Microcalorimetric Studies on the Formation of Magnesium Complexes with 5′ Ribonucleotides of Guanine, Uracil, and Hypoxanthine[1a]

J. C. Sari[1b] and J. P. Belaich*

Contribution from the C.N.R.S., Laboratoire de Chimie Bactérienne, 31, chemin J.-Aiguier, 13009—Marseille, France. *Received March 30, 1973*

Abstract: Thermodynamic data are reported for magnesium complex formation of GTP, ITP, UTP, GDP, UDP, GMP, and UMP. The ΔG, ΔH, and ΔS values were determined at 30°, pH 8.50, and $\mu = 0.2$ with a microcalorimetric technique using an isothermic Calvet apparatus. In all cases the ΔH values are proportional to the length of the phosphate chains. On the other hand, the ΔG and ΔS values are dependent on the nature of the ring moiety, especially in the case of the nucleotide triphosphates. The affinity of the nucleotide triphosphates for magnesium increases in the order ATP > UTP > ITP, GTP. Moreover, the pK's of the rings of magnesium-complexed species are shifted to lower pH values. These facts are in good agreement with the hypothesis that the ring moiety of the nucleotides interacts weakly with Mg^{2+}.

The adenine nucleotide–magnesium complexes, because of their importance in a great many biological reactions, have been extensively studied. Much work has been done on the determination of the stability constant and thermodynamic quantities associated with complex formation. Unfortunately, research concerning the magnesium chelates of the other 5′ ribonucleotides is very rare. The only work reported in this field is that of Walaas,[2] whose results show no change in the stability constant for either magnesium or manganese ions, if uridine or guanosine triphosphate replaces ATP. Since that time, it has been routinely assumed that the stability constants are the same for all the homologous nucleotides; however, the values presently used, *e.g.*, by Shramm and Morrison,[3] are not those measured by Walaas, but those recently obtained for the formation of the adenine nucleotide–Mg^{2+} complexes.

In this study, the thermodynamic quantities (ΔG, ΔH, ΔS) of magnesium complex formation of GTP, UTP, ITP, GDP, UDP, GMP, and UMP have been estimated by a microcalorimetric technique. (The usual abbreviations of adenosine, guanosine, uridine, and inosine mono-, di-, and triphosphate are used when there is no need to specify the ionic species precisely.)

Experimental Section

Apparatus. The experimental procedure and the microcalorimeter used are the same as those previously described by Belaich and Sari.[4] However, for the mixing of reagents, the experimental "siphon cell" previously described is replaced by a cell which is equipped with a closing device (Figure 1). The opening and the mixing artefacts observed when 1 ml of buffer contained in this cell is injected into the Pyrex calorimetric cell containing 5 ml of the same buffer are less than 3×10^{-4} cal.

The pH stat titrations were made with a Radiometer apparatus 26 equipped with the glass electrode Type G 202C.

The pH measurements were carried out under pure nitrogen gas at 30°. The ionic strength was maintained at $\mu = 0.2$ with tetramethylammonium bromide (TMABr) as supporting electrolyte.

All calorimetric experiments were made at 30°, pH 8.50, and at ionic strength 0.2 maintained with TMABr, the concentration of which varies with the concentration of other reagents. The concentration of triethanolamine buffer (TEA) was equal to 0.15 *M*.

Reagents. All nucleotides were purchased from Boehringer and repurified. For this purpose, the top of a column of DEAE-Sephadex A-25 (60 mm × 10 mm) was loaded with 50 mg of sodium salt of nucleotide at pH 3. The column was eluted with a linear NaCl gradient (0–0.25 *M*). The fraction containing the pure nucleotide was twice recrystallized with 6 volumes of cold acetone. The last pellet of centrifugation is dried under vacuum. After purification each nucleotide contained approximately one sodium per phosphorus. The electrophoresis at 2000 V showed only one molecular species. The nucleotide was redissolved with TEA and TMABr solution, the pH adjusted to 8.50 with HCl or tetramethylammonium hydroxide (TMAOH), and the concentration determined from the ultraviolet absorbance. The extinction coefficients (ϵ) at 260 nm and pH 7.0 used were: UTP, UDP, UMP = 9.9 $cm^2/\mu mol$; GTP, GDP, GMP = 11.8 $cm^2/\mu mol$; ITP = 7.4 $cm^2/\mu mol$.

$MgSO_4 \cdot 7\ H_2O$ was purchased from Prolabo or J. Matthey, and the Mg checked by EDTA analysis.

TEA and TMABr were purchased from Merck.

