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"pH STAT" TITRATION METHOD FOR THE DETERMINATION OF THE THERMODYNAMIC QUANTITIES ASSOCIATED WITH THE FORMATION OF ATP–MAGNESIUM COMPLEXES*

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SUMMARY

A new technique of pH titration for the study of $ATPMg^{2-}$ complex formation reactions is described. The results obtained with this technique are in good agreement with the thermodynamic quantities obtained with calorimetric measurements. The stability constant, obtained at 30 °C and ionic strength 0.2, is $45\,700 \pm 2000$ M^{-1}. The pK value of $ATPH^{3-}$ ionization has been found to be equal to 6.96 in these experimental conditions. The Van 't Hoff isochore shows that no significant ΔC_p variation occurs between 3 and 39 °C; over this temperature range the ΔH is equal to 4.50 ± 1.34 kcal/mole.

INTRODUCTION

A great deal of work has been done on the thermodynamic studies of the reactions of complex formation between adenine nucleotides and divalent metals. Many techniques have been used for this purpose, such as pH titration, spectroscopic techniques and techniques using competitive ligands such as ion-exchange resins or dyes. Recently, two of us[1] have described a microcalorimetric technique suitable for these studies. A few years ago, Phillips[2] pointed out the existence of very big discrepancies in the thermodynamic values obtained for the $ATPMg^{2-}$ complex formation reaction; thus, the log of the stability constant varies from 2.92 to 4.35 at 25 °C and ionic strength 0.2, and there are similar variations in the enthalpy and the entropy (ΔH and ΔS) values reported.

Our results[1] are not in agreement with the data of many authors and more especially our ΔH calorimetric value is different from the ΔH values obtained under practically the same physicochemical conditions by the Van 't Hoff method[3–5].

The purpose of this paper is to describe a new pH-titration technique and to compare the thermodynamic quantities obtained by this technique with the calorimetric values reported previously[1].

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MATERIALS AND METHODS

The pH titrations were made with a Radiometer apparatus 26 equipped with the glass electrode type G202C. All the experiments were carried out under pure N_2. The sodium salt of ATP was purchased from Boehringer and repurified by ion-exchange chromatography (DEAE-Sephadex). Na^+ was exchanged by tetramethylammonium ion. The purity of the $MgSO_4$ used was checked by titrimetry. The ionic strength in all experiments was maintained at I 0.2 with tetramethylammonium bromide as the supporting electrolyte. The ATP and Mg^{2+} comprised only 5% of the total ionic strength. The protons evolved during the complex formation reaction were neutralized using tetramethylammonium hydroxide ($2.63 \cdot 10^{-3}$ M). The experimental temperature was maintained by means of a precision thermostat. The volume of the liquid phase used for all the experiments was 5 ml with only negligible variation (<2%, w/v) occurring during the titration. All the calculations were made with a Programma 101 Olivetti computer.

The experiments were done at constant pH using either discontinuous or continuous addition of Mg^{2+}. For discontinuous addition, the Mg^{2+} was added as two sequential portions into the reaction vessel. In this case the quantity of protons evolved was estimated after each addition. The continuous additions were performed using a micropump injecting the Mg^{2+} solution at a constant rate into the reaction vessel. With this device the protons evolved were continuously neutralized by the pH stat. The curves obtained by this method give directly the quantity of tetramethylammonium hydroxide added for neutralization *versus* the time. From these curves it is easy to calculate the quantity of protons evolved corresponding to the quantity of Mg^{2+} added.

RESULTS

The method used is based on the shift of the pK of the second ionization of the γ-phosphate group of ATP when $ATPH^{3-}$ is complexed with Mg^{2+}. This shift was estimated with precision by Phillips *et al.*[5]. The pK of $ATPH^{3-}$ is approximatively equal to 7 and the pK of $ATPHMg^-$ is 5.26 at I 0.2. So for pH values high enough (pH >7.5), we can assume that the quantity of protons evolved during the complex formation reaction is proportionnal to the quantity of $ATPMg^{2-}$ formed according to the scheme:

$$ATP^{4-} + Mg^{2+} \rightleftharpoons ATPMg^{2-}$$

$$ATPH^{3-} \rightleftharpoons ATP^{4-} + H^+$$

Discontinuous procedure

Two sequential Mg^{2+} additions as described in Materials and Methods were made into the reaction vessel containing ATP^{4-} and $ATPH^{3-}$. Let us call:

a the total nucleotide concentration, *i.e.* $[ATP^{4-}]+[ATPH^{3-}]$ or during the titration $[ATP^{4-}]+[ATPH^{3-}]+[ATPMg^{2-}]$.

