cytosine base pairs would also have different dimensions, and these differences might result in considerable distortions in the secondary structure of nucleic acids containing thioguanine.[5] Structural distortions caused by the substitution of thioguanine for guanine might be of sufficient magnitude to disrupt the normal biological functioning of nucleic acids, and to account for the antimetabolite activity of thioguanine. The thio bases of tRNA might also disrupt the regularity of hydrogen-bonded regions by affecting hydrogen bond lengths, thus contributing to the control of tRNA conformation.

Interactions of Divalent Metal Ions with Inorganic and Nucleoside Phosphates. I. Thermodynamics

Cheryl Miller Frey and John E. Stuehr*

Contribution from the Department of Chemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received October 19, 1971*

Abstract: Thermodynamic data are reported for 11 nucleotide and phosphate systems: orthophosphate, pyrophosphate, and tripolyphosphate; ribose phosphate; adenosine 5′-mono-, di-, and triphosphates; adenosine 2′-monophosphate; cytidine 5′-mono-, di-, and triphosphates; pK_a values of the free acids and stability constants with Ni(II) and Mg(II) are reported at 15° and $I = 0.1$. Magnesium behaves similarly with all systems containing the same number of phosphates, indicating at the most a very weak interaction between Mg(II) and the nucleotide ring. For nickel, a number of differences are noted, including ring specificity and the appearance of 1:2 as well as 1:1 complexes in some instances. These results are interpreted on the basis of various structural possibilities.

Due in part to the role of nucleotides and their metal ion complexes as substrates in enzyme-catalyzed reactions, considerable interest in recent years has focused upon the stability of metal ion complexes formed with the adenine nucleotides.[1-11] Unfortunately, various investigations have been carried out under differing conditions of temperature, ionic strength, and supporting electrolyte. As a consequence, it is often difficult to make detailed comparisons of the results from different investigations. In addition, nucleotides other than the adenine series are only sparcely represented in the literature.[12,13]

We report here pK_a values and stability constants for nickel and magnesium for a series of inorganic phosphates, adenine nucleotides, and cytosine nucleotides in 0.1 *M* KNO_3 at 15°. The metal ions Mg(II) and Ni(II) were chosen for study because they might be expected to behave differently with respect to ring binding.[14-16] The purpose of this research was to study the influence of the following parameters on the thermodynamic behavior: (1) length of phosphate backbone; (2) base composition, adenine series *vs.* cytosine series; (3) nucleoside phosphates *vs.* inorganic phosphates; (4) Ni(II) *vs.* Mg(II). The supporting electrolyte was chosen primarily to have results to compare with the large body of data already available for the adenine nucleotides. In addition, titrations were carried out at several different metal:ligand ratios.

Critical to an analysis of potentiometric data is a consideration of the possible existence of various species, including not only those of the ligand, but also the metal–ligand complex. The formation of ML and MHL (protonation on the phosphate)[14] is well known in the adenine nucleotides and the inorganic phosphates.[17,18] In addition, there is considerable evidence for self-association, or base stacking (*i.e.*, L_2 formation), in many nucleotides and nucleosides.[19-25] Recently there has also been speculation about the formation of M_2L_2 and ML_2 complexes involving base-stacked ligands in the adenine nucleotides.[26,27] We have focused our attention on the possibility of these species in the analysis of our potentiometric data.

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Experimental Section

Materials. Nucleoside phosphates were purchased principally from the Sigma Chemical Co. and were used without further purification (quoted purities >98%). These compounds were stored as solids in a desiccator below 0°. The free acid form of the ligand was used for potentiometric titrations. Where only the sodium salt was available (ribose phosphate, CDP, CTP, and ATP), the compounds were quantitatively converted to the free acid form by column chromatography on Dowex 50-X2-400 (hydrogen form) resin. Titration of the columned sample was performed the same day. The diphosphates are known to decompose 3–6%/month even when stored frozen We routinely checked the diphosphates for decomposition by thin-layer chromatography[28] and rejected lot numbers that had undergone observable hydrolysis. In addition, ADP was stored as the barium salt.

Orthophosphate solutions were prepared by dilution of Fisher Certified H_3PO_4 and concentrations checked by titration. Fisher Certified sodium pyrophosphate ($Na_4P_2O_7 \cdot 10H_2O$) was purified by the method of Quimby[29] to remove other phosphate impurities. Ethyl alcohol was added to an aqueous solution of 12–15% phosphate and stirred for 30 min, then washed and filtered several times with a 1:1 mixture of EtOH–H_2O. The damp crystals were redissolved and the above procedure was repeated at least five times. Conversion to the free acid form was again achieved by Dowex 50-X2-400 column chromatography.

Tripolyphosphate ($Na_5P_3O_{10}$) was prepared by column fractionation.[30] Contamination by orthophosphate and pyrophosphate was not visible when checked for by thin-layer chromatography. The purified $Na_5P_3O_{10}$ was then columned on Dowex 50-X2-400 and stock solutions of the free acid were obtained.