Results

A. Ionic Species and Equilibrium Constants. Compared with adenine nucleotides, the guanine, uracil, and hypoxanthine nucleotides have, at our experimental pH, one additional ionizable hydrogen on the ring moiety (pK_r = 9–9.5). Therefore, in the pH range 7–9, four ionic species must be taken into account for each complexed or uncomplexed nucleotide studied.

The reaction of complex formation of a divalent metal M with these hydroxylated nucleotides can be written.

$$AHr + A + AHrHp + AHp + M \rightleftharpoons MAHr + MA + MAHrHp + MAHp$$

The meanings of the symbols used in this equation are given in Figure 2. The apparent stability constant measured for this equilibrium (K_{app}) at a finite ionic strength can be written using brackets for the concentrations of the ionic species.

$$K_{app} = \frac{[MAHr] + [MA] + [MAHrHp] + [MAH_p]}{([AHr] + [A] + [AHrHp] + [AHp])[M]} \quad (1)$$

(1) (a) With the technical assistance of P. Simonpietri. (b) This work was done in partial fulfillment of the requirements for the degree of Doctor es Sciences for J. C. Sari at the University of Aix-Marseille.

(2) E. Walaas, *Acta Chem. Scand.*, **12**, 528 (1958).

(3) V. L. Schramm and J. F. Morrison, *Biochemistry*, **10**, 2272 (1971).

(4) J. P. Belaich and J. C. Sari, *Proc. Nat. Acad. Sci. U. S.*, 64, 763 (1969).

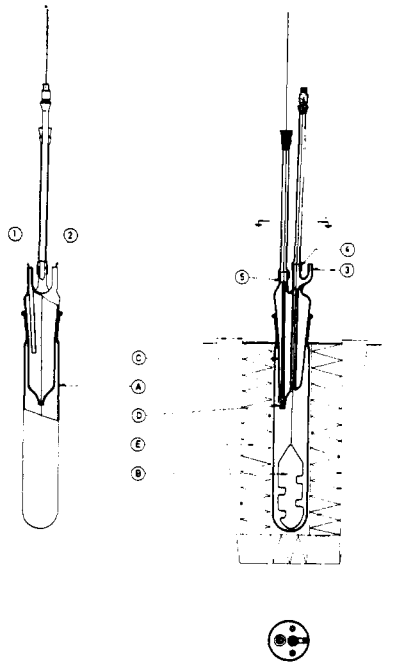

Figure 1. (A) Pyrex calorimetric cell; (B) stirrer in Teflon; (C) small Pyrex cell which is held by a ground-glass stopper on the calorimetric cell. The lower aperture of this cell has a closing device (D); (E) thermocouples surrounding the calorimetric cell: (1) tube for introducing the reactant, (2) tube for equilibrating the pressure between the calorimetric cell and the exterior, (3) tube for equilibrating the pressure between the calorimetric cell and the exterior, (4) access for the stirrer support, and (5) access for the closing device.

The apparent constants for each equilibrium are

$$K_1 = [MAHr]/[AHr][M] \quad (2)$$

$$K_2 = [MA]/[A][M] \quad (3)$$

$$K_3 = [MAHrHp]/[AHrHp][M] \quad (4)$$

$$K_4 = [MAHp]/[AHp][M] \quad (5)$$

For the apparent ionization constants, we defined a mixed equilibrium constant as $K = [A]H/[HA]$, where A is the conjugate base of the acid specie HA and H is the activity of H^+ ion. Therefore, for each ionization reaction of Figure 2 we can write

$$K_r = \frac{[AHp]H}{[AHrHp]} = \frac{[A]H}{[AHr]} = \frac{[AR]H}{[ARH]} \quad (6)$$

with [AR] = [AHP] + [A] and [ARH] = [AHrHp] + [AHr];

$$K_p = \frac{[AHr]H}{[AHrHp]} = \frac{[A]H}{[AHp]} = \frac{[AP]H}{[APH]} \quad (7)$$

with [AP] = [A] + [AHr] and [APH] = [AHrHp] + [AHp];

$$K_{mr} = \frac{[MAHp]H}{[MAHrHp]} = \frac{[MA]H}{[MAHr]} = \frac{[MAR]H}{[MARH]} \quad (8)$$

with [MAR] = [MAHp] + [MA] and [MARH] = [MAHrHp] + [MAHr]

$$K_{mp} = \frac{[MAHr]H}{[MAHrHp]} = \frac{[MA]H}{[MAHp]} = \frac{[MAP]H}{[MAPH]} \quad (9)$$

with [MAP] = [MAHr] + [MA] and [MAPH] = [MAHrHp] + [MAHp]. From relationships 1–9 one obtains