b the Mg^{2+} concentration corresponding to the first Mg^{2+} addition.

c the Mg^{2+} concentration corresponding to the sum of the first addition and the second addition $c = 2b$.

x and y the concentration of $ATPMg^{2-}$ at equilibrium after the first and the second Mg^{2+} additions, respectively.

α_i, with $i = 0$, 1, or 2, the concentration of ATP^{4-} before the Mg^{2+} additions and after the first and the second Mg^{2+} additions, respectively.

α_i' the concentration of $ATPH^{3-}$ with the same convention for i as above.

Z_1 and Z_2, the number of equivalents of H^+ evolved after the first and the second Mg^{2+} addition, respectively.

ϕ_1 and ϕ_2, the number of equivalents of H^+ per 1 corresponding to the first and the second Mg^{2+} addition, respectively ($\phi_1 = Z_1/V$, $\phi_2 = Z_2/V$, V=volume of the solution in the reaction vessel).

So if k is the ionization constant of $ATPH^{3-}$ we can write:

$$\alpha_i' = \alpha_i \frac{[H^+]}{k} \tag{1}$$

$$a = \alpha_0 + \alpha_0' = \alpha_0 + \alpha_0 \frac{[H^+]}{k} \tag{2}$$

$$a = \alpha_1 + \alpha_1' + x = \alpha_1 + \alpha_1 \frac{[H^+]}{k} + x \tag{3}$$

$$a = \alpha_2 + \alpha_2' + y = \alpha_2 + \alpha_2 \frac{[H^+]}{k} + y \tag{4}$$

$$\phi_1 = \alpha_0' - \alpha_1' = (\alpha_0 - \alpha_1) \frac{[H^+]}{k} \tag{5}$$

$$\phi_2 = \alpha_0' - \alpha_2' = (\alpha_0 - \alpha_2) \frac{[H^+]}{k} \tag{6}$$

From relationships (2) (3) and (5) we have:

$$x = \alpha_0 - \alpha_1 + (\alpha_0 - \alpha_1) \frac{[H^+]}{k} = \frac{\phi_1}{[H^+]} \cdot k + \phi_1 = \phi_1 \left(\frac{k}{[H^+]} + 1 \right) = \phi_1 \varepsilon \tag{7}$$

with $\varepsilon = (k/[H^+]) + 1$. From relationships (2) (4) and (6) we have

$$y = \phi_2 \varepsilon \tag{8}$$

The apparent stability constant (K_{app}) of the $ATPMg^{2-}$ formation reaction at definite ionic strength is:

$$K_{app} = \frac{x}{\alpha_1(b - x)} = \frac{y}{\alpha_2(c - y)} \tag{9}$$

The apparent measured stability constant ($K_{app,\,measd}$) of the $ATPMg^{2-}$ formation reaction when the concentration of $ATPH^{3-}$ is not negligible is:

$$K_{app,\,measd} = \frac{x}{(\alpha_1 + \alpha_1')\,(b - x)} = \frac{x}{(a - x)\,(b - x)} \tag{10}$$

or

TABLE I

RESULTS OBTAINED WITH THE DISCONTINUOUS PROCEDURE

Results obtained using the means of all values of Columns III and VI: $K_{\mathrm{app,\,measd}} = 45\,800\ \mathrm{M}^{-1}$. p$K$ = 6.96. It should be noticed that the values are very close to the means calculated in the table. In all experiments $a = 5.73 \cdot 10^{-4}$ M, $b = 6 \cdot 10^{-4}$ M, $c = 12 \cdot 10^{-4}$ M, pH 8.00, I 0.2, t = 30 °C, tetramethylammonium hydroxide (TMAOH) titer = $2.63 \cdot 10^{-3}$ M.