The metal salts KNO_3, $Ni(NO_3)_2$, 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. Stock solutions of $Ni(NO_3)_2$ were standardized with EDTA and salicylic acid with $NaNO_2$.

Titrations were carried out with 0.1 *N* KOH (Fisher Certified) standardized with potassium hydrogen phthalate. All solutions were prepared in deionized water (Stokes).

Methods. Determination of pK_a values and stability constants was carried out by potentiometric titration in a 15-ml jacketed-cell thermostated at 15 ± 0.05°. The pH combination electrode (Thomas 4858-L-60) was previously equilibrated at the same temperature. The titration solutions were mechanically stirred and nitrogen was bubbled through to exclude carbon dioxide. A pH meter precise to ±0.002 (Beckman Research) was standardized at three pH values (4, 7, and 10) by means of Sargent buffers or buffers prepared from National Bureau of Standards chemicals.

Additions of KOH and stock metal ion solutions were made with a 2-ml Gilmont microburet. Ionic strength was maintained at 0.1 *M* with KNO_3. The concentration of ligand ranged from 2 to 5×10^{-3} *M*. All ligands were in the free-acid form as previously discussed. A stock solution of 50 ml of free acid was prepared, 10-ml aliquots of which were titrated. Each experiment consisted of two titrations for pK_a determination and two complexation titrations at different metal:ligand ratios. As a consequence, it was not necessary to separately determine the end point for a titration with added metal ion. (Metal–hydroxy formation frequently interferes with a precise determination of the end point.) The pK_a determinations were carried out in the absence of metal ions. Stability constant determinations were accomplished by titration of the ligand with added metal ion $Ni(NO_3)_2$ or $Mg(NO_3)_2$ at three different metal:ligand ratios (1:1, 1:2, and 2:1). All titrations were repeated at least twice and as many as five times for the monophosphates. Stability constants determined in separate experiments differed by no more than 5–10%.

Treatment of Data

The activity of the hydrogen ion at 0.1 *M* ionic strength was converted to concentration by the use of $\gamma_H = 0.83$. For nonoverlapping pK_a values a standard graphical procedure was used. For pK_a values separated by less than 2.5 pH units an algebraic method was used to calculate the separate pK_a values. The procedure employed is outlined by Albert and Sargent,[31] and was programmed on a Wang 362 calculator. Constancy was usually to within ±0.015 pK unit for about ten titration points.

Monophosphates. Equations for calculation of stability constants for various species have been derived for all the systems in this paper. We will first consider the derivation for the monophosphates, where no MHL has been observed. The species in solution could conceivably be: H_2L, HL^-, L^{2-}, ML, $(ML_2)^{2-}$, $(L_2)^{4-}$, H^+, OH^-, and K^+ (from the titration with KOH), where ML and ML_2 represent the mono and bis complexes, respectively, and L_2 a base-stacked species. We used the value[27] $K_3 = 5\ M^{-1}$. Constants are defined as follows (charges omitted for simplicity)

$$\begin{aligned} K_{a1} &= (H)(HL)/(H_2L) \\ K_{ML} &= (ML)/[(M)(L)] \\ K_3 &= (L_2)/(L)^2 \\ K_{a2} &= (H)(L)/(HL) \\ \beta_2 &= (ML_2)/[(M)(L)^2] \end{aligned} \tag{1}$$

Solution is effected by solving the mole balances on (L^0), (H^0), and (M^0)

$$(L) = \frac{2(L^0) - K - (H) + (OH)}{(H)/K_{a2} + 2(H^2)/(K_{a1}K_{a2})} \tag{2}$$

where (L^0) = total ligand concentration, (M^0) = total metal ion concentration, and (H^0) = total proton concentration. The above value for L is the same whether ML_2 or L_2 is included in the species. Algebraic manipulation of the mole balance relations in conjunction with eq 1 and 2 yields

$$\frac{A - 2K_3(L)}{[2(M^0) + 2K_3(L^2) - A(L)](L)} = K_{ML}\frac{(M^0) + 2K_3(L^2) - A(L)}{[2(M^0) + 2K_3(L^2) - A(L)](L)} + \beta_2 \tag{3}$$

where

$$A = \left[\frac{(L^0)}{(L)}\right] - \left[1 + \frac{(H)}{K_{a2}} + \frac{(H^2)}{K_{a1}K_{a2}}\right] \tag{4}$$

Equation 3 is the equation for a straight line: $Y = K_{ML}X + \beta_2$, where

$$\begin{aligned} Y &= \frac{A - 2K_3(L)}{[2(M^0) + 2K_3(L^2) - A(L)](L)} \\ X &= \frac{(M^0) + 2K_3(L^2) - A(L)}{[2(M^0) + 2K_3(L^2) - A(L)](L)} \end{aligned} \tag{5}$$

The correction for stacking (*i.e.*, the term involving K_3) was found to be only 1–3% and could be neglected. A plot of Y *vs.* X yields K_{ML} as the slope and $\beta_2 = (K_{ML}K_{ML_2})$ as the intercept. MgAMP5′ (Figure 1) is illustrative of a system where no detectable ML_2 is formed and consequently $\beta_2 = 0$. However, for NiAMP5′ (Figure 1) the stability constants K_{ML} and K_{ML_2} are of comparable magnitude.

