Interactions of Divalent Metal Ions with Inorganic and Nucleoside Phosphates. II. Kinetics of Magnesium(II) with $HP_3O_{10}^{4-}$, ATP, CTP, $HP_2O_7^{3-}$, ADP, and CDP[1]

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Abstract: Kinetic data are reported at 15° for the interaction of Mg^{2+} with six inorganic and nucleoside phosphates: $HP_3O_{10}^{4-}$, ATP, CTP, $HP_2O_7^{3-}$, ADP, and CDP. Complexation of magnesium with tripolyphosphate and pyrophosphate yields kinetic results consistent with a single rate-determining step involving the expulsion of water molecule(s) from the inner hydration shell of the magnesium ion. Reaction with the nucleotides yields the following results. First, the adenine and cytosine nucleotides of a given charge type exhibit the same kinetic behavior with magnesium. Second, the mechanism which quantitatively fits the data for all Mg–nucleotide systems involves the formation of a 1:1 complex (ML) coupled to 2:1 complex (M_2L). Finally, the rate-determining step in the formation of both complexes is the expulsion of water molecule(s) from the inner hydration shell of the magnesium ion.

A number of temperature-jump kinetic investigations of complex formation with various metal ions and ATP,[3,4] ADP,[5] and the corresponding inorganic phosphates ($HP_3O_{10}^{4-}$, $HP_2O_7^{3-}$)[6] have indicated that the mechanism of complexation involves formation of an ion pair (solvent separated) followed by the rate-determining dissociation of one or more water molecules from the inner hydration shell of the metal ion. The rate constants characterizing the dissociation of water molecules from the completely aquated metal ion have been found to be essentially independent of the particular ligand. That is, the kinetics of the interactions of various metal ions with these ligands seemed to be merely a reflection of the charge type of the ligand and the water exchange rate of the particular metal ion.

Up until this time, however, there has been no comparison of the influence of different ring systems (purine *vs.* pyrimidine) on metal complexation kinetics. This paper describes a systematic study of the kinetics of Mg^{2+} with six di- and triphosphates ($HP_3O_{10}^{4-}$, $HP_2O_7^{3-}$, ATP, CTP, ADP, and CDP). By studying these six particular ligands we sought to indentify and systematically characterize the effects of (1) metal–phosphate interactions (the inorganic phosphates); (2) the length of the phosphate backbone and charge (diphosphates *vs.* triphosphates); and (3) the influence of two different ring systems (adenine and cytosine, Figure 1). In addition kinetic experiments were carried out over a wide range of concentrations (10^{-4} to $\sim 10^{-2}$ *M*).

Experimental Section

Materials. Nucleoside phosphates were purchased principally from the Sigma Chemical Co. and were used without further purification. These compounds were stored as solids in a desiccator below 0°. For kinetic experiments, solutions were prepared daily by dissolving carefully weighed amounts of material in 0.1 *M* KNO_3 in a volumetric flask and adding the desired amount of stock metal solution. Tripolyphosphate ($Na_5P_3O_{10}$) was prepared by column fractionation.[7] Contamination by orthophosphate and pyrophosphate was not detectable by thin-layer chromatography.[8] Concentrations were verified by titration with standardized KOH. Pyrophosphate ($Na_4P_2O_7$) was purified by the method of Quimby.[9]

The metal salts KNO_3 and $Mg(NO_3)_2$ were obtained from Fisher Scientific. Stock solutions of $Mg(NO_3)_2$ were standardized volumetrically with EDTA and the indicator Eriochrome Black T. Chlorophenol Red (CPR) and Phenol Red (PR) were used as rapid pH indicators in the kinetic experiments. All solutions were prepared in triply distilled water.

Methods. All kinetic data were obtained at 15° on a temperature jump spectrometer (Messanlagen Studiengesellschaft). Concentration changes following the temperature jump were monitored by transmittance at 500–570 mμ, depending on the indicator used. Prior to a kinetic determination, the temperature jump cell compartment was equilibrated at 10° for at least 2 hr, and the thermostated cell of a pH meter adjusted to 15°. The pH of the experimental solution was adjusted by dropwise addition of solutions of KOH and/or HNO_3. The pH was measured at 15° on a Sargent digital or Beckman pH meter and the solution then transferred to the T-jump cell. After about 30 min, the solution was jumped 5 ± 0.3° by means of a calibrated high voltage discharge (35 kV) and the resultant relaxation trace photographed. At least 20 min were allowed between jumps to ensure thermal equilibrium. The pH of the solution was checked after jumping to ensure that there had been no change. Subsequent solutions were dilutions of a stock metal–ligand solution, rather than the initial solution, to guard against decomposition by repeated electrical discharge. All solutions were free of turbidity. In all cases metal–indicator and ligand–indicator systems were tested independently to be sure that metal–ligand interactions were being measured. Proton transfer reactions of the various ligand and indicator systems were observed at 20–50 μsec, but were not studied.

Treatment of Data. The relaxation times were computed from at least three oscilloscope traces, photographed with a Polaroid camera. All traces were enlarged on graph paper and then plotted on semilog paper to determine the relaxation times. Table I lists the equilibrium constants. Equilibrium concentrations of all species, as well as various concentration functions, were calculated from the constants and overall metal ion and ligand concentrations and pH by means of a Univac 1108 computer.