Expt No.	*I* *µl TMAOH*	*II* $Z_1 \times 10^6$	*III* $\phi_1 \times 10^4$	*IV* *µl TMAOH*	*V* $Z_2 \times 10^6$	*VI* $\phi_2 \times 10^4$	*VII* ε	*VIII* *pK*	*IX* $K_{app,\,measd}$ (M^{-1})	*X* K_{app} (M^{-1})
1	75.3	0.1980	0.3960	87.1	0.2290	0.4580	12.049	6.957	40 500	44 100
2	76.8	0.2020	0.4040	88.3	0.2320	0.4641	11.924	6.962	44 600	48 700
3	76.7	0.2017	0.4034	88.2	0.2319	0.4639	11.934	6.961	44 300	48 300
4	77.4	0.2035	0.4071	88.9	0.2338	0.4676	11.847	6.965	45 200	49 400
5	75.5	0.1986	0.3971	87.7	0.2306	0.4613	11.927	6.961	37 700	41 100
6	76.1	0.2001	0.4003	88.0	0.2314	0.4629	11.926	6.961	40 700	44 400
7	76.2	0.2004	0.4008	88.1	0.2377	0.4634	11.909	6.962	40 700	44 400
8	77.4	0.2035	0.4071	89.8	0.2361	0.4723	11.664	6.972	38 700	42 300
9	77.2	0.2030	0.4061	88.8	0.2335	0.4671	11.843	6.965	43 900	47 900
10	77.3	0.2033	0.4066	89.3	0.2349	0.4697	11.754	6.969	41 200	45 000
11	77.1	0.2027	0.4055	88.8	0.2335	0.4671	11.850	6.963	43 500	47 500
Mean 95% confidence			0.4031			0.4652		6.963		45 700
			±0.0026			±0.0027		±0.003		± 2 000

$$K_{\text{app, measd}} = \frac{y}{(\alpha_2 + \alpha'_2)\,(c - y)} = \frac{y}{(a - y)\,(c - y)} \tag{11}$$

with

$$K_{\text{app}} = K_{\text{app, measd}}\left(1 + \frac{[\mathrm{H}^+]}{k}\right) \tag{12}$$

from relationships (7), (8), (10) and (11) it becomes:

$$\frac{\phi_1\varepsilon}{(a - \phi_1\varepsilon)(b - \phi_1\varepsilon)} = \frac{\phi_2\varepsilon}{(a - \phi_2\varepsilon)\,(c - \phi_2\varepsilon)} \tag{13}$$

and

$$\varepsilon = \frac{(c - b)\phi_1\phi_2 - \sqrt{\phi_1{}^2\phi_2{}^2(b - c)^2 - 4a(\phi_1\phi_2{}^2 - \phi_1{}^2\phi_2)\,(\phi_1 c - \phi_2 b)}}{2(\phi_1\phi_2{}^2 - \phi_1{}^2\phi_2)} \tag{14}$$

Experimental determination of ϕ_1 and ϕ_2 gives ε, and from ε, provided that the pH is known, it is easy to calculate k, $K_{\text{app, measd}}$, and, from relationship (12), K_{app}.

Table I shows the results obtained in a series of 11 experiments done at 30 °C where $a = 5.73 \cdot 10^{-4}$ M, $b = 6 \cdot 10^{-4}$ M, and $c = 12 \cdot 10^{-4}$ M. The results reported in Columns I, II and III correspond to the first Mg^{2+} addition. The values in Column I are the μl of tetramethylammonium hydroxide added for the neutralization of protons evolved after the addition of Mg^{2+}. These values are corrected for the slight acidification produced by the addition of the same quantity of magnesium under the same condition but without nucleotides in the reaction vessel. The results of Columns II and III are calculated from the values of Column I; these are Z_1 and ϕ_1, respectively.