Di- and Triphosphates. In addition to all the species formed by the monophosphates, the higher phosphates

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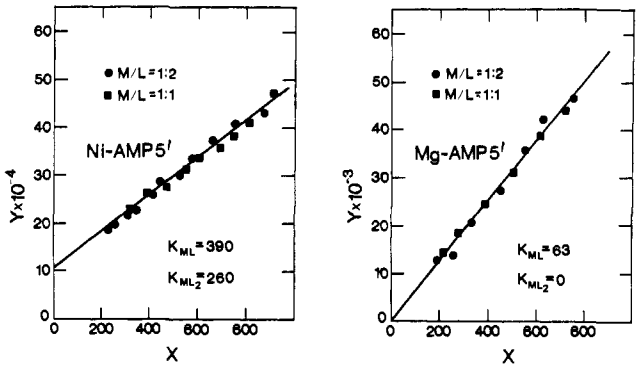


Figure 1. Graphical representation of potentiometric data for determining stability constants according to eq 3. Results of titrations carried out with metal:ligand ratios of 1:2 and 1:1 are superimposed.

also form an MHL complex, where the proton is associated with one of the phosphates.[14] For this derivation, the constants are the same as defined for the monophosphates, except $K_3 = (L_2)/(L^2)$ will be omitted because of its negligible contribution. The additional constant for MHL is defined as

$$K_{MHL} = (MHL)/[(HL)(M)]$$

An explicit solution for L which does not involve knowing the value of the stability constant K_{MHL} is not possible. One can combine the (M^0) and (L^0) balances for a solution for (M)

$$(M) = \frac{[(M^0) - (L^0)] + (L)\left[1 + \frac{(H^2)}{K_{a1}K_{a2}} + \frac{(H)}{K_{a2}}\right]}{1 - \beta_2(L^2)} \quad (6)$$

Because of the addition of MHL to the (H^0) balance, (L) must be solved for by a cubic equation

$$\beta_2\left[\frac{2(H^2)}{K_{a1}K_{a2}} + \frac{(H)}{K_{a2}}\right](L^3) - \left\{\beta_2 E + K_{MHL}\frac{(H)}{K_{a2}}\left(1 + \frac{(H^2)}{K_{a1}K_{a2}} + \frac{(H)}{K_{a2}}\right)\right\}(L^2) - \left(\frac{2(H^2)}{K_{a1}K_{a2}} + \frac{(H)}{K_{a2}}\{1 + K_{MHL}[(M^0) - (L^0)]\}\right) \times (L) + E = 0 \quad (7)$$

where $E = 2(L^0) - (H) - K + (OH)$. In order to solve for L, values of K_{MHL} and β_2 must be known. K_{MHL} values were obtained from low pH data at 1:1 metal:ligand ratios *via* the procedure described by Khan and Martell.[1] Many values of β_2 were then chosen and resulting L's, M's, etc., calculated by the following equations

$$(ML) = (M^0) - (M)\left\{1 + (L)\left[K_{MHL}\frac{(H)}{K_{a2}} + \beta_2(L)\right]\right\} \quad (8)$$

$$K_{ML} = \frac{(ML)}{(M)(L)}$$

$$K_{ML_2} = \frac{\beta_2}{K_{ML}}$$

Values of K_{ML} and K_{ML_2} were printed out for each titration point. If ten values of β_2 were used then ten values of K_{ML_2} and K_{ML} would be printed out for each pH reading. When an estimate for β_2 was too large, no positive root for L could be obtained from the cubic. The most sensitive parameters in the calculation of the stability constants were the pK_a values. Typical data for K_{ML} and K_{ML_2} as a function of β_2 are displayed in Table IV.

The stability constants defined above are "effective" values in that they do not take into account the possibility that some of the ligands may interact with the cation of the supporting electrolyte (0.1 *M* K^+ in the present work). Recently Rechnitz[32] has shown that potassium ion interacts considerably more strongly with free ATP^{4-} than had been previously thought. His value of K_{KATP} at $I = 0$ (230) may be estimated to be about 30 at $I = 0.1$. The effect that this interaction will have on a metal ion stability constant is easily calculated. If, in the presence of an excess constant concentration of K^+, one measures the stability constant for another metal ion without taking into account the amount of KL present, then the "effective" constant K_{ML}' is

$$K_{ML} = \frac{(ML)}{(M)(L + KL)} = \frac{K'_{ML}}{1 + K_{KL}(K^+)} \quad (9)$$

As a consequence, the "true" equilibrium constants (*i.e.*, which would be measured in the presence of a 0.1 *M* hypothetical supporting electrolyte which did not interact with the ligand) can be obtained by multiplying the effective values by $1 + K_{KL}(K^+)$. This is about 4 for ATP complexes. The correction factors for systems with fewer phosphate groups will be considerably less. The stability constants reported in this paper are K_{ML} values, rather than K'_{ML}.

