Thermodynamic Studies of the Formation and Ionization of the Magnesium(II) Complexes of ADP and ATP over the pH Range 5 to 9[1]

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Abstract: The formation and ionization of the Mg^{2+} complexes of ADP and ATP have been studied over the pH range 5–9 by resin competition and pH titration methods, respectively. Using empirical activity coefficient equations the data have been extrapolated to zero ionic strength, and $\Delta F°$, $\Delta H°$, and $\Delta S°$ have been calculated. The complexes $(adp)Mg^-$ and $(atp)Mg^{2-}$, and their conjugate acids (adp)HMg and $(atp)HMg^-$, are stable solely by virtue of very favorable entropy changes which outweigh quite unfavorable enthalpy changes. However a comparison of their partial molal entropies relative to those of the parent ionic species indicates that charge cancellation is far from complete in the complexes, *i.e.*, they are of the ion-pair association type—a feature that may well be an important factor in the further binding of the complexes to their specific enzymes.

The influence of metal ions on the hydrolysis of ATP and other high energy phosphates has long been known. Interest has centered on Mg^{2+} because it is an essential ion for many phosphorylation reactions. Further, since Mg^{2+} ion forms complexes of varying stability with atp^{4-}, $(atp)H^{3-}$, adp^{3-}, $(adp)H^{2-}$, and PO_4^{3-}, it might be expected to play an important role in the thermodynamics of ATP hydrolysis.

We have therefore carried out a systematic investigation of the formation of the Mg^{2+} complexes of ADP and ATP and their ionization over a wide range of experimental conditions to serve as the basis for a more detailed examination of the factors governing the hydrolysis reaction and to provide the requisite data for calculating the variation of ΔF_{obsd} with pH and Mg^{2+} ion concentration in terms of the ionization and complex formation equilibria.

Abbreviations and Symbols. The usual abbreviations ADP and ATP for adenosine di- and triphosphate are used when there is no need to specify the ionic species precisely. When this is essential, adp^{3-} and atp^{4-} are used to designate the nucleotide species in which the phosphate groups are fully ionized: likewise the completely unionized forms are denoted by $(adp)H_3$ and $(atp)H_4$, and the partially ionized forms by $(adp)H_2^-$ and $(atp)H_3^-$, etc.

In the same way glu-1-p^{2-}, gly-1-p^{2-}, and gly-2-p^{2-} are used to denote the fully ionized forms of glucose-1-phosphate, glycerol-1-phosphate, and glycerol-2-phosphate, respectively.

The constants for the secondary ionization of the terminal phosphate group in ADP and ATP are denoted by K_3 and K_4 since this is the third and fourth ionization, respectively, of the anhydride group as a whole in these molecules. The constants for the ionization of the conjugate acid species of the Mg^{2+} complexes of ADP and ATP are denoted by K_{1c} and K_{2c} since these are, respectively, the first (and only) and the second ionization with respect to the anhydride group that these species can undergo. Following general practice[2] the stability constants for the formation of the Mg^{2+} complexes of the nucleotides are referred to as K_{ML} in the case of the fully ionized ligands, adp^{3-} and atp^{4-}, and as K_{MHL} when the ligands are in their conjugate acid forms.

Both ionization and stability constants are written without superscripts unless there is need to make a distinction between values at finite ionic strength and the value at zero ionic strength, when the superscripts, *i.e.*, K' and K^0 are employed, respectively.

The abbreviations tma^+ and tpa^+ stand for the tetramethyl- and tetra-*n*-propylammonium cations, respectively.

Materials

Resin. Dowex 1-X8 in the Cl^- form for use in the complex formation studies was stirred in a large excess of 5% NaCl for 6 hr to ensure complete conversion to the Cl^- form. It was then washed thoroughly and allowed to dry at room temperature and humidity.

Tetraalkylammonium Compounds. tpaBr and tmaCl salts and tpaOH (10% in water) were obtained from Eastman Kodak Co. and used without further purification.

Phosphates. ATP and ADP were obtained from Sigma Chemical Co. in the following forms: disodium salt of ATP extracted from rabbit muscle and monosodium salt of ADP extracted from horse muscle. The Sigma Co. assay for inorganic phosphate indicated that the ATP and ADP were 98 and 98–100% pure, respectively. Titration curves also indicated high purity. The ATP was used within 1 month of arrival, the ADP immediately because it decomposes 1–3% per month even at a storage temperature of 0°.

Before studying the various ionization and Mg^{2+} complex formation equilibria, the sodium ion in the ATP and ADP was replaced by the tetrapropylammonium ion, tpa^+. This was essential because complex formation with Na^+ is perceptible even at the dilutions employed in these experiments, as shown in section A below. The procedure, which involves an exchange

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[1] This work was undertaken as part of a research program on the thermodynamics of phosphate reactions, supported by USPHS Grant AM 04764. A preliminary report of this work was presented before the Biological Chemistry Section at the 48th National Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 1964; P. George, R. C. Phillips, and R. J. Rutman, *Federation Proc.*, 23, 265 (1964).

[2] J. Bjerrum, G. Schwarzenbach, and L. G. Sillén, "Stability Constants, Part I, The Chemical Society, London, 1957, pp XII and XIII.

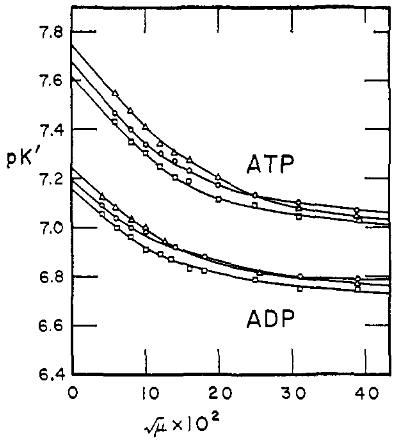

Figure 1. Plots of pK' against $\sqrt{\mu}$ for the secondary phosphate ionization of ATP and ADP at 10°, □; 25°, O; and 37°, △; tpaBr is the supporting electrolyte.

reaction with Dowex 50 W-X8 in its tpa$^+$ form, has been outlined in a previous paper.[3]

Experiments and Results

A. Thermodynamic Data for the Secondary Phosphate Ionizations of ATP and ADP, (*i.e.*, (atp)H^{3-} $\rightleftharpoons$ atp^{4-} + H$^+$, and (adp)H^{2-} $\rightleftharpoons$ adp^{3-} + H$^+$ with Ionization Constants K_4 and K_3, Respectively). The method employed to study these ionizations has been described at length in a previous publication[3] and the values obtained for the standard free energies, enthalpies, and entropies of ionization are given later in Tables I and IV and Figure 1.

Table I. pK' Functions for the Secondary Phosphate Ionization of ATP and ADP, with Tetra-*n*-propylammonium Ion as the Cation[a]

Temp, °C	Function for the ATP ionization $(\mathrm{atp})\mathrm{H}^{3-} \overset{K_4}{\rightleftharpoons} \mathrm{atp}^{4-} + \mathrm{H}^+$	Function for the ADP ionization $(\mathrm{adp})\mathrm{H}^{2-} \overset{K_3}{\rightleftharpoons} \mathrm{atp}^{3-} + \mathrm{H}^+$
10	$7.62 - 3.48\sqrt{\mu} + 4.95\mu$	$7.15 - 2.48\sqrt{\mu} + 3.85\mu$
25	$7.68 - 3.56\sqrt{\mu} + 4.90\mu$	$7.20 - 2.54\sqrt{\mu} + 3.84\mu$
37	$7.73 - 3.64\sqrt{\mu} + 4.25\mu$	$7.24 - 2.60\sqrt{\mu} + 3.34\mu$

[a] The error is estimated to be ±0.04 pH units in all cases.

However, the results of some additional experiments are reported below because they have an important bearing on the other equilibria studied in this paper, and because they illustrate the extent to which thermodynamic data for these ionizations would be in error if Na$^+$ salts were used, or if values were calculated from the results using tpa$^+$ salts without extrapolation to zero ionic strength. The principle of the method depends very simply on identifying the pH of a solution half-neutralized with respect to the secondary phosphate ionization as pK'. The method is justifiable for these ionizations since pK' fulfills the condition $4 < \mathrm{p}K' < 10$ and, in addition, there are no other groups in the nucleotides which ionize in the same pH range.[4] Starting with the most dilute solution that gives reproducible pH measurements, the variation of pK' with ionic strength is determined by the addition of neutral salt comprised of electrostatically weak ions, namely tpaBr.