In the interpretation of data we will have need for the outer-sphere equilibrium constant (K_{os}), which describes the complex or ion pair in which the reacting species are separated by a solvent molecule. The value of K_{os} depends on interacting charge types

(1) A preliminary account of this work was presented at the North East Regional Meeting of the American Chemical Society, Buffalo, New York, Oct 1971.
(2) NIH Career Development Awardee (17834).
(3) H. Diebler, M. Eigen, and G. Hammes, *Z. Naturforsch. B*, 15, 554 (1960).
(4) M. Eigen and G. Hammes, *J. Amer. Chem. Soc.*, 83, 2786 (1961).
(5) M. Eigen and G. Hammes, *ibid.*, 82, 5951 (1960).
(6) G. Hammes and M. Morrell, *ibid.*, 86, 1497 (1964).
(7) R. H. Kolloff, *ASTM Bull.*, No. 237,74 (1959).
(8) K. Randerath and E. Randerath, *J. Chromatogr.*, 16, 111 (1964).
(9) O. T. Quimby, *J. Phys. Chem.*, 58, 603 (1954).

Table I. Equilibrium Constants for Metal Complex and Ligand Ionization Equilibria in 0.1 M KNO_3 at 15° [a]

Ligand	pK_{a_1}	pK_{a_2}	Complex	Log K_{MHL}	Log K_{ML}	Log K_{M_2L} [e]
ATP	4.18	6.57	Mg-ATP	2.18	4.05	1.77
CTP	4.85	6.63	Mg-CTP	2.18	4.03	
$H_5P_3P_{10}$ [b]	5.50	7.93	Mg-$HP_3O_{10}^{4-}$ [c]	4.00	5.75	
ADP	4.05	6.41	Mg-ADP	1.55	3.21	
CDP	4.56	6.38	Mg-CDP	1.60	3.22	
$H_4P_2O_7$ [b]	6.02	8.36	Mg-$HP_2O_7^{3-}$ [d]	3.18	5.37	

[a] C. M. Frey and J. Stuehr, *J. Amer. Chem. Soc.*, **94**, 8898 (1972). [b] pK_a's refer to the last two phosphate ionizations. [c] L ≡ P_3O_{10}. [d] L ≡ P_2O_7. [e] Reference 24.

and statistical corrections, as well as the ionic strength and nature of the reaction medium.[10] At finite ionic strength, the outer-sphere association constant is given by

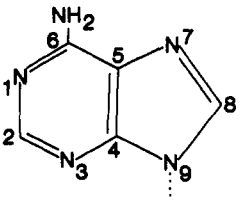
$$K_{os} = \left(\frac{\gamma_M\gamma_L}{\gamma_{ML}}\right)(4\pi Na^3S/3000)\exp(-Z) \qquad (1)$$

where $Z = z_Mz_Le^2/\epsilon akT$, N is Avogadro's number, a is the distance of closest approach of the two ions, S is a statistical factor, and ϵ is the bulk solvent dielectric constant. For the interactions of Mg^{2+} with the triphosphates ($z_Mz_L = -8$), K_{os} at ionic strength 0.1 M may be estimated to be 330 at $a = 5.3$ Å; the corresponding value for the diphosphates is 67. The needed activity coefficients were computed from the extended Debye-Hückel equation. Similar computations can be made for the other charge types.

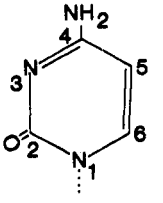
In addition to the medium effect resulting from a finite ionic strength, equilibrium (and rate constants) may be influenced by the choice of supporting electrolyte. The reason is that many supposedly "inert" cations themselves complex with inorganic polyphosphates and the corresponding nucleotides. The cation K^+ is known[11,12] to interact with ATP. The effect of a supporting electrolyte interaction with the ligand under study is to reduce the true free ligand concentration by the amount tied up by the cation of the supporting electrolyte (*i.e.*, as $KATP^{3-}$). The effect this will have on the value of K_{os} is easily shown to be

$$K_{os}' = \frac{K_{os}}{1 + K_{KATP}\bar{K}} \qquad (2)$$

where K_{os}' is the effective equilibrium constant, K_{KATP} is the K-ATP association constant and $\bar{K}$ is the equilibrium potassium concentration (≅0.1 M). The finite ionic strength value of K_{KATP} may be estimated as 31 at $I = 0.1$; the corresponding value for ADP, about 10. As a consequence, the values of K_{os}' relevant to this study are 34 and 80 for di- and triphosphate interactions with divalent metal ions. These values were used in the calculation of k_{H_2O} listed in Table IV.



Adenine



Cytosine

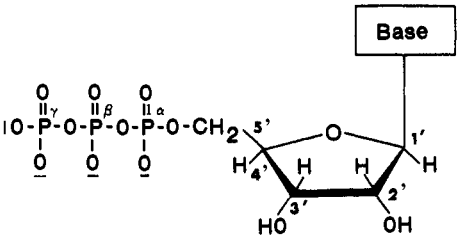


Figure 1. Structure of adenine and cytosine nucleotides.

Table II. Equilibrium Concentrations and Kinetic Data for Mg-ATP at 15°, 0.1 M KNO_3 [a]

$M°$,[b] $M \times 10^2$	$L°$,[b] $M \times 10^2$	pH[c]	$\bar{M}$, $M \times 10^4$	$\overline{ML}$, $M \times 10^4$	$\overline{MHL}$, $M \times 10^4$	$\overline{M_2L}$, $M \times 10^4$	$\bar{L}$, $M \times 10^4$	$\overline{HL}$, $M \times 10^4$	$1/\tau$, sec^{-1} Expt[d]	$1/\tau$, sec^{-1} Calcd
1.00	0.996	7.64	7.59	84.7	0.117	3.79	9.96	1.02	9,945	9,808
0.686	0.684	7.41	6.77	57.1	0.134	2.28	7.54	1.31	9,934	9,388
0.500	0.498	7.44	5.94	41.1	0.090	1.44	6.18	1.00	8,878	8,897
0.300	0.299	7.50	4.67	23.9	0.046	0.660	4.58	0.649	8,379	7,847
0.200	0.199	7.15	4.09	15.1	0.065	0.365	3.30	1.05	6,540	6,849
0.100	0.100	7.08	2.85	6.89	0.035	0.116	2.16	0.805	4,810	5,042
0.060	0.060	7.12	2.10	3.78	0.0173	0.047	1.61	0.546	4,100	3,950

[a] Kinetic data obtained with 4-8 × 10^{-5} M Phenol Red indicator. [b] Overall concentrations. [c] a_H values converted to C_H by $\gamma_H = 0.83$. [d] ±5% deviation in the average of at least three traces.