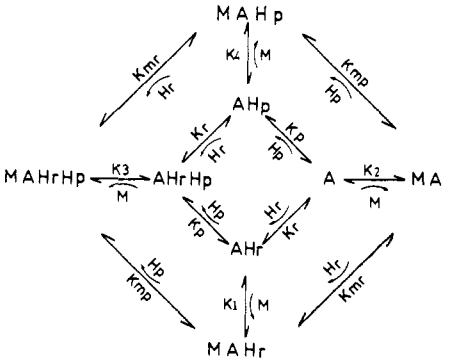

Figure 2. Complex formation reactions of the different ionic species of hydroxylated nucleotides with magnesium. A and MA are the completely ionized nucleotide and the divalent metal complex, respectively; AHp, AHr, and AHrHp are the different ionized forms of the nucleotides; Hp and Hr symbolize the ionizable hydrogen of the phosphate chain and of the ring moiety, respectively; MAHp, MAHr, and MAHrHp are the divalent metal complexes of these ionic species.

$$K_{app} = K_1 \frac{1 + H/K_{mp} + K_{mr}/H + K_{mr}/K_{mp}}{1 + H/K_p + K_r/H + K_r/K_p} \quad (10)$$

$$K_{app} = K_2 \frac{1 + H/K_{mr} + H/K_{mp} + H^2/K_{mp}^2}{1 + H/K_r + H/K_p + H^2/K_p^2} \quad (11)$$

$$K_{app} = K_3 \frac{1 + K_{mp}/H + K_{mr}/H + H^2/K_{mp}K_{mr}}{1 + K_p/H + K_r/H + H^2/K_pK_r} \quad (12)$$

$$K_{app} = K_4 \frac{1 + H/K_{mr} + H/K_{mp} + K_{mp}/K_{mr}}{1 + H/K_r + H/K_p + K_p/K_r} \quad (13)$$

The knowledge of K_p, K_r, K_{mr}, and K_{mp} and the determinations of K_{app} and H give K_1, K_2, K_3, and K_4.

B. Microcalorimetric Method for the Determination of K_{app}. From two heat determinations, it is possible to calculate K_{app}. These determinations were performed as previously described by Belaich and Sari.[4] Let us recall: a is the total initial nucleotide concentration; b and c, the total Mg^{2+} corresponding respectively to the first and the second experiment; V, the volume of the liquid phase; x_i with $i = 1, 2, 3, 4$, corresponding respectively to [MAHr], [MA], [MAHrHp], and [MAHp] at equilibrium after the first experiment; y_i with $i = 1, 2, 3, 4$, the same concentrations after the second experiment; Q_1 and Q_2, the heat quantity measured after the first and the second experiment, respectively; ΔH_i with $i = 1, 2, 3, 4$, the enthalpy variation corresponding to the reaction of formation of MAHr, MA, MAHrHp, and MAHp, respectively; ΔH_p, ΔH_r, and ΔH_n, the enthalpy of ionization of the secondary phosphate, of the ring, and of the TEA buffer, respectively.

Corrections of Q_1 and Q_2 for the Neutralization of Protons. The heat quantities corresponding to the Mg^{2+} complex formation must be corrected for the neutralization of protons evolved during the reaction. If the subscript 0 is employed for the initial concentrations

$$a = [AHr]_0 + [A]_0 + [AHrHp]_0 + [AHp]_0 = [APH]_0 + [AP]_0 = [ARH]_0 + [AR]_0$$

Using relationships 6 and 7, the concentrations of

Table I. Experimental Values of Q_1 and Q_2[a] and Thermodynamic Data

Complex	No. of expt	a, mM	b, mM	c, mM	V, ml	Q_1, mcal	Q_2, mcal	Log K_{app}[b]	ΔH_{app}, kcal
GTP–Mg	15	2.30	2.50	5.00	6	46.7 ± 0.8	53.9 ± 1.0	3.934 ± 0.047	4.08 ± 0.03
GDP–Mg	13	2.50	2.50	5.00	5	27.1 ± 1.7	35.3 ± 0.9	3.420 ± 0.112	3.30 ± 0.12
GMP–Mg	18	4.00	4.32	25.90	6	7.0 ± 0.2	22.3 ± 0.6	1.761 ± 0.017	1.69 ± 0.03
UTP–Mg	14	2.48	2.50	5.00	6	52.1 ± 1.9	60.0 ± 2.1	4.153 ± 0.095	4.17 ± 0.07
UDP–Mg	10	2.45	2.70	5.40	5	27.2 ± 0.8	33.9 ± 0.9	3.448 ± 0.058	3.08 ± 0.06
UMP–Mg	12	4.00	4.32	25.90	6	6.6 ± 0.2	22.9 ± 0.3	1.696 ± 0.020	1.76 ± 0.04
ITP–Mg	10	2.50	2.50	5.00	6	50.8 ± 0.9	60.3 ± 1.1	3.930 ± 0.059	4.21 ± 0.06