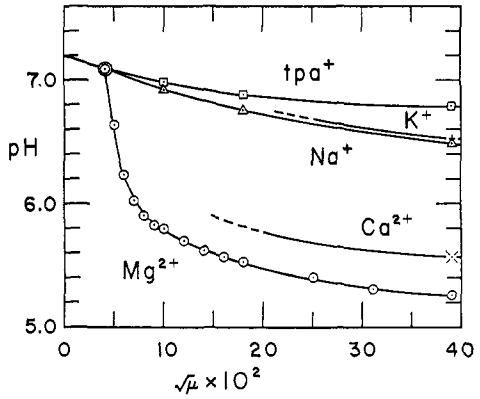

Figure 2. Data illustrating the influence of complex-forming cations on the pH of an ADP solution at 25°, half-neutralized with respect to the secondary phosphate ionization. The value in the double circle indicates the pH of the original ADP–tpa$^+$ solution, and the families of curves the values resulting from the addition, in separate experiments, of tpa$^+$, K$^+$, Na$^+$, Ca^{2+}, and Mg^{2+} salts. For simplicity the pH values have been plotted against $\sqrt{\mu}$ uncorrected for complex formation.

The need to use a salt containing an electrostatically weak cation, and indeed to replace the alkali metal ions in the original ATP and ADP, is demonstrated very clearly by the data in Figure 2 for a typical experiment carried out with ADP. The point enclosed by the double circles at $\sqrt{\mu} = 4 \times 10^{-2}$ gives the pH of the initial ADP–tpa$^+$ solution, half-neutralized with respect to the secondary phosphate ionization. From top to bottom, the family of curves give the pH values resulting from the addition of tpa$^+$, K$^+$, Na$^+$, Ca^{2+}, and Mg^{2+} salts, respectively, plotted against the ionic strength of the solutions calculated on the basis that no complex formation occurs. The divergence of the curves for the singly and doubly charged monatomic cations from that for tpa$^+$ demonstrates that complex formation is an important factor even at these dilutions, the affinity of the nucleotide for the cation increasing in the order $\mathrm{K}^+ < \mathrm{Na}^+ < \mathrm{Ca}^{2+} < \mathrm{Mg}^{2+}$, in agreement with other results in the literature.[5]

B. Thermodynamic Data for the Formation of the Mg^{2+} Complexes of ATP and ADP (*i.e.*, atp^{4-} + Mg^{2+} $\rightleftharpoons$ (atp)Mg^{2-}, and adp^{3-} + Mg^{2+} $\rightleftharpoons$ (adp)Mg$^-$). The resin competition method developed by Schubert[6] was used in which an anion-exchange resin competes with Mg^{2+} for the nucleotide. The basic equation for this method is

$$\frac{1}{D} = \frac{K'_{\mathrm{ML}}}{D_0} \times [\mathrm{Mg}^{2+}] + \frac{1}{D_0} \qquad (1)$$

where D is the distribution coefficient of atp^{4-} between the resin and solution and D_0 is the distribution coefficient at zero concentration of Mg^{2+}. By measuring D at different Mg^{2+} concentrations, K'_{ML} can thus be obtained from the slope and intercept of the plot of $1/D$ against [Mg^{2+}].

The procedure employed was as follows. Ten samples of resin large enough to adsorb about 70% of the nucleotide from solution (*i.e.*, 10 to 500 mg, depending on the nucleotide used, the pH, and the ionic

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(3) R. C. Phillips, P. George, and R. J. Rutman, *Biochemistry*, **2**, 501 (1963).

(4) W. D. Kumler, *J. Am. Chem. Soc.*, **60**, 859 (1938).

(5) R. M. Smith and R. A. Alberty, *ibid.*, **78**, 2376 (1956).

(6) J. Schubert, *J. Phys. Chem.*, **56**, 113 (1952).

strength) were weighed out to within 0.3 mg and added to each of ten screw-cap culture tubes.

A nucleotide solution was prepared so as to give a final concentration of 0.07 m*M* nucleotide, 0.01 *M* tris buffer (adjusted to pH 8.7 with HCl), and 0.07, 0.10, or 0.17 *M* tpaBr to give a range of ionic strength. Ten milliliters of this solution was pipetted into eight of the ten tubes. The other two tubes were filled with 10 ml of an otherwise identical solution, but omitting the nucleotide, to serve as blanks in the optical density measurements to follow.

From a stock solution of $MgCl_2$, which had been standardized by the Mohr method and checked by Fajan's method, varying amounts of $MgCl_2$ of less than 1.5 ml were added to three pairs of the nucleotide solutions such that for the pair of solutions containing the most Mg^{2+} ion (~0.1 m*M* for ATP and ~1.2 m*M* for ADP) only about 30% of the nucleotide would be adsorbed by the resin.

The final volumes of all ten tubes were then brought to 11.5 ml with a solution of tmaCl which was twice the molarity of the $MgCl_2$ solution used. This made the concentration of Cl^- ion in all ten tubes the same.

A blank solution and a nucleotide solution containing no resin were prepared to give a measure of total nucleotide (adsorbed, complexed, and free) in each of the eight solutions containing resin and nucleotide.

The two resin-containing blanks and the eight resin-containing nucleotide solutions were then brought to equilibrium over 2–4 hr (depending on the temperature) in a specially designed, temperature-controlled, rotary mixer.

The ten resin-containing tubes were then removed and filtered immediately. The optical density of each solution at 260 mμ was determined with a Beckman DU spectrophotometer. Since the optical density of the nucleotide solution without resin gave the total nucleotide remaining in solution (complexed and free), *D* was readily calculated.

The Mg^{2+} concentration was obtained by subtracting the Mg^{2+} complexed by the nucleotide from the total amount added, the amount of Mg^{2+} complexed being calculated by successive approximations.

This procedure was carried out in triplicate at ionic strengths of 0.07, 0.10, and 0.17 and at temperatures of 5, 25, 45, and 65°. As a check on the possibility of ATP and ADP hydrolysis occurring at the higher temperatures one set of samples was allowed to equilibrate for 2.5 hr and another for 4 hr at 65°. No significant differences in the results were detectable.

$1/D$ was plotted against $[Mg^{2+}]$ for each set of samples. The stability constants derived from the slopes and intercepts of these plots according to eq 1 are recorded in logarithmic form in Table II.

It is clear that these stability constants do not vary enough to serve as a basis for an extrapolation to $\mu = 0$ in order to determine pK^0_{ML}. An attempt was made to carry out similar measurements at lower ionic strengths, but the nature of the exchange reaction made it impossible.

Table II. Stability Constants for the Formation of the Mg^{2+} Complexes of ATP and ADP in Their Fully Ionized Forms at Various Ionic Strengths[a]

	Log K'_{ML} for the reaction $atp^{4-} + Mg^{2+} \longleftrightarrow (atp)Mg^{2-}$			Log K'_{ML} for the reaction $adp^{3-} + Mg^{2+} \longleftrightarrow (adp)Mg^{-}$		
Temp, °C	$\sqrt{\mu} \times 10^2 = 25.5$	$\sqrt{\mu} \times 10^2 = 32.1$	$\sqrt{\mu} \times 10^2 = 41.6$	$\sqrt{\mu} \times 10^2 = 19.8$	$\sqrt{\mu} \times 10^2 = 32.8$	$\sqrt{\mu} \times 10^2 = 42.3$
5	4.46	4.45	4.38	3.48	3.24	3.14
25	4.60	4.63	4.54	3.65	3.44	3.33
45	4.78	4.81	4.70	3.83	3.60	3.46
65	4.96	4.92	4.85	4.00	3.76	3.64

[a] The values are averages of three separate determinations of K'_{ML} in each case.