Results and Discussion

Monophosphates. The five monophosphates studied were selected to explore the influence of the ribose ring, base structure, and phosphate position on the pK_a values and metal binding properties. The results include a reinvestigation of several monophosphates which were presumably well characterized[2] (AMP5', AMP2'), and measurements of others which have not been previously studied (ribose 5'-phosphate (RP) and CMP5').

Table I lists the pK_a values for the monophosphates. The pK_a values for ring ionization are around 4 and pK_a values for terminal phosphate ionization are around 6. Data are in good agreement with previously determined values. One observes a lowering of pK_{a_n} when a ribose group or a nucleoside is attached to the phosphate. This effect has been attributed to the electron withdrawing tendency of the ribose. Khan and Martell feel that the difference in the acidities of AMP2', AMP3', and AMP5' must be due to a steric effect involving differences in the proximity to the ribose hydroxyl groups and the ring nitrogens.[2]

The determination of stability constants K_{ML} and β_2 *via* eq 3 for the monophosphates was straightforward. If one attempts to compute K_{ML} values without including

(32) G. A. Rechnitz and M. S. Mohan, *Science*, **168**, 1460 (1970).

Table I. pK_{a_n} Values[a] at 15° and I = 0.1 KNO_3 for $H_nL \rightleftharpoons H^+ + H_{n-1}L$

Ligand	$pK_{a_{n+1}}$	pK_{a_n}	pK_{a_r}[b]
$H_2PO_4^-$	>11	6.79 (6.70)[c]	
H_2ribose 5′-phosphate		6.22	
H_2AMP5′		6.25 (6.28)[e]	3.96 (3.93)[e]
H_2AMP2′		6.07 (6.05)[e]	3.86 (3.83)[e]
H_2CMP5′		6.27 (6.30)[d]	4.52 (4.50)[d]
$H_3P_2O_7^-$	8.36 (8.45)[c]	6.02 (6.08)[c]	
H_3ADP		6.41 (6.47)[e]	4.05 (4.05)[e]
H_3CDP		6.38 (6.40)[d]	4.56 (4.60)[d]
$H_4P_3O_{10}^-$	7.93 (7.87)[c]	5.50 (5.43)[c]	
H_4ATP		6.57 (6.59)[f]	4.18 (4.17)[f]
H_4CTP		6.63	4.85

[a] $pK_{a_{n-1,2,3}} \leq 2$ for all systems; previously measured values are in parentheses. [b] $pK_{a_r} = pK_a$ for ring ionization. [c] 0.1 *M* KCl, 20°, ref 5. [d] 0.1 *M* NaCl, 25°, R. Bock, N. Ling, S. Morell, and S. Lipton, *Arch. Biochem. Biophys.*, **62**, 253 (1956). [e] 0.1 *M* KNO_3, 15°, ref 2. [f] 0.1 *M* KNO_3, 15°, ref 1.

β_2, a trend with pH is found in systems where ML_2 is present. A simple analysis will show why. At low pH's the dominant complexed species is ML, and additional lowering of the pH due to ML_2 formation will be small. At high pH's (where the ligand is principally in the deprotonated form) the dominant species will still be ML, but there will now be excess L available for the formation of ML_2. The pH reading at high pH will be lower than expected for just ML formation if ML_2 is also formed. This will cause the calculated K_{ML} to increase with increasing pH. The procedure we used, therefore, was to graph the data according to eq 3 from which the two constants K_{ML} and β_2 were obtained.

Stability constants for Ni(II) and Mg(II) complex formation with the monophosphates are listed in Table II. We were particularly interested in the differences, if any, in the binding of Ni(II) and Mg(II) to the various monophosphates. All ligands are −2 charge types (in the pH region where metal complexation occurs) and showed no evidence for formation of a complex with the HL species. If there is metal binding only to the phosphate oxygens, one would expect the stability constants to be identical within experimental error.[33]

Table II. Summary of Stability Constants for the Monophosphates[a]

Complex	K_{ML}	K_{ML_2}
$NiHPO_4$	100 (120)[b]	0
NiRP	80	0
NiAMP5′	390 (724)[d] (450)[b]	260
NiAMP2′	120 (680)[d]	160
NiCMP5′	80	0
$MgHPO_4$	60 (63)[c]	0
MgRP	50	0
MgAMP5′	63 (64)[d]	0
MgAMP2′	56 (64)[d]	0
MgCMP5′	56	0

[a] Previously measured values in parentheses. [b] 0.1 *M* $NaClO_4$, 25°, ref 34. [c] 0.16 *M* NaCl, 38°, L. Holt, J. Pierce, and C. Kajdi, *J. Colloid Sci.*, **9**, 409 (1954). [d] 0.1 *M* KNO_3, 15°, ref 2.