The results reported in Columns IV, V and VI correspond to the second Mg^{2+} addition. The values in Column IV represent the total μl of tetramethylammonium hydroxide added for the neutralization of all the protons evolved after the two successive Mg^{2+} additions. So the Z_2 and ϕ_2 correspond to $12 \cdot 10^{-4}$ M of Mg^{2+} added.

Columns VII, VIII, IX and X show the ε, pK, $K_{\text{app, measd}}$, and K_{app} values. The average of all the important values are reported in the last line of Table I and, with a confidence level equal to 0.95, the means pK and K_{app} values are 6.963 ± 0.003 and $45\,700 \pm 2000\ \mathrm{M}^{-1}$, respectively.

Table II shows the results obtained for K_{app} at the same ionic strength but at five different temperatures.

Continuous procedure

Fig. 1 shows a titration curve obtained by this procedure. Experimental results can be plotted according to a linear transform. Therefore, if x_i becomes the concentration of ATPMg^{2-} in the reaction vessel and b_i the concentration of total Mg^{2+}, the relationship (10) becomes:

$$x_i = \frac{\dfrac{1}{K_{\text{app, measd}}} + a + b_i - \sqrt{\left(\dfrac{1}{K_{\text{app, measd}}} + a + b_i\right)^2 - 4ab_i}}{2} \tag{15}$$

TABLE II

ln K_{app} VALUES AND K_{app} MEANS *VERSUS* TEMPERATURE (pH 8.00, *I* 0.2)

Number of experiments	*Temp. (°C)*	*ln K_{app}*	*K_{app} (M^{-1})*
8	3	10.022, 10.006, 10.067, 10.002, 9.859, 9.981 10.018, 9.908	21 700 ± 1700
10	17	10.645, 10.377, 10.389, 10.139, 10.466, 10.621, 10.204, 10.502, 10.410, 10.521	34 100 ± 4000
6	26	10.609, 10.653, 10.662, 10.687, 10.605, 10.640	41 900 ± 2100
11	30	10.694, 10.793, 10.785, 10.808, 10.624, 10.701, 10.701, 10.652, 10.777, 10.714, 10.768	45 700 ± 2000
8	39	11.038, 11.082, 10.859, 10.908, 10.884, 10.822, 10.889, 10.961	55 800 ± 6300

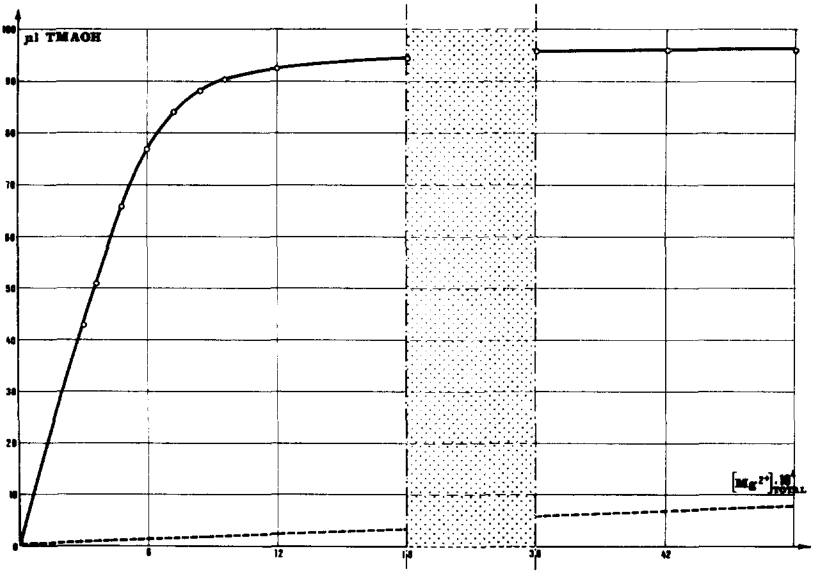

Fig. 1. Experimental curve obtained with the continuous procedure. $a = 6.28 \cdot 10^{-4}$ M, $[Mg^{2+}]$ in the pump $= 12 \cdot 10^{-2}$ M, rate of pumping = 1.2 ml/h. Dotted line corresponds to the control but without ATP. Continuous line curve is obtained by substraction of dotted line from direct recording. ○ correspond to each Mg^{2+} concentration chosen for the establishment of Fig. 2.