[a] Each experimental Q_1 value was tested against each Q_2 value giving K_{app} and ΔH_{app}. Thus if n values of Q_1 and n' values of Q_2 were obtained from experiments, this method gives nn' K_{app} values and nn' ΔH_{app} values. [b] We give log K_{app} instead of K_{app} in order to obtain normally distributed values. All the results are given with their confidence interval of the mean ($p = 0.05$).

nucleotide which have one ionizable hydrogen on the phosphate chain or on the ring before Mg^{2+} addition can be written respectively

$$[APH]_0 = aH/K_p/(1 + H/K_p) \quad (14)$$

$$[ARH]_0 = aH/K_r/(1 + H/K_r) \quad (15)$$

After the firt Mg^{2+} addition (subscript 1)

$$a = [AHr]_1 + [A]_1 + [AHrHp]_1 + [AH]_1 + \Sigma x_i$$

The relationships 14 and 15 become

$$[APH]_1 = \frac{(a - \Sigma x_i)H/K_p}{1 + H/K_p} \quad (16)$$

$$[ARH]_1 = \frac{(a - \Sigma x_i)H/K_r}{1 + H/K_r} \quad (17)$$

Therefore, the concentrations of the complexed and uncomplexed nucleotides which still have ionizable hydrogen on the phosphate chain or on the ring after the first Mg^{2+} addition can be written respectively using the relationships 6–9, 16, and 17

$$[APH]_1 + x_3 + x_4 = \frac{aH/K_p - x_1\beta H/K_p}{1 + H/K_p} + x_1\frac{H}{K_{mp}} + x_1\frac{K_{mp}}{K_{mr}} \quad (18)$$

$$[ARH]_1 + x_1 + x_3 = \frac{aH/K_r - x_1\beta H/K_r}{1 + H/K_r} + x_1 + x_1\frac{H}{K_{mp}} \quad (19)$$

with

$$\beta = 1 + K_{mr}/H + H/K_{mp} + K_{mr}/K_{mp} \quad (20)$$

We express all the terms as a function of x_1 because at experimental pH, MAHr is the predominant ionic form. The heat quantity corresponding to the protons evolved and neutralized after the first Mg^{2+} addition, using relationships 14, 15, 18, and 19, is

$$Q_{n_1} = Vx_1\left(\frac{\beta H/K_p}{1 + H/K_p} - \frac{H}{K_{mp}} - \frac{K_{mp}}{K_{mr}}\right)(\Delta H_p - \Delta H_n) + Vx_1\left(\frac{\beta H/K_r}{1 + H/K_r} - 1 - \frac{H}{K_{mp}}\right)(\Delta H_r - \Delta H_n) \quad (21)$$

The relationship giving the heat quantity corresponding to the protons evolved and neutralized during the second Mg^{2+} addition (Q_{n_2}) is the same as relationship 21 but replacing x_1 by y_1.

Mathematical Treatment for the Determination of K_{app}. The heat quantities evolved after each Mg^{2+} addition can be expressed as

$$Q_1 = V\Sigma x_i\Delta H_i + Q_{n_1} \quad (22)$$

$$Q_2 = V\Sigma y_i\Delta H_i + Q_{n_2} \quad (23)$$

Rearranging the terms, one obtains from relationships 21–23

$$Q_1 = Vx_1\theta \quad (24)$$

$$Q_2 = Vy_1\theta \quad (25)$$

with

$$\theta = \Delta H_1 + (K_{mr}/H)\Delta H_2 + (H/K_{mp})\Delta H_3 + (K_{mr}/K_{mp})\Delta H_4 + \left(\frac{\beta H/K_p}{1 + H/K_p} - \frac{H}{K_{mp}} - \frac{K_{mr}}{K_{mp}}\right) \times (\Delta H_p - \Delta H_n) + \left(\frac{\beta H/K_r}{1 + H/K_r} - 1 - \frac{H}{K_{mp}}\right) \times (\Delta H_r - \Delta H_n) \quad (26)$$

The temperature, ionic strength, and pH being constant during the two experiments, the relationships of K_{app} corresponding to the first and the second experiment can be identified.

$$K_{app} = \beta x_1/(a - \beta x_1)(b - \beta x_1) = \beta y_1/(a - \beta y_1)(c - \beta y_1) \quad (27)$$