Inspection of Table II shows that the Mg(II) binding constants for all five systems are around 50–60, that is, identical. Presumably Mg(II) sees no difference in the ligands and binds only to the phosphate oxygens. In addition, we could not find evidence for bis complexation with magnesium and any of the monophosphates.

The situation for nickel complexation is, however, quite different: $NiHPO_4$, NiRP, and NiCMP5′ all show identical behavior—$K_{ML} \sim$ 80–100, and there is no evidence for bis complexation. However, when an adenine ring is present (AMP5′ and AMP2′), interesting and striking differences appear. First, the adenosine monophosphates show a greater stability with Ni(II) than do the corresponding inorganic phosphate or ribose phosphate. Stability constants for the mono complex with nickel also differ substantially from values previously in the literature. Second, variation of the phosphate position produces a marked effect on the stability constant: K_{ML}(AMP5′) > K_{ML}(AMP2′). Finally, substantial amount of bis complexation is present with Ni(II) and the adenosine monophosphates. The effect of the phosphate location is also evident in the magnitude of the stability constants for the bis complexes. We shall discuss each of these observations separately.

Sigel, *et al.*,[34] have also noted the elevation of K_{ML}(NiAMP5′) over $K_{ML}(NiHPO_4)$ and suggested the difference may be due to an increased amount of outer-sphere association with AMP5′. But both AMP5′ and HPO_4 are the same charge type (2−) and would presumably have the same amount of outer-sphere association. The answer rather seems to be the presence of the adenine ring in AMP5′. A back-bound ring interaction with the metal ion has been proposed by a number of workers for NiATP.[35] Such an additional interaction in NiAMP5′ would cause the complex to be more stable than the simple $NiHPO_4$ complex.

We consider next the difference in the binding constants of AMP2′ and AMP5′ with Ni^{2+}. There is a notable difference in the magnitude of binding in the two systems (K_{ML}(NiAMP5′) = 390 and K_{ML}(NiAMP2′) = 120) of over a factor of three which is clearly outside the realm of experimental uncertainty. One obvious answer is the orientation of the adenine ring. Nmr studies[25] of the effect of the phosphate ionization on AMP5′ and AMP2′ have indicated the 5′-phosphoryl group (but not the 2′) to have a specific deshielding effect on the H-8 proton on the ring at pH's >6 (*i.e.*, secondary ionization of the phosphate). A back-bound interaction with the metal ion and the ring may be more favorable with AMP5′ than AMP2′. That is, not only is there strong evidence for inteactions between the phosphate and the ring, but the degree of the interaction is dependent on the phosphate position.

The magnitude of the bis binding constant (K_{ML_2}) compared to K_{ML} is quite interesting. In both instances in which we detected the presence of the bis complex, the magnitude of its stability constant was comparable to that for the mono complex. This is contrary to the situation ordinarily encountered, *i.e.*, successive stability constants decrease. In the adenosine monophosphates there must be a bis complex configuration with

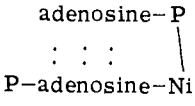
(33) We estimate the error to be ~10% for systems with $K_{ML} \leq 100$ and ~5% for systems with $K_{ML} \sim$ 50–500.

(34) H. Sigel, K. Becker, and D. McCormick, *Biochim. Biophys. Acta*, **148**, 655 (1967).

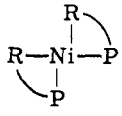
(35) R. Izatt, J. Christensen, and J. Rytting, *Chem. Rev.*, **71**, 439 (1971).

Ni^{2+} that is particularly favorable, more so than would result from the interaction of Ni(II) with the phosphate moieties of two nucleotides in the configuration adenosine–P–Ni–P–adenosine (P = phosphate residue). In addition CMP5′ shows no tendency to form this sort of bis complex.

There are a number of additional possibilities for the structure of the bis complex. For example, it is well known that the bases self-associate ("stack") in solution. If stacking persists in the bis complex, the structure could be



where the metal ion binds simultaneously to the ring of one nucleotide and the phosphate oxygens of another in a stacked arrangement. The conformational possibilities of the bis complex in NiAMP5′ and NiAMP2′ could therefore be quite dependent on the *stacking* capabilities of the two nucleotides.[25] An alternate possibility is the structure drawn below, where R = adenosine



In this case the strength of the complex would reflect the ease of formation of two back-bound complexes. This could be quite dependent on the phosphate position. There are doubtless other possible structures; unfortunately stability constant data alone are not sufficient to permit assignment of the configuration of the bis complex.