Results

The kinetics of magnesium interactions with $HP_3O_{10}^{4-}$, CTP, ATP, $HP_2O_7^{3-}$, CDP, and ADP were studied at 15° and 0.1 M KNO_3 at metal-ligand ratios of approximately 1:1. Tripolyphosphate and pyrophosphate were run at pH values ≤6 so that the complexes formed involved the $HP_3O_{10}^{4-}$ and $HP_2O_7^{3-}$ species. The terminal hydrogen therefore substitutes for the ribose linkage present in the nucleotides; as a consequence, the charges on the inorganic phosphates were the same as those of the corresponding nucleotides. Tabulation of concentration data, equilibrium concentrations, and relaxation times for MgATP is given in Table II. Table III contains just the initial concentration data and relaxation times for the remaining five systems. The measured relaxation times are estimated to be reliable to ±5%. The concentration ranges studied were typically 5 × 10^{-4}–10^{-2} M for the nucleotides. The upper limit was lower for the inorganic phosphate systems due to solubility problems.

Kinetic data for the triphosphates in terms of $1/\tau$ *vs.* the appropriate function of concentration, $f(C)$, for an association reaction are displayed in Figures 2 and 3. The analogous diphosphate systems exhibit the same morphology as the data for the triphosphates (*i.e.*, $MgHP_2O_7$ is linear over the entire concentration range and MgCDP and MgADP deviate from linearity at high concentrations). A summary of observed slopes and intercepts for all systems is given in Table IV.

(10) M. Eigen, *Z. Phys. Chem. (Frankfurt am Main)*, **1**, 176 (1954); R. Fuoss, *J. Amer. Chem. Soc.*, 80, 5059 (1958).
(11) R. Phillips, *Chem. Rev.*, **66**, 501 (1966).
(12) M. S. Mohan and G. A. Rechnitz, *J. Amer. Chem. Soc.*, **92**, 5839 (1970).

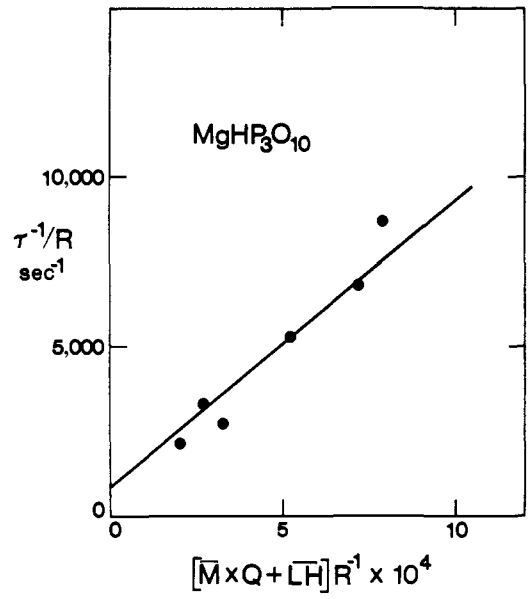


Figure 2. Concentration dependence of τ^{-1}/R for $MgHP_3O_{10}$.

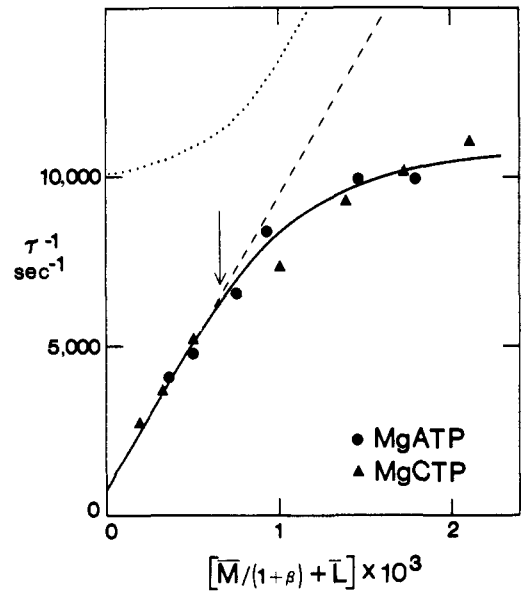


Figure 3. Concentration dependence of the reciprocal relaxation time for MgCTP and MgATP. Dotted line refers to τ_+ root, solid line to τ_- root. Limiting slope is indicated by the dashed line. Arrow indicates highest concentration limit of early T-jump data for MgATP (ref 3 and 4).

Two of the six systems[13] for which we here present data over a wide concentration range have been studied previously in more dilute solutions: MgADP[5] and MgATP.[3,4] Where comparisons can be made, our low concentration results (limiting slopes as $f(C) \rightarrow 0$, and intercepts) are in good agreement with earlier work (see Table IV).

Distinctly different behavior is found for the nucleotides at high concentrations as compared to the inorganic phosphates. Since the latter represent the simplest type of interaction possible with the nucleotides, 1:1 phosphate complexes alone, their kinetic behavior will be described first.