Since values of pK'_{ML} for the complex formation reactions could not be obtained at sufficiently low ionic strengths to permit extrapolation to $\mu = 0$, the empirical extrapolation procedure described in an earlier paper was used.[7] It is based on the assumption that to a first approximation nucleotide species of the same charge type have the same activity coefficients. Taking the formation of the ATP complex as the example

$$atp^{4-} + Mg^{2+} \overset{K_{ML}}{\longleftrightarrow} (atp)Mg^{2-} \tag{2}$$

the expression for the thermodynamic stability constant is

$$K^0_{ML} = K'_{ML}\frac{\gamma_{(atp)Mg^{2-}}}{\gamma_{atp^{4-}}}\frac{1}{\gamma_{Mg^{2+}}} \tag{3}$$

Multiplying numerator and denominator by $\gamma_{(atp)H^{3-}}$ gives

$$K^0_{ML} = K'_{ML}\frac{\gamma_{(atp)Mg^{2-}}}{\gamma_{(atp)H^{3-}}}\frac{\gamma_{(atp)H^{3-}}}{\gamma_{(atp)^{4-}}}\frac{1}{\gamma_{Mg^{2+}}}$$

and making the assumptions that $\gamma_{(atp)Mg^{2-}} \approx \gamma_{(adp)H^{2-}}$ and $\gamma_{(atp)H^{3-}} \approx \gamma_{adp^{3-}}$, this expression becomes

$$K^0_{ML} = K'_{ML}\frac{\gamma_{(adp)H^{2-}}}{\gamma_{adp^{3-}}}\frac{\gamma_{(atp)H^{3-}}}{\gamma_{atp^{4-}}}\frac{1}{\gamma_{Mg^{2+}}} \tag{4}$$

The activity coefficient terms $\gamma_{(adp)H^{2-}}/\gamma_{adp^{3-}}$ and $\gamma_{(atp)H^{3-}}/\gamma_{atp^{4-}}$ are quite familiar because they have appeared previously for the secondary phosphate ionizations of ADP and ATP relating pK' to pK^0. In Table I these terms are given in a logarithmic form as functions of $\sqrt{\mu}$ and μ, and a similar expression for $\gamma_{Mg^{2+}}$ has been worked out using the modified Debye–Hückel theory from the data of Robinson and Stokes.[8] Making the various substitutions, the following equation is thus obtained for the ATP reaction

$$pK'_{ML} = pK^0_{ML} + 6.10\sqrt{\mu} - 8.74\mu + \frac{2.04\sqrt{\mu}}{1 + 6.02\sqrt{\mu}} \tag{5}$$

with an estimated error of 0.06 in pK'_{ML}.

Substituting the pK'_{ML} values given in Table II for 25° and ionic strengths of 0.065, 0.10, and 0.17 gives pK^0_{ML} values of −5.80, −5.90, and −5.80, respectively, with a mean of −5.83 and an estimated uncertainty of ±0.10 ($\Delta F^0_{ML} = -7.95 \pm 0.13$ kcal/mole). Using this mean value of pK^0_{ML} the entire variation of pK'_{ML} with ionic strength may be calculated over the range $\mu = 0$ to 0.17 using eq 5. $(\Delta H_{ML})_{\mu \approx 0.1}$ was obtained by plotting the change in pK'_{ML} averaged over the three ionic strengths against $1/T$ (see Figure 3). No

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(7) P. George, R. C. Phillips, and R. J. Rutman, *Biochemistry*, **2**, 508 (1963).

(8) R. A. Robinson and R. H. Stokes, *Trans. Faraday Soc.*, **36**, 733 (1940).

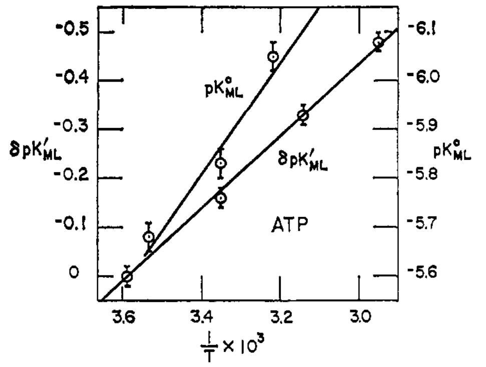

Figure 3. van't Hoff isochore plots of stability constant data for the formation of the Mg^{2+} complex of the fully ionized species of ATP, *i.e.*, atp^{4-}. The $\delta pK'_{ML}$ plot refers to data at $\mu \approx 0.1$ and the pK^0_{ML} plot to extrapolated values at $\mu = 0$.

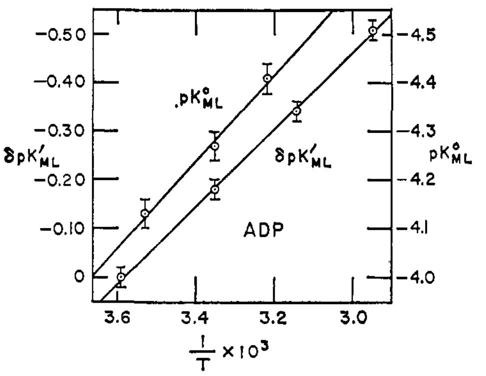

Figure 4. van't Hoff isochore plots of stability constant data for the formation of the Mg^{2+} complex of the fully ionized species of ADP, *i.e.*, adp^{3-}. The $\delta pK'_{ML}$ plot refers to data at $\mu \approx 0.1$ and the pK^0_{ML} plot to extrapolated values at $\mu = 0$.

distinction was made between the increments at the various ionic strengths because within experimental error they are identical. The value of $(\Delta H_{ML})_{\mu \approx 0.1}$ obtained in this way is 3.3 ± 0.3 kcal/mole. pK'_{ML} values for 10 and 37° were read off the plot of $\delta pK'_{ML}$ against $1/T$ in Figure 3 and extrapolated to $\mu = 0$ by the same extrapolation procedure using the appropriate activity coefficient data for 10 and 37° listed in Table I. A plot of pK^0_{ML} against $1/T$ (see Figure 3) gave $\Delta H^\circ_{ML} = 5.1 \pm 0.3$ kcal/mole. The substantial difference from the value at $\mu = 0.1$ is a consequence of the convergence of the curves relating pK'_4 to $\sqrt{\mu}$ at high ionic strength that ultimately results in the curves at 25 and 37° crossing over (see Figure 1). ΔS°_{ML} for the ATP reaction at 25°, calculated from the equation $\Delta F^\circ_{ML} = \Delta H^\circ_{ML} - T\Delta S^\circ_{ML}$, is 43.7 ± 1.5 eu.

Similar calculations from the data in Table II for the ADP reaction gave the following results: $pK^0_{ML} = -4.14$, -4.27, and -4.39, with a mean of -4.27 ± 0.10; $\Delta F^\circ_{ML} = -5.83$ kcal/mole; $(\Delta H_{ML})_{\mu \approx 0.1} = 3.6 \pm 0.3$ kcal/mole; $\Delta H^\circ_{ML} = 4.3 \pm 0.3$ kcal/mole; and $\Delta S^\circ_{ML} = 34.0 \pm 1.5$ eu. The van't Hoff isochore plots from which $(\Delta H_{ML})_{\mu \approx 0.1}$ and ΔH°_{ML} were obtained are shown in Figure 4.

C. Thermodynamic Data for the Ionization of the Conjugate Acids of the Mg^{2+} Complexes of ATP and ADP (*i.e.*, $(atp)HMg^- \rightleftharpoons (atp)Mg^{2-} + H^+$, and $(adp)HMg \rightleftharpoons (adp)Mg^- + H^+$ with Ionization Constants K_{2c} and K_{1c}, Respectively). The complications and difficulties involved in getting thermodynamic data for the ionization of the conjugate acid species of the Mg^{2+} complexes of ATP and ADP can best be appreciated by reference to the cycle of equilibria that are important in the pH range 5 to 9, *i.e.*

$$\begin{tikzcd}
(atp)H^{3-} + Mg^{2+} \arrow[r, leftrightarrow, "K_4"] \arrow[d, leftrightarrow, "K_{MHL}"] & atp^{4-} + H^+ + Mg^{2+} \arrow[d, leftrightarrow, "K_{ML}"] \\
(atp)HMg^- \arrow[r, leftrightarrow, "K_{2c}"] & atpMg^{2-} + H^+
\end{tikzcd} \qquad (6)$$

It is self-evident that the study of the secondary phosphate ionization and the formation of the Mg^{2+} complexes by the fully ionized ATP and ADP, as described in sections A and B, was greatly facilitated by the opportunity to isolate these equilibria, working with solutions free from complexing cations in the first case and with a relatively alkaline solution of pH 8.7 in the second case.