From relationships 24, 25, and 27, one obtains

$$\frac{\theta}{\beta} = \Delta H_{app} = \frac{(c - b)Q_1Q_2 \pm \sqrt{\Delta}}{2(Q_1ac - Q_2ab)V} \quad (28)$$

with $\Delta = Q_1^2Q_2^2(b^2 - 2bc + c^2) - 4(Q_1ac - Q_2ab) \times (Q_1Q_2^2 - Q_2Q_1^2)$. This relationship is the same as those previously published in a simpler case.[4]

Table I shows the calorimetric experimental results and K_{app} and ΔH_{app} values obtained for all the nucleotides studied. The determination of θ and β requires the knowledge of K_r, K_p, K_{mr}, and K_{mp}. As Phillips, *et al.*,[5] have demonstrated that the K_p's are practically identical for all the homologous nucleotides studied, it can be assumed that the K_{mp}'s are also identical, in other words, that the shifts of pK of the secondary phosphate occurring when Mg^{2+} is complexed with nucleotide are practically the same. The K_r of the hydroxylated bases have been reported by Sigel[6] and the influence of the length of the phosphate chain on the

(5) R. C. Phillips, P. Eisemberg, P. George, and R. J. Rutman, *J. Biol. Chem.*, 240, 4393 (1965).
(6) H. Sigel, *Eur. J. Biochem.*, 3, 530 (1968).

nucleoside's pK_r values is negligible. A shift of the pK_r has been observed by Sigel[6] in the case of complexes of Cu^{2+} and hydroxylated nucleotides. This phenomenon results from the interaction of Cu^{2+} with the bases of the hydroxylated nucleotides.[7] Nothing has been published for the shift of pK_r during the hydroxylated nucleotides–magnesium complex formation. Therefore, if such a phenomenon arises with Mg^{2+}, the quantity of protons evolved during the reaction must be greater than the quantity of protons expected from the phosphate chain.

C. Determination of the pK_r Shift during Mg^{2+} Complex Formation. To test such an eventuality, the quantity of protons released during Mg^{2+} complex formation has been measured at pH 8.0 and 8.5 by a pH stat titration method (Table II).

Table II. pH Stat Titration Results[a]

	ϕ (equiv of H^+/l.) $\times 10^4$	$[AP]_0/[APH]_0$	pK_p
ATP			
pH 8.00	0.5034	10.91	6.96
pH 8.50	0.1680	34.7	6.96
GTP			
pH 8.00	0.6636	8.03	7.09
pH 8.50	0.4038	13.85	7.36
UTP			
pH 8.00	0.7224	7.30	7.13
pH 8.50	0.4458	12.45	7.40
ITP			
pH 8.00	0.7230	7.30	7.13
pH 8.50	0.5562	9.78	7.51

[a] These data showed a shift of pK_r in the case of hydroxylated nucleotide triphosphates. In each experiment the concentration of nucleotide is equal to 6×10^{-4} *M*.

Since all the protons of the phosphate chain are released at pH >8 (Sari and Belaich[8]), the pK_p is easily calculated according to

$$\phi = [APH]_0 \qquad (29)$$

where ϕ is the number of H^+ equivalents per liter corresponding to the addition of excess Mg^{2+}. In this case the nucleotides are entirely complexed. The logarithmic form of (7) is

$$pK_p = pH - \log ([AP]_0/[APH]_0) \qquad (30)$$

It can be seen that in the case of ATP whose pK_r is equal to 4, the pK_p calculated with the relationships 29 and 30 are the same for the two experimental pH's. This is not the case for the hydroxylated nucleotides and it must be concluded that for GTP, ITP, and UTP an excess of protons is released from the ring as a result of a pK_r shift during Mg^{2+} complex formation.

This shift has been calculated from Sigel's pK_r values and the results of Table II. Hence, with the excess of protons evolved in the case of the hydroxylated nucleotides, the relationship 29 becomes

$$\phi = [APH]_0 + [ARH]_0 - [MARH]_e \qquad (31)$$

$[MARH]_e$ is the concentration of Mg^{2+} complex which still has an ionizable hydrogen on the ring after the addition of excess Mg^{2+} (subscript e). From relationship 31, it is possible to estimate $[MARH]_e$ (Table III).

Table III. Determination of ΔpK_r

	pK_r[a]	$[AR]_0/[ARH]_0$	$\phi - [APH]_0$, (equiv of H^+/l.) $\times 10^4$	$[MAR]_e/[MARH]_e$	pK_{mr}	ΔpK_r
GTP	9.6					
pH 8.00		0.025	0.1602	0.050	9.30	0.30
pH 8.50		0.079	0.1938	0.126	9.41	0.19
UTP	9.5					
pH 8.00		0.031	0.2190	0.072	9.15	0.35
pH 8.50		0.100	0.2778	0.156	9.31	0.19
ITP	9.2					
pH 8.00		0.063	0.2196	0.106	8.98	0.23
pH 8.50		0.199	0.3882	0.293	9.03	0.17

[a] Sigel's[5] values. In all experiments the concentration of nucleotides is equal to 6×10^{-4} *M*.