(13) The interaction of Mg^{2+} with tripolyphosphate has been studied by Hague and Eigen (*Trans. Faraday Soc.*, **62**, 1236 (1966)) at high pH's where the complex is MgP_3O_{10} (*i.e.*, a +2, −5 charge interaction).

Table III. Kinetic Data[a] at 15°, 0.1 *M* KNO_3

M°,[b] $M \times 10^2$	L°,[b] $M \times 10^2$	pH[c]	$1/\tau$, sec^{-1} Expt[d]	$1/\tau$, sec^{-1} Calcd
		1. MgCTP		
1.00	0.981	6.40	11,102	10,798
0.700	0.686	6.32	10,127	10,344
0.500	0.490	6.38	9,284	9,570
0.300	0.294	6.45	7,290	8,403
0.101	0.099	6.62	5,250	5,164
0.100	0.098	6.50	5,300	5,160
0.053	0.050	6.44	3,688	3,684
0.025	0.025	6.51	2,762	2,483
		2. $MgHP_3O_{10}$		
0.500	0.533	5.72	11,000	9,574
0.400	0.426	5.75	8,432	8,625
0.200	0.213	5.81	6,317	6,267
0.075	0.080	5.91	3,005	3,995
0.050	0.052	5.95	3,500	3,298
0.025	0.027	6.03	2,045	2,402
		3. MgADP		
1.00	1.04	6.55	12,070	11,800
0.700	0.728	6.24	12,600	11,711
0.490	0.510	6.39	10,266	11,503
0.343	0.357	6.48	9,962	10,808
0.240	0.250	6.64	8,338	9,162
0.100	0.112	6.23	5,782	5,885
0.050	0.056	6.36	5,479	4,428
		4. MgCDP		
1.00	1.14	6.16	11,836	10,773
0.700	0.796	6.26	10,250	10,546
0.490	0.577	6.34	10,021	10,361
0.196	0.220	6.10	7,211	8,021
0.098	0.110	6.22	6,855	5,753
0.059	0.066	6.28	5,646	4,626
0.019	0.022	6.40	3,241	3,198
		5. $MgHP_2O_7$		
0.505	0.547	6.12	11,800	11,212
0.404	0.438	6.16	10,147	10,163
0.303	0.328	6.18	8,451	9,010
0.202	0.215	5.94	7,522	7,960
0.101	0.107	5.92	6,237	5,923
0.081	0.086	6.12	5,544	5,263
0.061	0.064	6.01	4,500	4,702

[a] Kinetic data obtained with 4–8 × 10^{-5} *M* Chlorophenol Red indicator. [b] Overall concentrations. [c] a_H values converted to C_H by $\gamma_H = 0.83$. [d] ±5% deviation in the average of at least three traces.

Inorganic Phosphates. The mechanism consistent with the data for the complexation of Mg^{2+} with $HP_2O_7^{3-}$ and $HP_3O_{10}^{4-}$ is the following

$$\begin{array}{ccc} & H_2L & \\ & \uparrow\downarrow K_a & \\ M + HL & \rightleftharpoons & MLH \\ & & \uparrow\downarrow K_M \\ & & ML + H^+ \end{array} \qquad (A)$$

$$HIn \overset{K_{In}}{\rightleftharpoons} H^+ + In^-$$

where all proton transfer reactions, including that of the indicator HIn, are taken as rapid preequilibria. This is the same mechanism that Hammes and Morrell[6] found for the complexation of these phosphates with Ni^{2+} and Co^{2+} The relaxation time for mechanism A is given by

$$\tau^{-1} = k_{1f}(\bar{M}Q + \bar{H}\bar{L}) + k_{1r}R \qquad (3)$$

Table IV. Summary of Kinetic Data for Interaction of Magnesium with Di- and Triphosphates at 15°, 1 *M* KNO_3 (Previously Determined Values in Parentheses)

	Diphosphates			Triphosphates		
	MgADP	MgCDP	$MgHP_2O_7$	MgATP	MgCTP	$MgHP_3O_{10}$
k_{1f}, M^{-1} sec^{-1}	3.8×10^6 (3×10^6)[a]	3.8×10^6	3.85×10^6	8.7×10^7 (1.2×10^7)[b]	8.7×10^6	8.5×10^6
k_{1r}, sec^{-1}	2.3×10^3 (2.5×10^3)[a]	2.3×10^3	2.55×10^3	7.8×10^2 (1.2×10^3)[b]	7.8×10^2	8.5×10^2
K_{os}', M^{-1}	34	34	34	80	80	80
k_{H_2O}, sec^{-1} (calcd)[c]	1.1×10^5	1.1×10^5	1.1×10^5	1.1×10^5	1.1×10^5	1.1×10^5
K_{M_2L} (calcd)	9	9		59	59	
k_{2f}, M^{-1} sec^{-1}	1×10^5	1×10^5		6×10^5	6×10^5	
k_{2r}, sec^{-1}	1×10^4	1×10^4		1×10^4	1×10^4	
K_{os}', M^{-1}	2.0	2.0		8.7	8.7	
k_{H_2O}, sec^{-1} (calcd)[c]	1.1×10^5	1.1×10^5		1.4×10^5	1.4×10^5	

[a] Data taken at 25°, 0.1 *M* KNO_3, ref 3 and 4. [b] Data taken at 25°, 0.1 *M* KNO_3, ref 5. [c] k_{H_2O} (exp) $\sim 10^5$, ref 26.