In order to isolate the ionization of the conjugate acid species of the Mg^{2+} complexes so that the pH titration method described in section A could be used, a Mg^{2+} concentration of about 0.05 and 0.10 *M* would be necessary for ATP and ADP, respectively, if the same low nucleotide concentrations were to be employed. These Mg^{2+} concentrations would contribute 0.15 and 0.30 to the total ionic strength, and thus make determinations at low ionic strength impossible. On the other hand, if low concentrations of Mg^{2+} were used, although the reaction could be studied at low ionic strength the participation of the other equilibria would necessitate calculating K'_{2c} (or K'_{1c}) from K'_4 (or K'_3), K'_{ML}, and the hydrogen ion and magnesium ion concentrations. Values obtained in this way would reflect the uncertainty of 0.06 in pK'_{ML} and the inaccuracy involved in estimating the free magnesium ion concentration from the total added. Inspection of the cycle of equilibria shows that as the total magnesium ion concentration is decreased, a greater fraction of the total nucleotide participates in the other equilibria, and as a consequence, K'_{ML} and K'_4 (or K'_3) become more significant in the calculation of K'_{2c} (or K'_{1c}). A formal proof of this can be set out by solving the equilibrium equations for K_{2c} (or K_{1c}) in terms of K_4 (or K_3), K_{ML}, $[H^+]$, and $[Mg^{2+}]$, and taking the derivative

$$\left(\frac{\partial K_{2c}\ (\text{or } K_{1c})}{\partial K_{ML}}\right)_{K_4(\text{or } K_3),\ [H^+],\ [Mg^{2+}]}$$

Furthermore, it can be shown that as the total concentration of Mg^{2+} is decreased, the percentage accuracy of the estimation of the free Mg^{2+} concentration decreases.

From these considerations it was decided that the most reliable determinations of pK'_{2c} and pK'_{1c} for extrapolation to $\mu = 0$ could be made by using Mg^{2+} concentration such that (a) the ionization of the conjugate acid species predominated to such an extent that the corrections arising from the participation of the other equilibria amounted to less than 0.15 p*K* units, and (b) the ionic strengths were low enough to fall well within the range of the activity coefficient data listed in Table I. Measurements were accordingly carried out using the pH titration method described in section A, but with 30.9 mM $MgCl_2$ present in all the solutions. K_{2c} (for the ATP reaction) was calculated from the following equation for the hydrogen ion concentration at the half-titration point

$$\frac{[H^+]_{1/2}}{0.5[\text{total ATP}]} = \frac{(K_4K_{ML}K_{2c}[Mg^{2+}] - K_4K_{ML}[Mg^{2+}][H^+]_{1/2} + K_4K_{2c})}{(K_4K_{ML}K_{2c}[Mg^{2+}] + K_4K_{ML}[Mg^{2+}][H^+]_{1/2} + K_{2c}[H^+]_{1/2} + K_4K_{2c})} \qquad (7)$$

which corresponds to Kumler's equation in its simplest form, $K = [H^+]_{1/2}$, for the case in which no Mg^{2+} was present.[9] The equation was used in this particular form, rather than one solved explicitly for K_{2c}, because it is more compact and easy to handle. A similar equation was used to calculate K_{1c} for the ADP reaction.

The experimental data and the values calculated for pK'_{2c} and pK'_{1c} at 10, 25, and 40° at the ionic strength of 0.09 are given in Table III. For the reasons outlined above it was impracticable to obtain equally reliable values over a range of ionic strengths, and the extrapolations to $\mu = 0$ have been based on these single values. For the ATP reaction the activity coefficient data for the secondary phosphate ionization of AMP were adopted because the charge change is identical, namely −1 to −2. For the ADP reaction the charge change is 0 to −1, and, in the absence of experimental activity coefficient data for this type of nucleotide ionization, an interpolation procedure was used based on the observation that for the other ionizations the simple Debye–Hückel relation holds up to an ionic strength of about 0.01, beyond which pK' rapidly becomes almost independent of μ. Although this is a rough approximation the error in the extrapolated value for pK^0_{1c} is not likely to be very great because the smallest charges are involved in this ionization, namely 0 and −1. The maximum pK increment estimated in this way only amounts to 0.08 unit.

Table III. Ionization Constants for the Conjugate Acid Species of the Mg^{2+} Complexes of ATP and ADP at 10, 25, and 40° and at $\mu = 0.09^a$

Temp, °C	pH half-neut	pK'
	$(atp)HMg^- \underset{}{\overset{K_{2c}}{\rightleftarrows}} (atp)Mg^{2-} + H^+$	
10	5.30	5.26
25	5.25	5.21
40	5.21	5.17
	$(adp)HMg \overset{K_{1c}}{\rightleftarrows} (adp)Mg^- + H^+$	
10	5.53	5.41
25	5.44	5.30
40	5.40	5.26

[a] In the ATP experiments the nucleotide and the $MgCl_2$ contributed 0.00324 and 0.0906 to the ionic strength, and in the ADP experiments 0.0018 and 0.0906, respectively.

Since it was found that ΔH for the secondary phosphate ionization of AMP was independent of ionic strength[10] it was assumed that the enthalpies of ionization for $(atp)MgH^-$ and (adp)MgH are also independent of ionic strength. From plots of pK'_{2c} and pK'_{1c} against $1/T$, ΔH°_{2c} and ΔH°_{1c} were thus obtained as 1.22 ± 0.55 and 2.02 ± 0.81 kcal/mole, respectively, and the corresponding entropies of ionization were calculated from the usual equation (see Table IV).

D. Thermodynamic Data for the Formation of the Mg^{2+} Complexes of the Conjugate Acid Species of ATP and ADP (*i.e.*, $(atp)H^{3-} + Mg^{2+} \leftrightarrow (atp)HMg^-$, and $(adp)H^{2-} + Mg^{2+} \leftrightarrow (adp)HMg$). Inspection of the cycle of equilibria set out at the beginning of section C (*i.e.*, eq 6) shows that the following simple additive relationship holds for the standard free energy changes

$$\Delta F_4^\circ + \Delta F^\circ_{ML} - \Delta F^\circ_{2c} = \Delta F^\circ_{MHL} \qquad (8)$$

with similar relationships for ΔH° and ΔS°. The values calculated from these equations for the formation of the Mg^{2+} complexes of the conjugate acid species, $(atp)H^{3-}$ and $(adp)H^{2-}$, are listed in Table IV, along with values for the thermodynamic stability constants, K^0_{MHL}.

It was decided, however, to undertake experimental determinations of K'_{MHL} as a check on the reliability of the values obtained by this indirect procedure. Since the same activity coefficient data in Table I which were used in extrapolating the equilibrium constants for the other three reactions to zero ionic strength would have to be used for this reaction too, nothing would be gained by an extrapolation of experimental values of K'_{MHL}. A direct comparison of the experimental values and the related apparent thermodynamic quantities at $\mu \approx 0.1$ has therefore been made with those calculated from the cycle of equilibria at the same ionic strength. These calculated values are listed in the second and fourth columns of Table V for the ATP and ADP reactions, respectively.

Values of the stability constants for the formation of the Mg^{2+} complexes of $(atp)H^{3-}$ and $(adp)H^{2-}$ were determined at $\mu = 0.1$ by essentially the same resin competition method as described in section B for the complexes of atp^{4-} and adp^{3-}. The only significant difference was that the solutions were buffered at pH 4.1 with a 0.01 M acetate buffer, the pH being adjusted with 10% (tpa)OH.

This pH was chosen so that as far as possible the formation of $(atp)HMg^-$ and (adp)HMg were the predominant equilibria, and as a consequence the other equilibria in each cycle did not have to be taken into account in calculating values for K'_{MHL}. This working pH is some 3 pH units less than the pK' values of 7.1 and 6.8 for the secondary phosphate ionizations of ATP and ADP, respectively, and about 2 pH units greater than those for the weakest primary phosphate ionizations. Hence, as far as the phosphate ionizations are concerned, $(atp)H^{3-}$ and $(adp)H^{2-}$ constitute at least 98% of the reactant species at equilibrium. The pK' values for the ionization of $(atp)MgH^-$ and (adp)MgH are 5.2 and 5.3, hence at pH 4.1 these conjugate acid species constitute 93 and 94% of the product species at equilibrium.