Multiple complex formation with metal ions and monophosphates has also been discussed by Childs,[36] who has extensively studied H_3PO_4 and binding with a series of divalent metal ions (Zn^{2+}, Mg^{2+}, Ca^{2+}, and Cu^{2+}). He reported not only the formation of ML, but also M_2L_2 and ML_2. We also analyzed our data for M_2L_2 formation according to eq 8, where A is

$$\frac{A}{C} = K_{ML} + K_{M_2L_2}(K_{ML})^2[2C(L)] \qquad (8)$$

identical with eq 4 and

$$C = (M^0) + \left[\frac{(H^2)}{K_{a_1}K_{a_2}} + \frac{(H)}{K_{a_2}} + 1\right](L) - (L^0)$$

where $K_{M_2L_2} = (M_2L_2)/(ML)^2$. This equation can be plotted in the form of a straight line, yielding K_{ML} as the intercept and $K_{M_2L_2}(K_{ML})^2$ as the slope. Our potentiometric data were not consistent with the presence of species M_2L_2. This species has also been proposed by Berger, *et al.*,[26] in their nmr study of Cu^{2+} with several adenosine monophosphates. However, they were working at $(M^\circ):(L^\circ)$ ratios $\sim 1:100$, which does not seem favorable for M_2L_2 formation, but rather ML_2 formation. Unfortunately, previous stability constant determinations for Cu^{2+} interactions with adenosine monophosphates were not analyzed for bis complexation.

It is worth emphasizing that caution should be exercised when information (*e.g.*, nmr, difference spectra, etc.) obtained at 1:1 metal:nucleotide ratios of the monophosphates is applied to the corresponding triphosphates.[27] Since some of the monophosphates form substantial amounts of bis complexes, erroneous conclusions may result from such an extrapolation. This problem is particularly critical when experimental conditions are such that the nucleotide concentration is much larger than that of the metal ion.

As a consequence of our findings we checked our experimental procedure to see if possible errors could lead to erroneous pH readings and subsequent analysis of several systems as having substantial ML_2 present. We used several different electrodes, pH meters, new buffers, and different lot numbers of the various ligands, varying one after the other or several at a time. In all instances, titration data were still consistent with ML_2 formation for Ni(II) with the adenosine monophosphates. Even when one takes into account extremes of possible error limits on the pK_a values (± 0.01–0.02) the data still indicate ML_2 formation.

Di- and Triphosphates. Table I lists the pK_a values for the di- and triphosphates. For pyrophosphate and tripolyphosphate one observes a lowering pK_{a_n} from the analogous nucleotide. This result is in agreement with that of previous workers.[5] With the nucleotides one observes an elevation of pK_{a_n} (terminal phosphate ionization[14]) as the number of phosphates is increased (*i.e.*, $pK_{a_n}(ATP) > pK_{a_n}(ADP) > pK_{a_n}(AMP5')$). The electron-withdrawing ability of the ring is less the further removed the terminal phosphate. In addition we are progressing from a 2− charge type (AMP5′) to a 4− charge type (ATP). The cytosine di- and triphosphates also have pK_{a_n} values similar to the adenine series; *i.e.*, the particular base structure does not have much influence on the phosphate ionization. This observation has been noted in a comparison of guanine nucleotides to adenine nucleotides.[13] In general, pK_a data from this study are in good agreement with previously measured values—especially when compared at the same temperature and ionic strength.

Table III contains the stability constants of Ni(II) and Mg(II) with the di- and triphosphates. The presence of the MHL species for the di- and triphosphates made the analysis of stability constants considerably more difficult. Any analysis for K_{ML} and

Table III. Summary of the Stability Constants of Di- and Triphosphates[a]

Complex	Log K_{MHL}	Log K_{ML}	Log K_{ML_2}
NiADP	2.30 (2.35)[b]	4.18 (4.55)[b]	2.30
NiCDP	1.87	3.48	1.99
NiP_2O_7	3.50 (3.81)[f]	6.22 (6.95)[f]	0
NiATP	2.78 (2.78)[e]	4.79 (5.09)[e]	0
NiCTP	2.68	4.41	0
NiP_3O_{10}	4.40 (5.01)[f]	7.20 (7.90)[f]	0
MgADP	1.55 (1.54)[b]	3.21 (3.08)[b] (3.15)[d]	0
MgCDP	1.60	3.22	0
MgP_2O_7	3.18	5.37	0
MgATP	2.18 (2.17)[e]	4.05 (4.13)[e] (4.04)[d]	0
MgCTP	2.18	4.03 (4.01)[e]	0
MgP_3O_{10}	4.00 (3.80)[d]	5.75 (5.80)[d]	0

[a] Previously measured values in parentheses. [b] 0.1 *M* KNO_3, 15°, ref 2. [c] 0.1 *M* KNO_3, 15°, ref 1. [d] 0.1 *M* KCl, 20°, ref 5. [e] 0.1 *M* NaCl, 23°, ref 12. [f] 0.1 *M* TMACl, 25°, ref 18.