where

$$Q = \frac{K_a\overline{ML} + (K_M + \bar{H})K_a(1 + \alpha) + \overline{LH}(2K_M + \bar{H})}{\overline{ML}(K_a + \bar{H}) + (K_M + \bar{H})[(K_a + \bar{H})(1 + \alpha) + \overline{HL}]}$$

$$R = \frac{\overline{ML}(K_a + 2\bar{H}) + \bar{H}[(K_a + \bar{H})(1 + \alpha) + \overline{HL}]}{\overline{ML}(K_a + \bar{H}) + (K_M + \bar{H})[(K_a + \bar{H})(1 + \alpha) + \overline{HL}]}$$

$$\alpha = \overline{In}/(K_{In} + \bar{H})$$

and the bars indicate equilibrium concentrations. The correction factor R results from the rapid proton transfer reaction involving MLH, and was close to unity for all systems studied. Equation 3 indicates that a graph of τ^{-1}/R *vs.* $(\overline{M}Q + \overline{HL})/R$ should give a straight line of slope and intercept k_{1f} and k_{1r}, respectively. The corresponding graph is shown in Figure 2 for the Mg–HP_3O_{10} system. The rate constants are summarized in Table IV.

Nucleotides. Analogous graphs of τ^{-1} *vs.* the appropriate concentration function for MgATP and MgCTP interactions are given in Figure 3. The low concentration data are linear, with slopes and intercepts identical with those for the corresponding inorganic phosphate (see Table IV). This is true for both the cytidine and adenosine nucleotides. At relatively high concentrations ($f(C) > 5 \times 10^{-4}$ *M*), however, the graphs for the nucleotide systems deviate substantially from linearity. It is thus apparent that (1) there are additional interactions with the metal ion when a nucleoside is present; (2) these interactions do not distinguish between an adenosine or cytosine moiety; (3) no effect is seen on the values of the complexation and dissociation rate constants, which are identical to those for the corresponding inorganic phosphates. Any postulated mechanism must be consistent with these observations.

An interpretation of the kinetic results must take into account all species which are known to exist in aqueous solution. The nucleotide itself can exist in various protonated states (L, HL, H_2L)[14] and conformational states (syn and anti).[15] The purines can readily adopt both syn and anti conformations whereas the pyrimidines exist predominantly in the anti form. It is also possible for the free nucleotide to self-associate or base-stack,[16] especially in concentrated solutions. There are a number of metal–nucleotide complexes possible: (1) outer- and inner-sphere complexes with the phosphate portion of the ligand (ML) as well as the protonated ligand (MHL);[14] (2) interaction of some metal ions with sites on the ring as well as the phosphates;[14] and (3) higher order complexes (*e.g.*, M_2L) due to the availability of multiple binding sites.[17,18]

Detailed consideration of all these facts leads to the conclusion that there might be a number of mechanistic possibilities which could explain the deviations from linearity seen in the metal–nucleotide systems. Several, however, could be immediately discarded.

First, we tested for the contributions of a kinetic pathway involving the protonated ligand *via* MHL. Analysis in detail of such mechanisms by the Castellan[19] determinantal technique clearly showed that they made negligible contributions at the pH's and concentrations employed for all systems. This is in agreement with all previous investigators, who concluded that above pH ~6, the MHL species could be neglected kinetically for metal–nucleotide systems.[3,4]

Second, the effect of base stacking of the nucleotides alone as a rapid preequilibrium was considered (2L = L_2). The inclusion of this preequilibrium with any reasonable value of the stacking equilibrium constant ($K \sim 5$–10 M^{-1})[20] had virtually no effect on the data displayed in Figure 3.

Third, the effect of the existence of two different forms of the nucleotide, *i.e.*, syn and anti,[15] was tested. Such a preequilibrium (L = L′) would change the observed forward rate constant for the magnesium–nucleotide systems, but not the functional dependence (*i.e.*, curvature at high concentrations).

It should be pointed out here that the Eigen–Tamm mechanism itself (C) (see Discussion) predicts deviations from linearity at sufficiently high concentrations. The relaxation time for (C) is given by

$$\tau^{-1} = \frac{k_{H_2O}K_{os}'(\bar{M} + \bar{L})}{1 + K_{os}'(\bar{M} + \bar{L})} + k_{1r} \qquad (4)$$

(14) R. Izatt, J. Christensen, and J. Rytting, *Chem. Rev.*, 71, 439 (1971), and references therein.

(15) L. Rhodes and P. Schimmel, *Biochemistry*, 10, 4426 (1971), and references therein.

(16) M. Schweizer, A. Broom, P. O. P. Ts'o, and D. Hollis, *J. Amer. Chem. Soc.*, 90, 1042 (1968), and references therein.

(17) C. Liebecq and M. Jacquemotte-Louis, *Bull. Soc. Chim. Biol.*, 40, 67 (1958).

(18) M. Mohan and G. Rechnitz, *J. Amer. Chem. Soc.*, 94, 1714 (1972).

(19) G. Castellan, *Ber. Bunsenges. Phys. Chem.*, 67, 898 (1963).

(20) A. Broom, M. Schweizer, and P. O. P. Ts'o, *J. Amer. Chem. Soc.*, 89, 3612 (1967).

With the outer-sphere equilibrium constants applicable to the present work, the denominator in eq 4 is never larger than about 1.1 for the triphosphates or 1.04 for the diphosphates. Thus, unless K_{os}' values were more than an order of magnitude larger than estimated for both diphosphates and triphosphates, eq 4 cannot account for the curvature in Figure 3. Increasing K_{os}' by an order of magnitude however would yield anomalously low values of k_{H_2O} for all systems. Finally, no such curvature is found in the inorganic phosphates, which have the same charge as the corresponding nucleotides. We conclude therefore that the denominator in eq 4 cannot account for the observed curvature in the nucleotide systems.