When the nucleotides are completely complexed we have

$$a = [MAR]_e + [MARH]_e$$

Therefore, according to the logarithmic form of (8)

$$pK_{mr} = pH - \log ([MAR]_e/[MARH]_e)$$

We can calculate pK_{mr} and ΔpK_r from Sigel's values.

Knowing the value of pK_{mr}, we can now take into account all the ionic species of nucleotide triphosphate for the determination of β. Using the values of K_p, K_{mp}, K_r, and K_{mr} and the relationships 10–13, one can determine K_1, K_2, K_3, and K_4 related to the reaction of formation of complexes between Mg^{2+} and the different ionic species of each nucleotide triphosphate studied. Table V shows the results obtained and Table IV gives the data used for the calculation of these equilibrium constants.

Discussion

The pK_r shifts observed at pH 8.5 are lower than those at pH 8.0; we have taken the lowest pK_r shift values

Table IV. Values Used for Thermodynamic Calculations for Nucleotides and Their Magnesium Complexes

	pK_p	pK_{mp}	pK_r	pK_{mr}
GTP	6.96[a]	5.26[b]	9.6[c]	9.41[d]
GDP	6.78[b]	5.31[b]		
GMP	6.47[b]			
UTP	6.96[a]	5.26[b]	9.5[c]	9.31[d]
UDP	6.78[b]	5.31[b]		
UMP	6.47[b]			
ITP	6.96[a]	5.26[b]	9.2[c]	9.03[d]

[a] Experimental value at $\mu = 0.2$ for $ATPH^{3-}$ ionization. [b] Values calculated at $\mu = 0.2$ for adenine nucleotides from Phillips's results: R. C. Phillips, P. George, and R. J. Rutman, *J. Amer. Chem. Soc.*, **88**, 2631 (1966). [c] Sigel's[5] values at $\mu = 0.1$. [d] Experimental values at pH 8.50.

Table V. Stability Constants of the Four Ionic Species Complexes of Each Nucleotide Triphosphate

	K_1, M^{-1}	K_2, M^{-1}	K_3, M^{-1}	K_4, M^{-1}
GTP–Mg	8,500	12,800	170	270
UTP–Mg	13,800	20,800	290	450
ITP–Mg	8,200	11,800	180	270

(7) A. T. Tu and C. G. Friederich, *Biochemistry*, **7**, 4367 (1968).
(8) J. C. Sari and J. P. Belaich, *Biochim. Biophys. Acta*, **305**, 1 (1973).

because at high pH the influence of the protons coming from the phosphate chain is slighter than at low pH. Those shifts are very close to those obtained by Hotta[9] and Watanabe[10] in the case of the adenine ring of ATP–Mg and ADP–Mg complexes. Since it is known that the Mg^{2+} binds to the phosphate chain,[11,12] our data support the model proposed by Hotta, *et al.*,[9] according to which Mg^{2+} forms a folded complex between the phosphate chain (primary binding site) and the ring (secondary binding site) of the nucleotides. The values of pK_r shifts observed in the case of Mg^{2+} complexes are about tenfold lower than Sigel's values[6] observed in the case of Cu^{2+} whose interactions with the ring were demonstrated unambiguously in the case of the nucleotides of guanine and hypoxanthine[7] or adenine.[13] The existence of interactions of Mg^{2+} with the nucleotide rings have been challenged as reviewed by Phillips.[14]

The relationships proposed here and the calorimetric technique allow the determination of the different stability constants (Table V). It is evident that the most interesting values are K_1 and K_2; it can be seen that the stability constants of the fully ionized species are highest in all cases.

Moreover, the K_1 values are close to the K_{app} determined at pH 8.50 because ΔH_r predominates in our experimental conditions. For this reason, it is possible to confuse K_1 and K_{app} at pH 8.50. For the nucleotide mono- and diphosphates, the stability constants directly determined at pH 8.50 have been taken to be equal to K_1.