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Table IV. Calculation of K_{ML} with and without β_2 for MgCDP and NiCDP

pH[a]	$\beta_2 = 0$ K_{ML}	$\beta_2 = 0$ K_{ML_2}	$\beta_2 = 8 \times 10^4$ K_{ML}	$\beta_2 = 8 \times 10^4$ K_{ML_2}	pH[a]	$\beta_2 = 0$ K_{ML}	$\beta_2 = 0$ K_{ML_2}	$\beta_2 = 3 \times 10^5$ K_{ML}	$\beta_2 = 3 \times 10^5$ K_{ML_2}
	System: MgCDP					System: NiCDP			
4.909	1600	0	1593	50	5.225	3041	0	2981	101
5.081	1680	0	1668	48	5.343	3038	0	2953	102
5.269	1618	0	1598	50	5.463	3209	0	3093	97
5.398	1613	0	1586	50	5.597	3130	0	2970	101
5.528	1633	0	1597	50	5.722	3307	0	3089	97
5.663	1619	0	1569	51	5.865	3222	0	2921	103
5.794	1656	0	1589	50	6.000	3379	0	2971	101
5.930	1646	0	1559	51	6.133	3748	0	3194	94
6.064	1667	0	1553	52	6.295	3558	0	2811	105
6.207	1624	0	1478	54	6.439	4179	0	3161	95
6.345	1708	0	1524	53	6.618	4785	0	3364	89
6.506	1624	0	1395	57					
6.672	1757	0	1474	54					

[a] Refers to data points of titration.

β_2 is dependent on knowledge of K_{MHL}. We therefore could not use the method of Khan and Martell[1] in systems where there was appreciable ML_2 present. Also in our procedure for the analysis of di- and triphosphates the ratio $(M^0):(L^0)$ could be varied and analysis carried out on 1:1, 1:2, and 2:1 titrations. Because our program directly printed out K_{ML} values over a range of about 15 pH points we could quickly spot trends with pH. An increase with pH in the calculated K_{ML} could be quantitatively accounted for by the presence of a small amount of ML_2. A series of β_2 values were chosen and resulting K_{ML_2} values calculated. With proper choice of K_{ML_2} the values of K_{ML} will now show a random variation over the entire pH range, rather than a trend with pH.

Table IV shows the results for MgCDP and NiCDP (1:2 metal:ligand titration). For MgCDP with $\beta_2 = 0$ (*i.e.*, no ML_2) the calculated values for K_{ML} are around 1650 (range 1760–1600) throughout the titration. If one assumes a small amount of ML_2 formation ($K_{ML_2} \sim 100$), calculated K_{ML} values decrease (range 1670–1395) with increasing pH (*i.e.*, points on the titration curve). It is important to note that our analysis cannot rule out ML_2 formation, but rather indicate upper limits for K_{ML_2}. In the case of MgCDP we can say that bis complexation is not detectable from our titration data ($K_{ML_2} < 50$).

For NiCDP we can see (Table IV) that values for K_{ML} calculated with $\beta_2 = 0$ range from ~3000 to 5000 over the course of the titration, with a definite increase in calculated K_{ML} as pH increases. The use of $\beta_2 = 3 \times 10^5$ produces the following change in the data: (1) Calculated K_{ML} values average ~3100, and do not increase significantly with pH. (2) Calculated K_{ML_2} values average around 95 and show a random variation with pH.

The titration data for the stability constants of all the di- and triphosphates were analyzed in the same manner—calculated K_{ML} values were studied as a function of β_2. Only two systems, NiCDP and NiADP, showed any indication of measurable ML_2 formation and the values of K_{ML_2} were quite small. Since the complexes themselves are not directly observed, these values should be regarded as upper limits, particularly in view of the fact that the diphosphates are known to hydrolyze in aqueous solution in the presence of divalent metal ions.

The stability constants for the magnesium complexes with HP_2O_7, CDP, ADP, HP_3O_{10}, CTP, and ATP are listed in Table III. We shall compare the K_{MHL} values for the inorganic phosphates with the K_{ML} values of the nucleotides. The charges on the ligands will therefore be identical, the terminal proton on the inorganic phosphates substituting for the ribose linkage. For the magnesium complexes the following can be noted. (1) Log K_{MHL} for the $MgHP_2O_7$ is identical with log K_{ML} for MgCDP and MgADP. (2) Log K_{MHL} for $MgHP_3O_{10}$ is identical with log K_{ML} for MgCTP and MgATP. (3) There is no evidence for bis complexation in any of the magnesium complexes.

By comparing a series of systems we can therefore conclude that the binding of magnesium is primarily to the phosphate backbone. Any additional stability conferred to the magnesium complex by interaction with the base rings is not evident; it may be the magnitude of experimental error.

As with the monophosphates, the results for Ni(II) complexation with the di- and triphosphates are quite different from the results with Mg(II). We shall again compare the K_{MHL} values for the inorganic phosphates with the K_{ML} values for the nucleotides. From data in Table III the following conclusions can be made. (1) Log $K_{MHL}(NiHP_2O_7) \sim$ log K_{ML}(NiCDP); log $K_{MHL}(NiHP_3O_{10}) \sim$ log K_{ML}(NiCTP). For the cytosine series, complexation with Ni(II) is primarily to the phosphate backbone. (2) Log K_{ML}(NiATP) > log $K_{MHL}(NiHP_3O_{10})$; log K_{ML}(NiADP) > log K_{MHL}(Ni-HP_2O_7). Nickel has a further interaction with ADP and ATP than just the phosphate backbone—the nature of the ring is important in the formation of the mono complex with Ni(II). (3) There is no evidence for bis complexation with Ni(II) and any of the triphosphates. (4) There is bis complexation in both NiCDP and NiADP; log K_{ML_2}(NiADP) > log K_{ML_2}(NiCDP). The nature of the ring is important in the stability of the bis complex.