Another possibility is that the concentration functions were in error because of significant amounts of KOH that were needed to adjust the pH at the higher nucleotide concentrations. This will affect the total potassium concentration (and hence the amount of $K\text{-}ATP^{3-}$) as well as the ionic strength. This was tested by an interative computer program which explicitly took into account the increased concentrations of K^+ and the accompanying ionic strength dependence of the rate and equilibrium constants. Only an approximate treatment was possible because the variation of activity coefficients with ionic strength for high charge types is difficult to assess. Nevertheless, we were able to show that if there were any changes in the values of $\bar{M}/(1 + \beta) + \bar{L}$, they were shifted slightly to *higher* values. We concluded therefore that the curvatures observed at high concentrations are not due to medium effects of this type.

On the other hand, there is now considerable evidence that both Ca^{2+} and Mg^{2+} form M_2L complexes with ATP. The species Mg_2ATP has been implicated in certain enzymatic transphosphorylation reactions. Kuby, Noda, and Lardy[21] found that the initial velocity of the forward reaction catalyzed by creatine kinase is dependent on the ratio Mg_t/ATP_t. Maximum relative initial velocity is reached when the ratio is about unity and decreases asymptotically to about 75% of the maximum when the ratio is increased above unity. The authors interpreted this result to indicate that the species Mg_2ATP is forming at high Mg_t/ATP_t ratios and that it is less reactive than the normal substrate MgATP. The same authors,[22] in a study of the equilibrium constant of the creatine kinase reaction, concluded that Mg_2ATP is necessary to account for the magnesium dependence of the apparent equilibrium constant.

More recently, Noat, *et al.*,[23] observed that high Mg^{2+} concentrations have an inhibitory effect on the forward reaction of yeast hexokinase. Since the free Mg^{2+} ion has no affinity for the enzyme, they concluded that the inhibition could only be accounted for by the formation of an inactive Mg_2ATP complex. From their kinetic data the authors estimated the value of the stability constant K_{M_2L} to be about 40 M^{-1} at $I = 0.1$ M. Rechnitz and coworkers[24] recently determined the value of K_{M_2L} by a direct potentiometric method using divalent specific ion electrodes. They obtained a value of $K_{M_2L} = 405$ M^{-1} for the Mg_2ATP system at 25° and zero ionic strength.

We found that the addition of the M_2L complex formation to the normal complexation mechanism resulted in a quantitative fit to our kinetic data. We used the extended Debye–Hückel equation to adjust Rechnitz' value of K_{M_2L} to 59 M^{-1} at 0.1 M ionic strength. The data for metal ion–nucleotide complexation are completely consistent with a two-step mechanism involving the formation of a 1:1 Mg–nucleotide complex coupled to a 2:1 complex as follows.

$$\begin{array}{ccccc} & & H & & \\ & & + \uparrow k_{1f} & & \\ 2M + L & \rightleftharpoons & ML + M & \overset{k_{2f}}{\rightleftharpoons} & M_2L \\ & & \uparrow\downarrow K_a & & \\ & & HL & & \end{array} \qquad \text{(B)}$$

$$HIn \overset{K_{1n}}{\rightleftharpoons} In + H$$

The two slow relaxation times for (B) are given by the following determinental equation

$$\begin{vmatrix} a_{11} - \dfrac{1}{\tau} & a_{12} \\ a_{21} & a_{22} - \dfrac{1}{\tau} \end{vmatrix} = 0 \qquad (5)$$

where

$$a_{11} = -\left[k_{1f}\left(\frac{\bar{M}}{1+\beta} + \bar{L}\right) + k_{1r}\right]$$

$$a_{12} = (k_{1f}\bar{L} - k_{1r})/(1 + \beta)$$

$$a_{21} = k_{2f}(1 + \beta)(\overline{ML} - \bar{M})$$

$$a_{22} = -[k_{2f}(\overline{ML} + \bar{M}) + k_{r2}]$$

$$\beta = \frac{\bar{H}}{K_a + \bar{L}/(1 + \alpha)}$$

and

$$\frac{1}{\tau_\pm} = -{}^1/_2[(a_{11} + a_{22}) \pm \sqrt{(a_{11} + a_{22})^2 - 4(a_{11}a_{22} - a_{12}a_{21})}] \qquad (6)$$

The two relaxation times have the following properties. The positive root (τ_+^{-1}) begins at a rather high value, and curves upward (see dotted line in Figure 3). The negative root (τ_-^{-1}) at low concentrations has the form

$$\frac{1}{\tau_-} = k_{1f}\left(\frac{\bar{M}}{1+\beta} + \bar{L}\right) + k_{1r} \qquad (7)$$

That is, it varies linearly with the concentration function for a simple complexation reaction. At high concentrations, the predicted behavior of τ_-^{-1} is to deviate downward from the linear initial slope, eventually transferring to a smaller slope corresponding to the concentration function for a_{22}. Since the value of K_{M_2L} is available experimentally, we were able to carry out a rigorous analysis for the MgATP system. The solid curve in Figure 3 is the predicted behavior of τ_-^{-1} with $\bar{M}/(1 + \beta) + \bar{L}$ for mechanism B. The values of k_{1f} and k_{1r} were obtained from the linear initial portion of the curve. These values, as stated

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earlier, are the same as for the inorganic tripolyphosphate system. Since K_{M_2L} is estimated to be 59 M^{-1} at $I = 0.1$, the rate constant k_{2f} can be obtained as the only variable in the high concentration region of Figure 3. The result is $k_{1f} = 8.7 \times 10^6$ and $k_{2f} = 6 \times 10^5$ M^{-1} sec^{-1}. The last column in Table II shows the relaxation times calculated for this mechanism as compared to the experimental values.