However pK' for the adenine ring ionization in ATP and ADP (and presumably in the Mg^{2+} complexes too) is about 4, and so with respect to this ionization both reactant and product species are present as approximately 1:1 mixtures with the adenine ring in its conjugate acid and conjugate base forms. It is in fact physically impossible to study the formation of the Mg^{2+} complexes of $(atp)H^{3-}$ and $(adp)H^{2-}$ under conditions

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(9) W. D. Kumler, *J. Am. Chem. Soc.*, 60, 860 (1938).

(10) R. C. Phillips, P. George, and R. J. Rutman, *Biochemistry*, 2, 502 (1963).

Table IV. Thermodynamic Data for the Ionization and Mg^{2+} Complex Formation Reactions of ATP and ADP at 25°, over the pH Range 5 to 9

Reaction	pK^0	$\Delta F°$, kcal/mole	$\Delta H°$, kcal/mole	$\Delta S°$, eu	pK' function
$(atp)H^{3-} \overset{K_4}{\rightleftarrows} atp^{4-} + H^+$	7.68 ± 0.01	10.48 ± 0.02	-1.68 ± 0.30	-40.7 ± 1.0	$\{7.68 - 3.56\sqrt{\mu} + 4.90\mu\} \pm 0.04$
$atp^{4-} + Mg^{2+} \overset{K_{ML}}{\rightleftarrows} (atp)Mg^{2-}$	-5.83 ± 0.10	-7.95 ± 0.10	5.1 ± 0.30	43.7 ± 1.5	$\{-5.83 + 6.10\sqrt{\mu} - 8.74 + (2.04\sqrt{\mu}))/1 + 6.02\sqrt{\mu})\} \pm 0.10$
$(atp)HMg^- \overset{K_{2c}}{\rightleftarrows} (atp)Mg^{2-} + H^+$	5.44 ± 0.05	7.42 ± 0.07	1.22 ± 0.55	-20.8 ± 2.0	$\{5.44 - 1.52\sqrt{\mu} + 2.52\mu\} \pm 0.03$
$(atp)H^{3-} + Mg^{2+} \overset{K_{MHL}}{\rightleftarrows} (atp)\text{-}HMg^{-}$ [a]	-3.59 ± 0.12	-4.89 ± 0.16	2.2 ± 1.25	23.8 ± 4.0	$\{-3.59 + 4.06\sqrt{\mu} - 6.36\mu + (2.04\sqrt{\mu})/(1 + 6.02\sqrt{\mu})\} \pm 0.12$
$(adp)H^{2-} \overset{K_3}{\rightleftarrows} adp^{3-} + H^+$	7.20 ± 0.01	9.83 ± 0.02	-1.37 ± 0.30	-37.5 ± 1.0	$\{7.20 + 2.54\sqrt{\mu} + 3.84\mu\} \pm 0.04$
$adp^{3-} + Mg^{2-} \overset{K_{ML}}{\rightleftarrows} (adp)\text{-}Mg^-$	-4.27 ± 0.10	-5.83 ± 0.14	4.3 ± 0.30	33.9 ± 1.5	$\{-4.27 + 4.06\sqrt{\mu} - 6.36\mu + (2.04\sqrt{\mu})/(1 + 6.02\sqrt{\mu})\} \pm 0.10$
$(adp)HMg \overset{K_{1c}}{\rightleftarrows} (adp)Mg^- + H^+$	5.38 ± 0.10	7.35 ± 0.14	2.02 ± 0.81	-17.9 ± 3.0	$\{5.38 - 0.51\sqrt{\mu} + 0.82\mu\} \pm 0.06$
$(adp)H^{2-} + Mg^{2+} \overset{K_{MHL}}{\rightleftarrows} (adp)\text{-}HMg$ [a]	-2.45 ± 0.20	-3.35 ± 0.27	0.9 ± 1.4	14.3 ± 5.0	$\{-2.45 + 2.03\sqrt{\mu} - 3.34\mu + (2.04\sqrt{\mu})/(1 + 6.02\sqrt{\mu})\} \pm 0.20$

[a] Values for these reactions have been calculated from the corresponding values for the three preceding reactions in each case, according to the reaction cycle set out in eq 6.

Table V. Stability Constants and Apparent Thermodynamic Data for the Formation of the Mg^{2+} Complexes of the Conjugate Acid Species of ATP and ADP at 25° and $\mu = 0.1$. A Comparison between Experimental Values and Those Calculated Indirectly from Data for the Three Other Reactions in the Cycle of Ionization and Complex Formation Equilibria Set out in Eq 6

	$(atp)H^{3-} + Mg^{2+} \rightleftarrows (atp)HMg^-$		$(adp)H^{2-} + Mg^{2+} \rightleftarrows (adp)HMg$	
	Exptl	Calcd	Exptl	Calcd
Log K'_{MHL}	2.86 ± 0.15	2.70 ± 0.12	2.00 ± 0.20	1.95 ± 0.20
ΔF_{MHL}, kcal/mole	-3.90 ± 0.20	-3.69 ± 0.16	-2.73 ± 0.27	-2.66 ± 0.27
ΔH_{MHL}, kcal/mole	1.9 ± 0.9	1.5 ± 1.25	2.0 ± 1.1	1.0 ± 1.5
ΔS_{MHL}, eu	19.4 ± 3.7	17.4 ± 5.0	15.9 ± 4.6	12.3 ± 5.8

where the equilibrium would correspond exactly to that completing the cycle of equilibria set out above, namely with the adenine ring entirely in its conjugate base form and with the phosphate groups ionized to give solely $(atp)H^{3-}$, $(adp)H^{2-}$, $(atp)MgH^-$, and $(adp)MgH$. The point at issue is thus the extent to which the charge on the adenine group in its conjugate acid form affects the bonding of the Mg^{2+} ion. Although there has been and still is a good deal of controversy, the balance of the evidence does not favor significant ring–chain interaction in either the Mg^{2+} complexes or in the acids themselves.[11] For example, Nanninga (1957) found that throughout the pH range of the adenine ring ionization the bonding of the Mg^{2+} ion was virtually unaffected,[12] and we have found that the thermodynamic data for the secondary phosphate ionization are virtually the same for the five monophosphates, AMP, CMP, UMP, GMP, and IMP, and also for the corresponding di- and triphosphate compounds.[13] A comparison of the values of K_{MHL} and the related thermodynamic quantities determined at pH 4.1 with those calculated from the cycle of equilibria would therefore appear to be quite valid.

Several determinations of K'_{MHL} were carried out at each of the four temperatures, 5, 25, 45, and 65°, at ionic strengths of 0.10 and 0.11 for the ATP and ADP reactions, respectively. The values obtained were less reproducible than those for the formation of the complexes with atp^{4-} and adp^{3-}, which is to be expected because $(atp)H^{3-}$ and $(adp)H^{2-}$ form much weaker complexes.

The values of pK'_{MHL} at all four temperatures were plotted against $1/T$ and slopes were determined by the least-squares method from which $(\Delta H_{MHL})_{\mu \approx 0.1}$ was calculated giving 1.9 ± 0.9 and 2.0 ± 1.1 kcal/mole for the ATP and ADP reactions, respectively. These values and those of $(\Delta S_{MHL})_{\mu \approx 0.1}$, obtained from the usual equation, are listed in the second and fourth columns of Table V along with those of pK'_{MHL} and $(\Delta F_{MHL})_{\mu \approx 0.1}$. The agreement between the calculated and experimental values of all the quantities is well within experimental uncertainty, and, with this limitation in mind, these results can be taken as additional evidence for the absence of any significant ring–chain interaction.

Discussion

The following topics will be dealt with in turn: firstly, a comparison of the present results with those reported in the literature; secondly, a discussion of the thermodynamic data and various correlations which are of a general character and in no way depend on an exact knowledge of the binding of the Mg^{2+} ion and the extent to which desolvation occurs; and thirdly, a discussion of the thermodynamic data in relation to the chemical structure of the complexes. A detailed treatment of the influence of Mg^{2+} complex formation on

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(11) S. Watanabe, L. Evenson, and I. Gulz, *J. Biol. Chem.*, **238**, 324 (1963).

(12) L. B. Nanninga, *J. Phys. Chem.*, **61**, 1144 (1957).