Determination of ΔH_1. From relationships 25, 27, and 28, ΔH_{app} can be written

$$\Delta H_{app} = \frac{\Delta H_1 + K_{mr}/H\Delta H_2 + H/K_{mp}\Delta H_3 + K_{mr}/K_{mp}\Delta H_4}{1 + K_{mr}/H + H/K_{mp} + K_{mr}/K_{mp}} + A + B \quad (32)$$

with

$$A = \frac{[(1 + K_{mr}/H + H/K_{mp} + K_{mr}/K_{mp})H/K_p]/(1 + H/K_p) - H/K_{mp} - K_{mr}/K_{mp}}{1 + K_{mr}/H + H/K_{mp} + K_{mr}/K_{mp}} (\Delta H_p - \Delta H_n) \quad (33)$$

$$B = \frac{[(1 + K_{mr}/H + H/K_{mp} + K_{mr}/K_{mp})H/K_r]/(1 + H/K_r) - 1 - H/K_{mp}}{1 + K_{mr}/H + H/K_{mp} + K_{mr}/K_{mp}} (\Delta H_r - \Delta H_n) \quad (34)$$

The two last terms of relationship 32 corresponding to the heat of ionization and neutralization of protons evolved during the experiments can be calculated without ambiguity. In the case of the nucleotide triphosphates, the B term is negligible with respect to A (Table VI). The first term of relationship 32 can be simplified. Thus, at our experimental pH, H/K_{mp} and K_{mr}/K_{mp} are very small with respect to 1. Therefore, we have

$$\Delta H_{app} - A - B = \frac{\Delta H_1 + K_{mr}/H\Delta H_2}{1 + K_{mr}/H}$$

Precise experimentation at extreme pH, *i.e.*, 7 and 9, would give the evaluation of ΔH_1 and ΔH_2. Nevertheless, for the purpose pursued here, we can consider that the error produced on the determination of ΔH_1, taking ΔH_1 close to ΔH_2, is negligible, since the term K_{mr}/H is always smaller than 1. In the most unfavorable cases studied here, that is, the case of ITP, $K_{mr}/H = 0.29$. Therefore we can write

$$\Delta H_1 = \Delta H_{app} - A - B$$

For the hydroxylated nucleotide mono- and diphosphates, it is possible to do all the same calculations as for the nucleotide triphosphates.

However, as has just been seen, K_1 is very close to K_{app}. Therefore, we have taken directly these experimental values equal to K_1. The A and the B terms of relationship 32 have been calculated assuming that all the shifts of pK_r are identical with those observed in the case of nucleotide triphosphates.

Table VII shows all the thermodynamic functions concerning the complexes of Mg^{2+} with hydroxylated nucleotide species AHr studied here. As can be seen on this table and on Figure 3, the ΔH values are proportional to the number of phosphate groups in all cases. Moreover, the values obtained for the hydroxylated nucleotides are very close to those obtained for the adenine nucleotides.

This is not the case for the ΔG values (Figure 4) which are practically identical for all the nucleotide monophosphates, slightly different for the nucleotide diphosphates, and significantly different for the nucleotide triphosphates in the order ATP > UTP > GTP, ITP.

These results show that the nature of the ring moiety influences the stability constant of the Mg^{2+} complexes.

Table VI. Correction Terms A and B Used for the Calculation of ΔH_1 from ΔH_{app}

	ΔH_p[a]	ΔH_r[b]	A, kcal	B, kcal
GTP	−1.75	8.6	−0.255	0.047
GDP	−1.48	8.6	−0.167	0.047
GMP	−1.45	8.6	−0.083	0.047
UTP	−2.02	8.0	−0.255	0.022
UDP	−1.08	8.0	−0.159	0.022
UMP	−1.12	8.0	−0.079	0.022
ITP	−1.61	7.2	−0.252	−0.024

[a] Values of Phillips, *et al.*[4] [b] Values of B. I. Sukhorukov, V. I. Poltev, and L. A. Blyumenfel'd, *Abh. Deut. Akad. Wiss. Berlin, Kl. Med.*, 381 (1964).

Table VII. Thermodynamic Values Related to All the Mg^{2+} Complexes Studied

	ΔH_1, kcal/mol	ΔG_1, kcal/mol	ΔS_1 eu
ATP-Mg^{2-}	4.46	−6.48	36.1
ADP-Mg^{-}	3.18	−5.08	27.3
AMP-Mg	1.77	−2.48	14.0
GTP-Mg^{2-}	4.29	−5.42	32.1
GDP-Mg^{-}	3.41	−4.72	26.8
GMP-Mg	1.72	−2.43	13.7
UTP-Mg^{2-}	4.40	−5.73	33.4
UDP-Mg^{-}	3.22	−4.76	26.4
UMP-Mg	1.82	−2.34	13.7
ITP-Mg^{2-}	4.48	−5.42	32.7

(9) K. Hotta, J. Brahms, and M. F. Morales, *J. Amer. Chem. Soc.*, **83**, 997 (1961).
(10) S. Watanabe, L. Evenson, and I. Gulz, *J. Biol. Chem.*, **238**, 324 (1963).
(11) M. Cohn and T. R. Hughes, *J. Biol. Chem.*, **235**, 3250 (1960).
(12) M. Cohn and T. R. Hughes, *J. Biol. Chem.*, **237**, 176 (1962).
(13) N. A. Berger and G. L. Eichhorn, *Biochemistry*, **10**, 1863 (1971).
(14) R. C. Phillips, *Chem. Rev.*, **66**, 501 (1966).

