cu(II) systems are dominated by formation and dimerization of hydroxy chelates. For ATP, the dimer is the primary species which contributed to the marked Cu(II) catalysis of dephosphorylation, and this may be true for ADP as well, although in this case the rates are

substantially lower. It is likely that a dimer is also kinetically dominant for Zn(II)–ATP and perhaps for Zn(II)—ADP. There are no structural data available for these particular hydroxylated dimeric species. Cohn and Hughes (1962) reported from nuclear magnetic resonance evidence that both Cu(II) and Zn(II) interact with the β- and γ-phosphates of ATP as well as with the adenine ring. Similarly Brintzinger (1963) found infrared spectral evidence for binding of Cu(II) and Zn(II) to the β- and γ-phosphates of ATP, while Schneider *et al.* (1964) deduced from ultraviolet spectra that binding of adenine to Cu(II) in the ATP chelate is predominant, and occurs to a lesser extent with Zn(II). In none of these studies were hydroxylated species considered, but they were probably present in the Cu(II) solutions used in the first two studies as indicated by the reported pH values. The primary product of cupric ion hydrolysis is $Cu_2(OH)_2^{2+}$ (Berecki-Biedermann, 1956) and a hydroxy–copper dimer has been reported coordinated to pyridine (Leussing and Hansen, 1957) and to substituted ethylenediamines (Pfeiffer and Glaser, 1938; Hatfield *et al.*, 1963). It is therefore reasonable to suppose that the ATP and ADP dimers are held together by double hydroxy bridges between the copper ions.

There is no obvious reason why these species are particularly susceptible to dephosphorylation. Apparently protonation is required, at least in the case of $[CuATP(OH)]_2^{6-}$. The proton might attack one of several phosphate sites, but it is difficult to see why the resulting species should be more reactive than the protonated chelate ($CuATPH^-$), which has the advantage of a lower charge. On the other hand one of the bridging hydroxyls of the dimer might be protonated producing, transiently, a dimer with a single bridge (eq 2). Subtle electronic changes in the bridging system produced by this reaction might indirectly labilize a phosphate linkage, but the details of such a process must remain conjectural. From Moll *et al.*'s (1964) finding that dephosphorylation of Cu(II)–ATP is accompanied by entry of solvent ^{18}O into the liberated phosphates, it appears that water attacks the terminal phosphate of the activated species.

Although the metal ion catalysis of adenosine polyphosphate dephosphorylation is evidently a complex phenomenon, a simplified model may tentatively be formulated from the present results, and set forth as follows. "Normal" chelation of ADP, as exemplified by Mn(II), tends to stabilize the diphosphate group toward water attack or elimination, although hydroxyl attack is accelerated. For ATP, "normal" chelation labilizes the terminal phosphate to water attack or elimination. Where the chelating metal can hydrolyze and dimerize, however, a more efficient pathway to dephosphorylation is provided by protonation of the dimeric chelate and water attack on this activated species. A prediction of this model is that, in the absence of dimerization, the rate law for the Cu(II) and Zn(II) systems should be comparable to those of the Mn(II) systems. Determination of rate–pH profiles for Cu(II) and Zn(II) in solutions sufficiently dilute so that the dimer concentration is negligible should provide a test of the prediction. More kinetic data are needed on metal ATP and ADP systems whose equilibria are well characterized, to determine whether the proposed model has any general validity.

Acknowledgments

The authors are indebted to Professors Richard Wolfenden and Neil Bartlett for valuable suggestions concerning this work.

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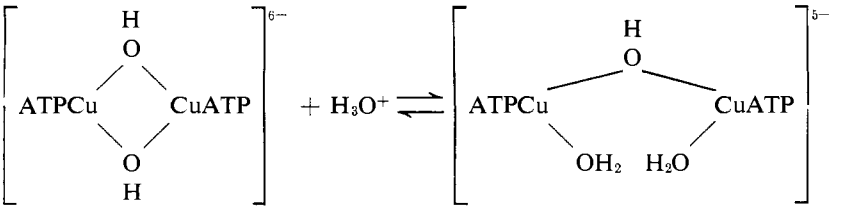

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Determination of Viral Plus and Minus Ribonucleic Acid Strands by an Isotope Dilution Assay*

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ABSTRACT: The determination of radioactive viral plus and minus strands in the presence of labeled host cell ribonucleic acid (RNA) by an isotope dilution method is described. With use of this procedure it is shown that the ratio of viral plus to minus strands is about 8 in cells infected with MS2 phage and 1.5 in cells infected with MU9, an amber mutant of MS2. The method has been used elsewhere to demonstrate that the virus-specific RNA polymerase, Q_β replicase, when primed with Q_β plus strands first synthesizes minus strands and later predominantly plus strands (Weissmann, C., and Feix, G. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1264), but when primed with Q_β minus strands, produces plus strands from the very outset of the reaction (Weissmann, C., Feix, G., Slor, H., and Pollet, R. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1870).

The study of viral RNA synthesis in bacteria infected with RNA phages is complicated by the concomitant synthesis of host cell RNA. Whereas the synthesis of host RNA can be specifically reduced by ultraviolet irradiation (Fenwick *et al.*, 1964) or, if *Escherichia coli* spheroplasts are used, by treatment with actinomycin (Haywood and Sinsheimer, 1963), these procedures give rise to quantitative and even qualitative alterations in viral RNA synthesis (Fenwick *et al.*, 1964; Kelly *et al.*, 1965; Nonoyama and Ikeda, 1964; Haywood and Harris, 1966). Viral RNA synthesis has been studied by infectivity assays (Paranchych, 1963; Engelhardt and Zinder, 1964; Pfeifer *et al.*, 1964; Delius and Hofschneider, 1964) or by measuring the amount of radioactivity recovered in phage particles after labeling the infected host cells for a limited time with RNA precursors (Cooper and Zinder, 1963; Lodish *et al.*, 1965); however, the amount of information obtained by these procedures is limited, since it is not possible, for example, to compare the rate of synthesis of viral plus strands[1] with that of viral minus strands, double-stranded RNA, and host RNA.

The double isotope specific dilution assay described in this paper allows the simultaneous determination of radioactive viral plus and minus strands in the presence of labeled host RNA. In principle, the method is a further elaboration of the specific dilution assay used to identify both DNA (Hoyer *et al.*, 1964) and RNA (Yankofski and Spiegelman, 1963; Weissmann *et al.*, 1964b; Robinson *et al.*, 1964) species or to determine the distribution of radioactivity between the plus and minus strands of virus-specific double-stranded RNA (Weissmann, 1966; Billeter *et al.*, 1966b).

Results

In the following section the specific dilution assay, as applied to the analysis of labeled, double-stranded viral RNA, will be discussed in more detail than has been the case hitherto, because it is the basis for the more elaborate double isotope specific dilution assay.

Specific Dilution Assay. When double-stranded MS2

* From the Department of Biochemistry, New York University School of Medicine, New York, New York. *Received August 4, 1967.* This work was supported by Grants AM-01845, AM-08953, and I-S01-Fr-05399 from the National Institutes of Health, U. S. Public Health Service, the Jane Coffin Childs Fund for Medical Research, and E. I. du Pont de Nemours, Inc.

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[1] The term "plus" strand is used to denote a viral RNA strand of the parental type; "minus" strand, to denote the strand with a base sequence complementary to that of the plus strands; SSC, 0.15 M sodium chloride–0.015 M sodium citrate; $n\times$ SSC, n-fold concentrated SSC; A_{260} unit, quantity of material resulting in an absorbance of 1.0 when dissolved in 1.0 ml and read ta wavelength 260 mμ (1.0-cm light path).