The positive root is impossible. The hyperbola described by the relationship (15) is not equilateral.

Now if we express x_i as a function $b_i' = b_i - x_i$ which is the free $[Mg^{2+}]$ of the medium, the relationship (10) becomes:

$$x_i = \frac{K_{\text{app, measd}} \cdot a \cdot b'_i}{1 + K_{\text{app, measd}} \cdot b'_i} \tag{16}$$

or

$$\frac{1}{x_i} = \frac{1}{K_{\text{app, measd}} \cdot a \cdot b'_i} + \frac{1}{a} \tag{17}$$

The volume μ_i of the titrant added tetramethylammonium hydroxide during the pumping of Mg^{2+} is:

$$\mu_i = F \cdot x_i \tag{18}$$

with

$$F = \frac{V}{\varepsilon \cdot [S]} \tag{19}$$

and $[S]$ = titer of the titrant.

For the determination of x_i the value of F can be estimated from k, or directly measured by an addition of a large excess of Mg^{2+} into the solution of nucleotide under the same experimental conditions. So, for a large excess of Mg^{2+} all the protons of $ATPH^{3-}$ are evolved, and under these conditions

$$x_i = a \quad \text{and} \quad \mu_{\max} = F \cdot a \tag{20}$$

From relationships (18) and (20):

$$x_i = \frac{\mu_i}{\mu_{\max}} \cdot a \tag{21}$$

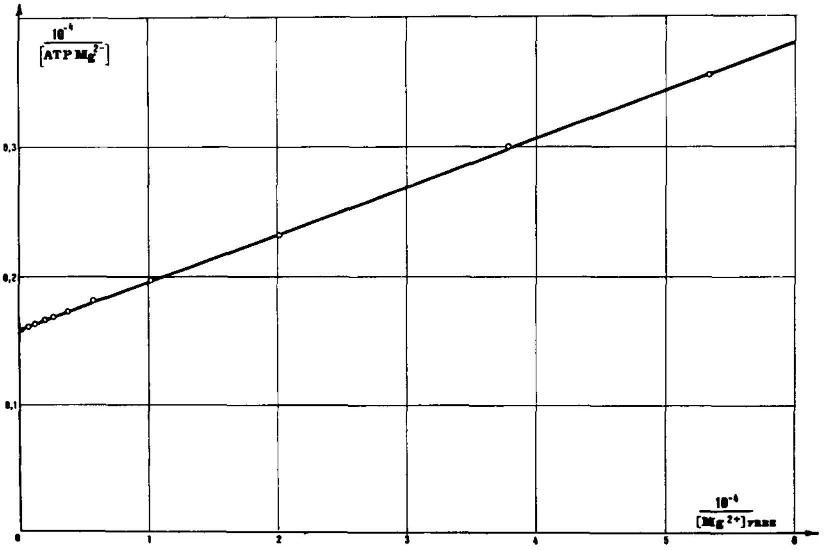

Fig. 2. $1/[ATPMg^{2-}]$ *versus* $1/[Mg^{2+}]$ corresponding to the experiment reported in Fig. 1. The slope of the straight line = $1/(K_{\text{app, measd}} \cdot a)$.

TABLE III

RESULTS OBTAINED WITH THE CONTINUOUS PROCEDURE

Expt No.	*pH*	$K_{app,\,measd}$ (M^{-1})	K_{app} (M^{-1})	r^*
1	8.00	43 400	47 300	0.999
2	8.00	40 500	44 100	0.999
3	8.00	45 700	49 800	0.999
4	8.50	43 400	44 700	0.987
5	8.50	38 700	40 000	0.999
Mean 95% confidence			45 100 ±3 600	

* Correlation coefficient corresponding to the straight line obtained on plotting $1/[\text{ATPMg}^{2-}]$ *versus* $1/[\text{Mg}^{2+}]$ (Fig. 2).

Relationship (21) and the curve of Fig. 1 give b_i'. Fig. 2 shows the plot of $1/x_i$ *vs* $1/b_i'$. The slope of the straight line obtained gives $1/K_{\text{app, measd}} \cdot a$ from which $K_{\text{app, measd}}$ can be calculated. Table III shows the results obtained by this procedure.