(13) R. C. Phillips, P. Eisenberg, P. George, and R. J. Rutman, *J. Biol. Chem.*, 240, 4393 (1965).

Table VI. p*K*′ Values Reported for the Various Ionization and Mg^{2+} Complex Formation Reactions of ATP and ADP

Method	Supporting electrolyte	Temp, °C	Log K_{ML} ATP	Log K_{ML} ADP	Ref
A. For the Reactions, $atp^{4-} + Mg^{2+} \longleftrightarrow atp(Mg)^{2-}$, and $adp^{3-} + Mg^{2+} \longleftrightarrow (adp)Mg^{-}$					
pH titration	0.1 *M* KCl	20	4.00 ± 0.04	3.11 ± 0.05	*a*
pH titration	0.2 *M* (tpa)Br	25	3.47 ± 0.03	3.00 ± 0.05	*b*
Resin competition	0.1 *M* KCl	23	4.04	3.15	*c*
Competition in soln with 8-hydroxy-quinoline	0.1 *M* (tbea)Br	25	4.58	3.34	*d*
	0.1 *M* (tbea)Br	35	...	3.48	*d*
	0.1 *M* (tbea)Br	64	4.99	3.84	*d*
	0.22 *M* (tbea)Br	25	4.35	3.23	*d*
Competition in soln with 8-hydroxy-quinoline	0.1 *M* tris buffer	30	4.30 ± 0.04	...	*e*
	0.1 *M* triethanol-amine buffer	30	4.89 ± 0.04	...	*e*
	0.1 *M* N-ethyl-morpholine buffer	30	4.93 ± 0.04	...	*e*
pH titration	0.1 *M* (tea)Br	30	5.02 ± 0.06	...	*e*
Resin competition	0.1 *M* (tea)Br	25	4.37 ± 0.03	...	*f*
pH titration	0.1 *M* (tea)Br	25	4.43 ± 0.03	...	*f*
pH titration	0.1 *M* KNO_3	25	...	3.19 ± 0.01	*g*
Competition in soln with 8-hydroxy-quinoline	0.1 *M* N-ethyl morpholine buffer	30	4.86 ± 0.04	3.60 ± 0.04	*h*
Resin competition	0.1 *M* (tpa)Br	25	4.60 ± 0.03	3.50 ± 0.03	This paper
B. For the Reactions, $(atp)MgH^{-} \longleftrightarrow (atp)Mg^{2-} + H^{+}$, and $(adp)MgH \longleftrightarrow (adp)Mg^{-} + H^{+}$					
pH titration	0.1 *M* KCl	20	4.5 ± 0.1	4.7 ± 0.2	*a*
pH titration	0.2 *M* (tpa)Br	25	5.2 ± 0.2	5.1 ± 0.1	*b*
Nmr titration	0.1 *M* ATP, 0.1 *M* $MgCl_2$	...	5.4 ± 0.2	...	*i*
pH titration	0.031 *M* $MgCl_2$	25	5.21 ± 0.01	5.30 ± 0.03	This paper
C. For the Reactions, $(atp)H^{3-} + Mg^{2+} \longleftrightarrow (atp)MgH^{-}$, and $(adp)H^{2-} + Mg^{2+} \longleftrightarrow (adp)MgH$					
pH titration	0.1 *M* KCl	20	2.0 ± 0.1	1.5 ± 0.2	*a*
pH titration	0.2 *M* (tpa)Br	25	1.49 ± 0.09	1.45 ± 0.06	*b*
pH titration	0.1 *M* KNO_3	25	...	1.6 ± 0.02	*g*
Resin competition	0.1 *M* (tpa)Br	25	2.86 ± 0.15	2.00 ± 0.20	This paper
Calcd	0.1 *M* (tpa)Br	25	2.70 ± 0.12	1.95 ± 0.20	This paper

[a] A. E. Martell and G. Schwarzenbach, *Helv. Chim. Acta*, **39**, 653 (1956). [b] R.M. Smith and R. A. Alberty, *J. Am. Chem. Soc.*, **78**, 2378 (1956). [c] E. Walaas, *Acta Chem. Scand.*, **12**, 528 (1958). [d] K. Burton, *Biochem. J.*, **71**, 388 (1959). [e] W. J. O'Sullivan and D. D. Perrin, *Biochim. Biophys. Acta*, **52**, 612 (1961). [f] L. B. Nanninga, *ibid.*, **54**, 330 (1961). [g] M. T. Kahn and A. E. Martell, *J. Am. Chem. Soc.*, **84**, 3037 (1962). [h] W. J. O'Sullivan and D. D. Perrin, *Biochemistry*, **3**, 18 (1964). [i] M. Cohn and T. R. Hughes, Jr., *J. Biol. Chem.*, **237**, 176 (1962).

the hydrolytic fission of ATP will be presented in a later publication.

i. Comparison with Previous Results. Although several groups of workers have studied the formation and ionization of the Mg^{2+} complexes of ADP and ATP, the stability constants and ionization constants were not extrapolated to zero ionic strength, so only a rough comparison with the present values can be made by choosing similar experimental conditions, *i.e.*, 0.1 *M* (tpa)Br at 25°. As can be seen from inspection of Table VI, the experimental difficulties discussed above have led to a variety of reported results.

ii. Discussion of the Thermodynamic Data. One of the most remarkable aspects of the formation of these magnesium complexes is the adverse enthalpy change. Even for orthophosphate and its monoesters, glucose 1-phosphate and glycerol 2-phosphate, the values are +2.9, +2.9, and +3.4 kcal/mole, respectively,[14] and for adp^{3-} and atp^{4-} they rise to +4.3 and +5.1 kcal/mole (see Table IV). With favorable standard free energies of formation amounting to several kcal/mole, these complexes owe their stability entirely to the large and favorable entropy changes. Desolvation of the ionic species must obviously contribute very substantially.

The present study has also provided thermodynamic data for the formation of the Mg^{2+} complexes by the conjugate acids, $(adp)H^{2-}$ and $(atp)H^{3-}$. The same considerations hold—the enthalpy changes are again unfavorable, and the entropy changes alone account for the stability of the complexes.

With the data now available for the secondary phosphate ionization and the formation of the Mg^{2+} complexes of ADP and ATP it is possible to look for various correlations with the corresponding data for the orthophosphate esters, glucose 1-phosphate, and glycerol 1- and 2-phosphate.[14–17] A familiar theme in coordination chemistry has been the linear relationship between the standard free-energy changes for proton and metal ion binding with structurally related ligands. Figure 5 shows that a linear relationship holds for proton and Mg^{2+} ion binding to the orthophosphate esters and the nucleoside di- and triphosphates. But it must be borne in mind that this comparison involves the binding of one and not two protons, so that for each ligand there is a difference in charge type between the two reactions, quite apart from the difference in charge type from one ligand to another.

(14) H. B. Clarke, D. C. Cusworth, and S. P. Datta, *Biochem. J.*, **58**, 146 (1954).

(15) J. H. Ashby, E. M. Crooke, and S. P. Datta, *ibid.*, **56**, 198 (1954).

(16) J. H. Ashby, H. B. Clarke, E. M. Crooke, and S. P. Datta, *ibid.*, **59**, 203 (1955).

(17) S. P. Datta and A. K. Grzybowski, *ibid.*, **69**, 218 (1958).

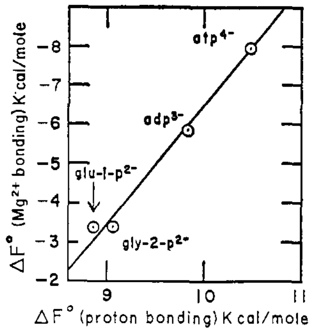

Figure 5. A relationship between the standard free-energy changes at 25° for proton and Mg^{2+} ion bonding by the phosphate esters glu-1-p^{2-} and gly-2-p^{2-} and the nucleotide species, adp^{3-} and atp^{4-}.