The interpretation of the MgCTP system was analogous. The values for k_{1f} and k_{1r} were obtained from the linear portion of the curve. Since k_{2f} for the Mg-ATP system appeared to be the "normal" constant for the Eigen–Tamm mechanism (see Discussion) for the charge type involved, k_{2f} was set equal to 6×10^5 M^{-1} sec^{-1} for the MgCTP system as well. The value of K_{M_2L} was adjusted until a fit was obtained to the data. The value of k_{2r} was obtained from the relationship $k_{2r} = k_{2f}/K_{M_2L}$. The value obtained for K_{M_2L} for MgCTP was identical to that for Mg–ATP within experimental error. Figure 3 shows that the two systems indeed superimpose.

The diphosphate nucleotides were analyzed in the same manner as Mg–CTP. The values of k_{2f} were computed on the basis of electrostatic considerations to be 1×10^5 M^{-1} sec^{-1} and the values of K_{M_2L} adjusted until a fit was obtained. Table IV shows that the values of the rate constants and K_{M_2L} are also identical for the two Mg^{2+} diphosphate systems.

A kinetic study of the MgATP system has also been carried out by Mg^{25} nmr spectroscopy. By studying the line broadening of the Mg^{25} resonance as a function of temperature, Bryant[25] was able to obtain an exchange rate of 2×10^4 sec^{-1} at pH 7.9 and ionic strength 4.5 (1.5 M $MgCl_2$, 2×10^{-2} M ATP) and 25°, which he attributed to the dissociation of the MgATP (1:1) complex. Bryant noted that this dissociation rate constant was a factor of 10 larger than the value of 1.3×10^3 sec^{-1} obtained in an earlier[3,4] T-jump study at $I = 0.1$. He attributed the difference to differences in ionic strength and pH between the two investigations. The dissociation of MgATP however is a (pseudo-) first-order process and consequently should not be so strongly ionic strength sensitive as would be required, nor should the pH be a factor at all if a true rate constant is involved. A far more likely explanation is that Bryant was observing the dissociation of the *Mg_2ATP* species. Under his experimental conditions (large excess of Mg^{2+}), one may show that the predominant complexed species is in fact Mg_2ATP, constituting about 98% of all the complexes in the system. Our own kinetic study shows the dissociation rate constant to be $k_{2r} = 1.0 \times 10^4$ sec^{-1} at 15°. A value of 2×10^4 sec^{-1} at 25° is completely consistent with this result.

Discussion

The usual formulation of the Eigen–Tamm mechanism for complex formation *via* an outer-sphere complex (MWL) may be represented by the following reaction[26]

$$M + L \overset{K_{os}'}{=\!=} MWL \overset{k_{H_2O}}{\rightleftharpoons} ML + H_2O \qquad (C)$$

where K_{os}' is the outer-sphere complex association constant (in the medium of 0.1 M KNO_3) and k_{H_2O} is the solvent exchange rate constant. The latter is related to the overall measured forward rate constant by $k_f = K_{os}'k_{H_2O}$.

Low-Concentration Data. Graphs of $1/\tau$ *vs.* the appropriate concentration functions are shown for $MgHP_3O_{10}$, MgATP, and MgCTP in Figures 2 and 3. The earliest kinetic studies of MgATP were carried out in the *linear* low-concentration region;[3,4] the maximum concentration in previous work is indicated by a small arrow in Figure 3. The same is true for the previous kinetic data for MgADP.[5]

As indicated in the Results Section, the data for $MgHP_3O_{10}$ and $MgHP_2O_7$ are consistent with a mechanism involving the complexed species MHL and ML. The forward rate constants (k_{1f}, Table IV) reflect essentially the increasing value of K_{os}' with increasing charge. Values of $k_{H_2O} \sim 10^5$ sec^{-1} are compatible with previously measured values for the water exchange rate of magnesium.[27]

The low concentration data for MgATP, MgCTP, MgADP, and MgCDP are consistent with the reaction scheme $M + L \rightleftarrows ML$ for which $1/\tau = K_{os}'k_{H_2O}f(C) + k_{1r}$, and $f(C) = \bar{M}/(1 + \beta) + \bar{L}$. This is identical to the treatment of the earliest studies of magnesium complexation with the adenine nucleotides.[3-5] Values of k_{1f} (obtained from the low concentration slope) and k_{1r} (intercept) are given in Table IV. Rate constants agree fairly well with those of previous investigations, particularly when differences in temperature are taken into account. Values of k_{1f} once again reflect only the charge of the ligand and do not show any influence of the particular nucleoside (*i.e.*, ATP *vs.* CTP) or, for that matter, the presence or absence of the entire nucleoside moiety (*i.e.*, $HP_3O_{10}^{4-}$ *vs.* ATP).

Unlike k_{1f}, k_{1r} is not a function of charge, and therefore should be indicative of the strength of the complex formed. For a given charge type, the values of k_{1r} are identical, indicating that the nucleoside moiety imparts *little*, if any, additional kinetic stability to the 1:1 complex (ML) over that found in just metal–phosphate interactions (*i.e.*, $MgHP_3O_{10}$ and $MgHP_2O_7$). As with the forward rate constant, the reverse rate constant is also independent of the nature of the nucleoside.