As with the monophosphates, nickel shows more interesting complexation with the di- and triphosphates relative to magnesium. There is evidence for some sort of ring interaction (*i.e.*, the increased stability of the adenine nucleotides over the cytosine nucleotides) and bis complexation is observable with CDP and ADP. For the adenine series, the stability constants for bis complexation follow the trend expected from electro-

static considerations: log K_{ML_2}(Ni(AMP5′)$_2$) (a -2,0 interaction) > log K_{ML_2}(Ni(ADP)$_2$) (a $-1,-3$ interaction) > log K_{ML_2}(Ni(ATP)$_2$)$_2$ (a $-4,-2$ interaction).

In conclusion, we have found several interesting features associated with the stability constants of magnesium and nickel with inorganic and nucleoside phosphates. They may be summarized as follows. First, for magnesium, potentiometric titration data show little or no difference between the presence or absence of a ring system for a given number of phosphates. Second, for nickel the interactions are more numerous, and several ligands show evidence for bis complexation. In addition, stability constants with nickel reflect considerable ring specificity. Third, all pK_a values and K values for magnesium agree well with previously published work, where comparisons can be made. Some differences, however, are obtained with nickel complexes, especially for systems which indicate substantial bis complexation.

Further work is under way in this laboratory on metal ion–nucleotide interactions, including thermodynamic studies as a function of temperature. The kinetics of complexation are also being studied. The kinetics show dramatic differences due to differences in binding strengths and sites. In addition these studies are being extended to other ring systems for which very little information is currently available (*e.g.*, inosine and guanosine).[37]

Acknowledgment. This work was supported by the National Institutes of Health in the form of a research grant to J. E. S. (GM-13116).

(37) NOTE ADDED IN PROOF. M. S. Mohan and G. A. Rechnitz (*J. Amer. Chem. Soc.*, **94**, 1714 (1972) have recently demonstrated the existence of a Ca_2 ATP complex by means of a specific ion electrode, and have suggested the possibility of an analogous complex (*i.e.*, Mg_2ATP) with magnesium. Their value of K_{M_2L} for Ca_2ATP was about three orders of magnitude smaller than their value for the monoc omplex (CaATP). If the same type of M_2L complex is formed with magnesium, it is unlikely that we would have been able to detect it under our experimental conditions. In addition, if no proton is liberated, pH titration methods will be insensitive to such a species.

Communications to the Editor

Exo–Endo Steric Impediment in Norbornene. Specification of the Transition State for the Reaction of Singlet Oxygen with 2-Methylnorborn-2-ene and 2-Methylenenorbornane

Sir:

One of the enduring problems of mechanistic organic chemistry stems from the extraordinary fact that electrophilic additions to norbornene occur overwhelmingly on the exo side of the molecule.[1] Nevertheless, appropriately located substituents can play a key role in controlling the approach of a reagent attacking the double bond.[2] Steric effects have been shown to be particularly important in the case of one-step cyclic additions.[2c]

These properties intrinsic to the norbornene skeleton should be eminently suitable for examining the topical question concerning the course of the reaction of singlet oxygen with monoolefins.[3] It should be possible to specify the optimum geometry of the transition state and to make a decision between the one-stage cyclic process[6] and the two-stage perepoxide mechanism.[7] With this aim in mind we have investigated the reactivity of 2-methylnorborn-2-ene (**1**) and 2-methylenenorbornane (**2**).[8]

Compounds **1** and **2** were photooxidized in acetonitrile at 10–12° using two 500-W tungsten projector lamps (Silvana FFX) as the light source and Methylene Blue as sensitizer.[9] The primary oxidation products were reduced either with triphenylphosphine[10] or with sodium borohydride in methanol,[11] and the resulting mixture was analyzed by vapor phase chromatography (20% FFAP, Chromosorb W). Compound **1** gave *exo*-2-methylenenorbornan-3-ol (**3**) and *endo*-2-methylenenorbornan-3-ol (**4**) in proportions of 98.5 and 1.5% with a yield of 75–85%.

A similar reaction of **2** for 5 min gave 2-hydroxymethylnorborn-2-ene (**5**) and norbornanone (**6**) in

(1) For the previous paper in this series and relevant references cited therein, see: C. W. Jefford and F. Delay, *J. Amer. Chem. Soc.*, **94**, 4794 (1972).

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(3) Despite an abundant literature[4] dealing with structurally simple olefins, few bridged bicyclic structures have been examined so far.[5]

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