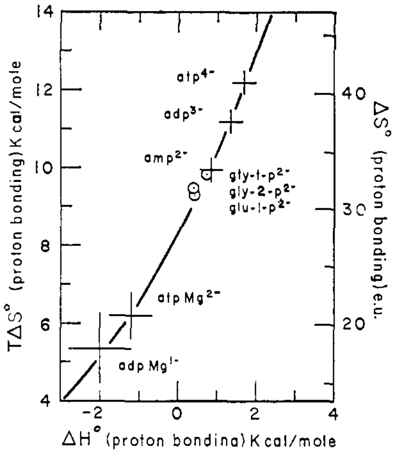

Figure 6. A relationship between the standard enthalpy and entropy changes at 25° for proton bonding by the phosphate esters glu-1-p^{2-}, gly-1-p^{2-}, and gly-2-p^{2-}, and the nucleotide species $(adp)Mg^-$, $(atp)Mg^{2-}$, amp^{2-}, adp^{3-}, and atp^{4-}.

Following on the observation that the major thermodynamic driving force in these reactions is the entropy change, and with the exception of proton addition to $(adp)Mg^-$ and $(atp)Mg^{2-}$, the sole driving force, it is of interest to enquire whether there is any systematic relationship between the favorable values of $\Delta S°$ and the (adverse) values of $\Delta H°$. $T\Delta S°$ and $\Delta S°$ have been plotted against $\Delta H°$ for proton combination in Figure 6, and for Mg^{2+} complex formation in Figure 7. The data show a regular trend in both cases. In plots of this kind, using the same scale for both axes, a slope of 45° would indicate a constant value for $\Delta F°$, *i.e.*, exact compensation between the effects responsible for the favorable $\Delta S°$ and unfavorable $\Delta H°$. In both cases, however, the "slopes" are greater than 45° since $\Delta F°$ becomes increasingly more favorable in going from the orthophosphate to the di- and triphosphate species. The "slope" is appreciably greater for Mg^{2+} complex formation in which there is nominally a greater cancellation of ionic charge; this is consistent with the hypothesis that contributions to the entropy change arising from desolvation of the ionic species are of paramount importance.

iii. **The Thermodynamic Data for the Mg^{2+} Complexes of ADP and ATP in Relation to Their Chemical Structure.** There is good evidence from studies of the infrared and nuclear magnetic resonance spectra that in the case of the Mg^{2+} complexes the metal ion is bonded only to the terminal and the adjacent phosphate group, with no additional bonding to a nitrogenous base group of the adenine.[18-20] Furthermore, in forming the conjugate acid species of the ATP complex, there is a pronounced chemical shift of the terminal phosphorus (γ) accompanied by a very slight shift of the adjacent phosphorus (β), indicating that the proton becomes attached to the terminal (γ) phosphate group in the complex and not to the adenine.[21] Titration curves of ATP and ADP obtained in the present study and in previous studies[22-24] bear this out, since the adenine ionization with $pK \approx 4$ is not significantly altered in the presence of 31 mM $MgCl_2$.

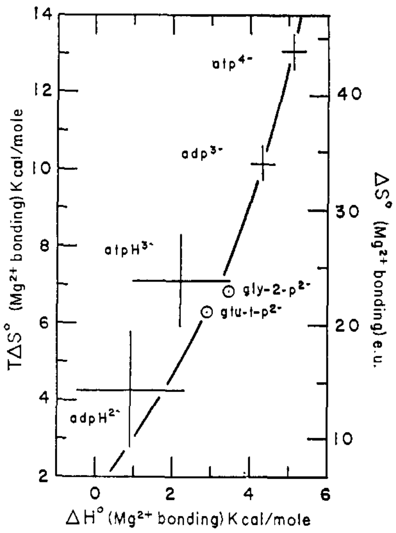

Figure 7. A relationship between the standard enthalpy and entropy changes at 25° for Mg^{2+} ion bonding by the phosphate esters glu-1-p^{2-} and gly-2-p^{2-}, and the nucleotide species $(adp)H^{2-}$, $(atp)H^{3-}$, adp^{3-}, and atp^{4-}.

In accord with these conclusions the similarity between the ionization data for $(atp)MgH^-$ and (adp)-MgH ($pK^0 = 5.44$, $\Delta H° = 1.22$ kcal/mole, and $\Delta S° = 20.8$ eu *vs.* $pK^0 = 5.38$, $\Delta H° = 2.02$ kcal/mole, and $\Delta S° = -17.9$ eu) indicate that the ionizing proton is leaving the same type of environment—such as that described above with the proton on the terminal phosphate group and Mg^{2+} linking the terminal and adjacent phosphate groups. It might have been expected that the pK values for $(atp)MgH^-$ and (adp)MgH would be much lower, because the values for the corresponding (*i.e.*, primary) ionizations in triphosphoric acid and pyrophosphoric acid are both about one[25] and those for $(atp)H_3^-$ and $(adp)H_3$

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[18] G. G. Hammes, G. E. Maciel, and J. S. Waugh, *J. Am. Chem. Soc.*, **83**, 2394 (1961).

[19] M. Cohn and T. R. Hughes, Jr., *J. Biol. Chem.*, **237**, 178 (1962).

[20] H. Brintzinger, *Biochim. Biophys. Acta*, **77**, 343 (1963).

[21] The usual designation of the phosphate groups in ATP with the terminal group γ, *e.g.*, adenosine-O-$P_\alpha O_2^-$-O-$P_\beta O_2^-$-O-$P_\gamma O_3^{2-}$ and in ADP with the terminal group β, *e.g.*, adenosine-O-$P_\alpha O_2^-$-O-$P_\beta O_3^{2-}$ tends to obscure the close similarity between the bonding sites for the Mg^{2+}, since, as far as ionic charge is concerned, the β and γ phosphate groups in ATP are identical with the α and β groups in ADP. Choice of the terminal group as α in both cases would be more in keeping with such important reactions as ionization, complex formation, hydrolytic fission, etc.

[22] H. G. Heis, *Biochim. Biophys. Acta*, **8**, 424 (1952).

[23] S. S. Spicer, *J. Biol. Chem.*, **199**, 301 (1952).

[24] R. M. Smith and R. A. Alberty, *J. Am. Chem. Soc.*, **78**, 2377 (1956).

[25] J. I. Watters, E. D. Loughran, and S. M. Lambert, *ibid.*, **78**, 4855 (1956).

are certainly in this region too. Apparently as far as the ionization of a proton from the terminal phosphate group is concerned the Mg^{2+} ion is by no means equivalent to two hydrogen ions. In fact its influence on this ionization is considerably less than that exerted by one hydrogen ion bound to the terminal group. This argues for bonding of a very different type in the case of the Mg^{2+} complexes, and, since the hydrogen is covalently bound, predominantly ionic bonding is indicated. While this conclusion could be regarded as self-evident and needing no justification, the lack of any appreciable charge cancellation as implied by predominantly ionic bonding has an important bearing in other respects.

If, as seems to be well established, the Mg^{2+} is bound to the β and γ phosphate groups in ATP and to the α and β groups in ADP, then, since these pairs of groups have identical charges in the two nucleotides it is not obvious why there should be such a difference in the thermodynamic data in relation to the orthophosphate complexes in which there is only a terminal group, $O{-}PO_3^{2-}$, to interact with the metal ion. In going from ADP to ATP the increment for the Mg^{2+} complex formation reaction with the fully ionized species is 10 eu in $\Delta S°$, 0.8 kcal/mole in $\Delta H°$, and 2.1 kcal/mole in $\Delta F°$, while in going from the orthophosphate esters to ADP the increments are only a little greater, about 12 eu in $\Delta S°$, 1.2 kcal/mole in $\Delta H°$, and 2.4 kcal/mole in $\Delta F°$ (see Table IV). The data for the secondary phosphate ionization show similar behavior although to a lesser extent, otherwise the correlations demonstrated in Figures 5, 6, and 7 would not exist. The only explanation would seem to be that even though the remaining (α) phosphate group in ATP is not bonded to the Mg^{2+} the presence of the metal ion profoundly alters the solvation of all the groups in the anhydride chain including the nonbonded group. To a lesser extent the presence of hydrogen on the terminal group evidently has the same effect. The configurations adopted by the phosphate groups in the pyrophosphate and triphosphate ions, $P_2O_7^{4-}$ and $P_3O_{10}^{5-}$, give a clue as to why this might happen. Structure determinations by X-ray analysis have shown that coulombic repulsion within the polyoxyanion, *i.e.*, charge separation, is a dominant factor. In $P_2O_7^{4-}$ the central P–O–P bond angle is 134° compared to the H–O–H bond angle of 105° in water; in $P_3O_{10}^{5-}$ the two corresponding angles are 122°, with the pair of charge-bearing oxygen atoms on the center group in a *trans* position with respect to pairs of oxygen atoms on each of the terminal groups.[26,27] With Mg^{2+} linking two groups, and with the bonding of hydrogen resulting in the cancellation of unit charge on one, the coulombic repulsion is substantially altered. This raises the possibility that there may be significant differences in the orientation of the phosphate groups with respect to each other in the various species. Hence considerable changes in solvation may be anticipated not only as a consequence of the new charge relationships set up, but also through a further effect on the configuration of the molecule as a whole.