As might be expected, the dissociation rate constants (k_{1r}) decrease with increasing phosphate chain length (*i.e.*, three possible coordination sites *vs.* two). The concept of three coordination sites with magnesium and ATP is, however, contrary to nmr ^{31}P data[28] which indicate that magnesium binds to only two phosphate oxygens (β and γ) in ATP and is therefore identical in number of binding sites to MgADP where the terminal phosphate is, of course, absent. Our results, which indicate that MgADP is nearly three times more labile than MgATP (k_{1r}(MgADP) $\cong 3k_{1r}$ (MgATP)), do not support the conclusions from nmr but rather argue that the additional phosphate group in ATP has some effect on the dissociative stability of the magnesium complex.

High-Concentration Data. The most unusual feature of the kinetic data at high concentrations is that the data for all *nucleotide* systems become virtually concentration independent at sufficiently high concentrations. Discern-

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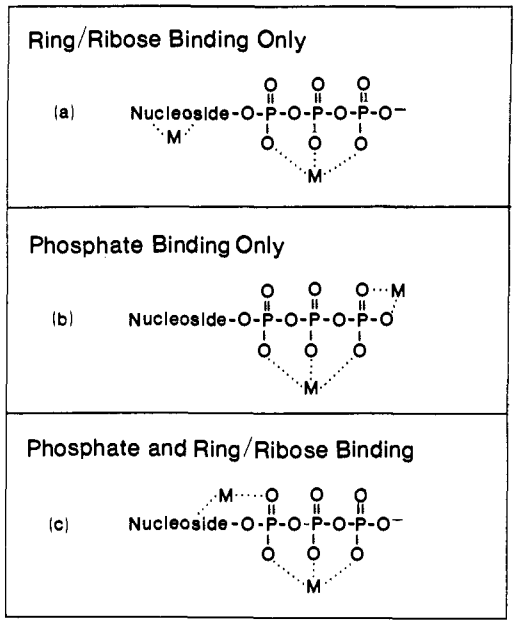

Figure 4. Possible structures of the M_2L complex.

ible deviations from linearity begin to occur at $f(C)$ values $\sim 5 \times 10^{-4}$ in all four magnesium–nucleotide systems. As discussed in the Results Section, this deviation from linearity could be quantitatively accounted for by the inclusion of the species M_2L in the reaction scheme. The rate constants k_{2f} listed in Table IV are for the formation of M_2L. As with k_{1f}, the values of k_{2f} reflect only the charge on the ligand (ML) and the incoming metal ion (M). The rate of formation of M_2L is also insensitive to the particular ring system present (adenine *vs.* cytosine). Calculated values for k_{H_2O} are on the order of 10^5 sec^{-1} when a steric factor S of ~ 0.5 is employed.

For the dissociation rate constant k_{2r}, however, all *four* systems have values $\sim 10^4$ sec^{-1}, that is, independent of the number of phosphates. This suggests that the complex may be similar in both di- and triphosphates and with either nucleoside. Several possibilities can be considered for the structure of the M_2L species (see Figure 4). (1) Involvement with just a ring or ribose position (Figure 4a). Since species M_2L is not detectable in a pH titration of magnesium plus adenosine[29] or magnesium plus cytidine,[30] it seems unlikely that the magnesium is binding at a protonated site (*i.e.*, N_1 in adenosine and N_3 in cytidine). This leaves only the NH_2 group identical in both structures, but it is seldom implicated in the binding of metal ions.[14] The additional possibility remains that M_2L involves N_3 in the adenine nucleotides and C=O in the cytidine nucleotides. An alternate possibility is that magnesium is chelating to only the ribose portion of the nucleotide; this, of course, would be identical in both structures. (2) Involvement with only the phosphate backbone (Figure 4b). (3) Involvment with the phosphate backbone and a ring or ribose position (Figure 4c).

Unfortunately it is not possible to unambiguously distinguish between structures b and c. They are both consistent with a phosphate-length independent value of k_{2r}. Our data would appear to support either (a) or (c) for the reason that we did not see any kinetic evidence for the presence of M_2L with the *protonated* inorganic phosphates (the completely ionized inorganic phosphates however are believed to form such complexes).[31,32] Even though our kinetic results in the protonated inorganic phosphates are not analogous to the results in the nucleotide systems (*cf.* Figures 2 and 3), we cannot exclude the presence of M_2L in the former. Further kinetic studies with ribose di- or triphosphate would be helpful in distinguishing among the various possibilities. Unfortunately these ligands are not readily available, nor are they stable to hydrolysis.[33] Additional studies under way in this laboratory with methyl triphosphate (both thermodynamic and kinetic) are aimed at clarifying these questions.

It is interesting to speculate on the possible regulatory role of the Mg_2ATP complex. Blair[34] points out that many magnesium-requiring enzymes have values for the Michaelis constant which are sensitive to magnesium concentrations in the physiological range, suggesting that magnesium may be of importance in metabolic control. Consider a closed system containing the following transphosphorylation reaction

$$ATP + X \overset{Mg}{\rightleftharpoons} ADP + X\text{-}P \qquad (D)$$

where $Mg_t \leq (ATP_t + ADP_t + X\text{-}P_t)$. Blair's computations for the adenylate kinase system have shown that, if K_{ML} varies in the order ATP > ADP $\gg$ X–P, the magnesium present will mostly be in the reactive complex MgATP if the equilibrium in reaction D is shifted far to the left. Shifting the equilibrium far to the right results in a decrease in ATP_t and a substantial increase in free magnesium concentration.

Under the conditions of decreasing ATP_t and increasing free magnesium the concentration of Mg_2ATP will rise relative to that of MgATP. If Mg_2ATP is not reactive, the rate of the forward reaction in reaction D will decrease, tending to shift the equilibrium back to the left. The analogous argument holds for ADP but since its K_{M_2L} value is considerably smaller than that for ATP, the effects would be weaker. It therefore appears possible that M_2L type complexes may play a role in the regulation of nucleotide metabolism.

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