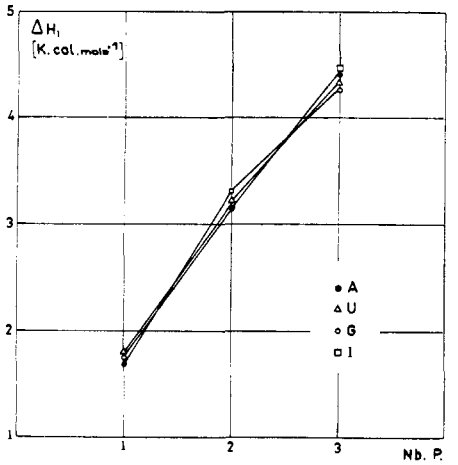

Figure 3. Plot of ΔH_i values *vs.* number of phosphate groups. Nucleotides of adenine, guanine, uracil, and hypoxanthine are symbolized by A, G, U, and I, respectively.

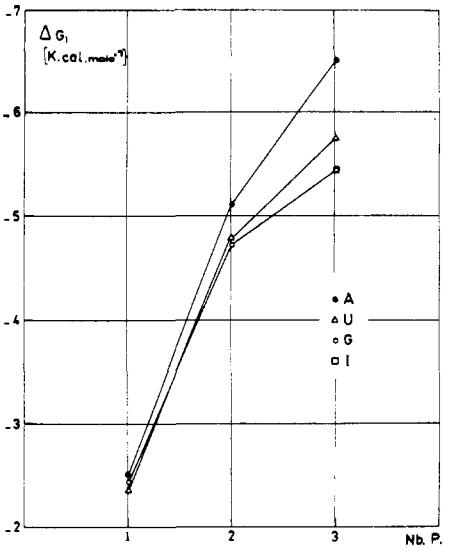

Figure 4. Plot of ΔG_i values *vs.* number of phosphate groups. The symbols used are the same as those of Figure 3.

Since the stability constant of ATP–Mg^{2-} is greater than the corresponding hydroxylated nucleotide triphosphates studied here and since the complex formation induces a pK ring shift it can be expected that the ring is affected by Mg^{2+}.

However, a direct binding of the Mg^{2+} to the ring of adenine nucleotides has not been demonstrated by direct spectroscopic techniques, *i.e.*, ORD,[15] nmr,[11,12,16,17] or uv.[18]

Yet, as suggested by Glassman, *et al.*,[19] and Kuntz, *et al.*,[20] if the metal nucleotide complexes contain a water molecule simultaneously coordinated to the metal ion and hydrogen bonded to the ring moiety, the spectroscopic studies would be inefficient, particulary in the case of a diamagnetic ion such as Mg^{2+}. Table VII shows that in all cases the driving force of the complex formation is the entropy increase accompanying the reaction.

However, the entropy increase is greater in the case of ATP–Mg^{2-} formation than in other example. These facts are in good agreement with the discussion of Glassman, *et al.*,[19] and with the possible structure of ATP–Mg^{2-} complex as suggested by the latter authors for the complexes of ATP with Co^{2+}, Ni^{2+}, and Mn^{2+}.

Acknowledgment. The authors are grateful to Dr. J. A. Fondarai, University Aix-Marseille II, for his help with statistical analysis.

(15) W. G. McCormick and B. H. Levedahl, *Biochim. Biophys. Acta*, **34**, 303 (1959).
(16) S. Hammes, G. E. Maciel, and I. S. Waugh, *J. Amer. Chem. Soc.*, **83**, 2394 (1961).
(17) J. A. Happe and M. Morales, *J. Amer. Chem. Soc.*, **88**, 2077 (1966).
(18) P. W. Schneider, H. Brintzinger, and H. Erlenmeyer, *Helv. Chem. Acta*, **47**, 992 (1964).
(19) T. A. Glassman, C. Cooper, L. W. Harrison, and T. J. Swift, *Biochemistry*, **10**, 843 (1971).
(20) G. P. P. Kuntz, T. A. Glassman, C. Cooper, and T. J. Swift, *Biochemistry*, **11**, 538 (1972).