The primary evidence for the dominant role of changes in solvation in these reactions, as pointed out above, is the very large favorable (positive) values of $\Delta S°$. In the gas phase the formation of both metal ion complexes and the conjugate acids would have negative values of $\Delta S°$ because of the loss in translational entropy associated with the decrease in the number of species in going from reactants to products. Favorable though the values are for the Mg^{2+} complexes of ATP and ADP in aqueous solution, the following approach suggests that the "effective" charge on the complexes is appreciably greater than that indicated by the net charge, *i.e.*, $(atp)Mg^{2-}$, $(adp)Mg^{-}$, etc., and as a consequence the values are not as favorable as they could be with more complete charge cancellation.

For this purpose any plot of the partial molal entropies or the relative values for each series of anions, *i.e.*, atp^{4-}, $(atp)H^{3-}$, $(atp)H_2^{2-}$, as a function of the ionic charge would suffice, the "effective" charge for the Mg^{2+} complex being read off according to its entropy value. Although the plot *vs.* Z would be the simplest, that *vs.* Z^2 has been adopted because Laidler (1956) and Laidler and Couture (1957) have shown that a Z^2 function, in keeping with the Born charging equation, is the better choice in the case of monatomic ions and simple oxyanions.[28,29] For the oxyanions, *e.g.*, ClO^-, HCO_3^-, SO_4^{2-}, HPO_4^{2-}, PO_4^{3-}, etc., they found the partial molal entropies to obey the empirical relationship

$$\bar{S}° = 40.2 + {}^3/_2 R \ln M - 108.8Z^2/nr \qquad (9)$$

where M is the molecular weight, Z the number of charges on the ion, n the number of charge-bearing oxygen atoms, and r the radius. In this treatment they employed "absolute" values of $\bar{S}°$, correcting the "practical" values which are based on the arbitrary choice of $\bar{S}°(H^+)$ as zero by appropriate multiples of −5.5 eu, the best value currently available for $\bar{S}°(H^+)$.

With the exception of the orthophosphate ions, practical (and hence absolute) values of $\bar{S}°$ are as yet unknown for all the other phosphate and polyphosphate ions. However it is a simple matter to calculate values

Table VII. Values of the Ionic Charge and the Absolute Partial Molal Entropies at 25° for Orthophosphate, Pyrophosphate, Triphosphate, Glycerol 2-Phosphate, ADP, and ATP, and Their Magnesium Complexes, Relative to the Values for the Species $H_2PO_4^-$, $H_2P_2O_7^{2-}$, $H_2P_3O_{10}^{3-}$, $(gly\text{-}2\text{-}p)H^-$, $(adp)H^{2-}$, and $(atp)H^{3-}$, Respectively[a]

Species	Rel $\bar{S}°_{abs}$	Z	Z_{eff}	Species	Rel $\bar{S}°_{abs}$	Z	Z_{eff}
$H_2PO_4^-$	0	1		$(gly\text{-}2\text{-}p)H^-$	0	1	
HPO_4^{2-}	−24.4	2		$gly\text{-}2\text{-}p^{2-}$	−26.3	2	
$MgHPO_4$	−41.4		2.5	$Mg(gly\text{-}2\text{-}p)$	−42.6		2.4
$H_2P_2O_7^{2-}$	0	2		$(adp)H^{2-}$	0	2	
$HP_2O_7^{3-}$	−23.7	3		$Mg(adp)H$	−24.9		2.8
$P_2O_7^{4-}$	−52.8	4		adp^{3-}	−32.0	3	
$MgP_2O_7^{2-}$	−56.2		4.1	$Mg(adp)^-$	−37.3		3.1
$H_2P_3O_{10}^{3-}$	0	3		$(atp)H^{3-}$	0	3	
				$Mg(atp)H^-$	−15.4		3.5
$HP_3O_{10}^{4-}$	−23.6	4		$Mg(atp)^{2-}$	−30.7		3.9
$P_3O_{10}^{5-}$	−51.7	5		atp^{4-}	−35.2	4	

[a] For the definition of Z_{eff} see the text.

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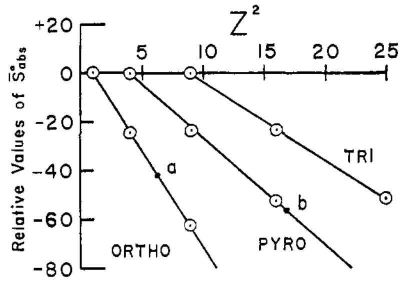

Figure 8. The absolute partial molal entropies at 25° for HPO_4^{2-} and PO_4^{3-}, $HP_2O_7^{3-}$ and $P_2O_7^{4-}$, and $HP_3O_{10}^{4-}$ and $P_3O_{10}^{5-}$ relative to the values for $H_2PO_4^-$ $H_2P_2O_7^{2-}$, and $H_2P_3O_{10}^{3-}$, respectively, plotted against ionic charge. The points a and b identify the entropy values for the magnesium complexes, $MgHPO_4$ and $MgP_2O_7^{2-}$, from which a measure of the "effective charge" on the complexes can be obtained.

for the ions and Mg^{2+} complexes in each series relative to any particular ion from the known values of ΔS° for ionization and complex formation. Values calculated in this way, taking $\bar{S}^\circ_{abs}(H^+) = -5.5$ eu and $\bar{S}^\circ_{pract}(Mg^{2+}) = -28.2$ eu, are listed in Table VII for the ions and Mg^{2+} complexes of ortho-, pyro-, and triphosphate, glycerol 2-phosphate, ADP, and ATP.

Comparison of the values for the Mg^{2+} complexes with those for the ions in each series immediately shows that they are far more negative than their net charges might lead one to expect. This is borne out by the plots of the relative values of $\bar{S}^\circ_{abs}$ *vs.* Z^2 in Figures 8 and 9. The effective charge on the Mg^{2+} complexes can readily be obtained from their entropy values, denoted by a, b, c, etc. Although this is a hypothetical quantity—the charge that could be attributed to the complexes if it were possible for them to be solvated in exactly the same way as the simple ions in each series—it gives a rough but striking demonstration of the lack of charge cancellation. The values are listed in Table VII; for Mg(gly-2-p), $Mg(adp)^-$, and $Mg(atp)^{2-}$, Z_{eff} is 2.4, 3.1, and 3.9, respectively; and for the conjugate acids, Mg(adp)H and $Mg(atp)H^-$, Z_{eff} is 2.8 and 3.5. The charge separation in the complexes thus has an effect on the partial molal entropy comparable on the average to about *two* discrete negative charges. This lack of charge cancellation might well be a very important factor in the further binding of the Mg^{2+} complexes to their specific enzymes.

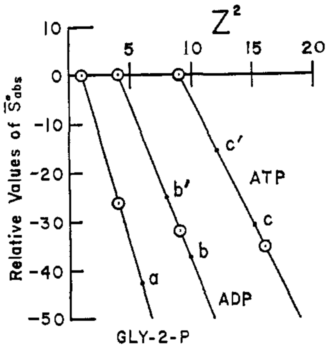

Figure 9. The absolute partial molal entropies at 25° for gly-2-p^{2-}, adp^{3-}, and atp^{4-} relative to the values for $(gly\text{-}2\text{-}p)H^-$, $(adp)H^{2-}$, and $(atp)H^{3-}$, respectively, plotted against ionic charge. The points a, b, and c identify the entropy values for the magnesium complexes Mg(gly-2-p), $Mg(adp)^-$, and $Mg(atp)^{2-}$, and b′ and c′ the values for the conjugate acid species Mg(adp)H and $Mg(atp)H^-$, from which a measure of the "effective charge" on the complexes can be obtained.