ΔH "Van 't Hoff"

From the results of Table II, it is possible to calculate the ΔH "Van 't Hoff" of ATPMg^{2-} formation. The statistical analysis of the 43 sets of experimental data

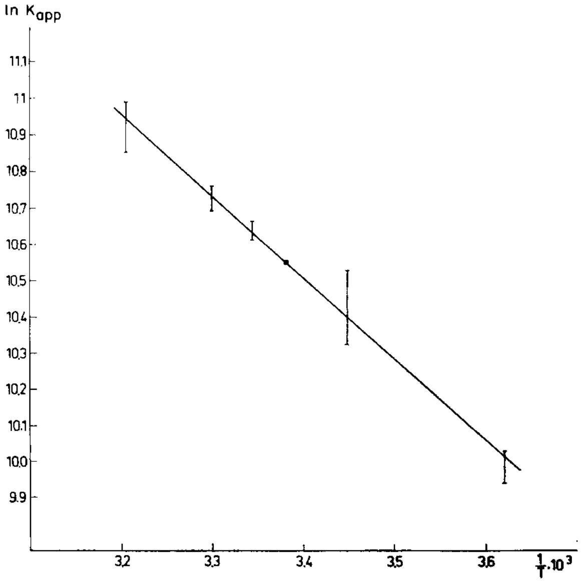

Fig. 3. Van 't Hoff isochore plots of stability constant data for the formation of ATPMg^{2-} at pH 8.00 and I 0.2. The regression line is obtained with 43 sets of experimental data of ln K_{app} *vs* $1/T$. For each experimental temperature the confidence intervals of ln K_{app} have been calculated with a confidence level of 0.95. The correlation coefficient is 0.958 and the linear Snedecor's F is 45.1. ●, means of ln K_{app} and $(1/T) \cdot 10^3$.

has been made using the analysis of variance of the regression. The slope of the regression line of ln K_{app} *vs* $1/T$ (Fig. 3) has been found to be equal to -2.252. The Snedecor's F coefficients for a linear and a non-linear term of the regression are 45.1 and 0.5, respectively. From the linear F value it can be calculated a confidence level for a straight line higher than 0.9995. The confidence intervals of the slope of the straight line have been calculated to be equal to -2.252 ± 0.671 with a confidence level of 0.95. The ΔH "Van 't Hoff" estimated from this analysis is equal to 4.50 ± 1.34 kcal/mole.

DISCUSSION

The apparent constant of $ATPMg^{2-}$ formation reaction obtained by this new titration method is in agreement with the value obtained previously by a microcalorimetric technique. Unfortunately, our data do not agree with all the results of the literature obtained under similar conditions of temperature and ionic strength. The closest values are those of Phillips *et al.*[5] who obtained, by a resin competition method, 40 000 M^{-1} at 25 °C and I 0.2.

If we neglect the slight correction for the ΔH of ionization of $ATPH^{3-}$, the ΔH "Van 't Hoff" obtained by the titration method is in good agreement with the ΔH "calorimetric" measured at the same ionic strength, but the precision is not so good. Moreover, the linear relation between ln K_{app} and $1/T$ indicates that the complex formation reaction does not significantly change the heat capacity of the solution over the temperature range 3–39 °C. This result is not in agreement with that of Alberty[6] who calculated an ΔC_p equal to 30 cal·mole^{-1}·degree^{-1} for the same reaction at 25 °C.

Finally, the pK value of $ATPH^{3-}$ obtained by our "pH stat" technique is in good agreement with the values reported or calculated for similar conditions of temperature and ionic strength[7–10]. Previously, some authors have used the pK shift of $ATPH^{3-}$ caused by addition of magnesium for the determination of the apparent constant formation of $ATPMg^{2-}$ complex; in almost all cases their K_{app} values are lower than ours[11–14] and rarely higher[15]. The closest figures being those of Nanninga[11] who obtained 26 700 M^{-1} at 25 °C with 0.1 M $(C_2H_5)_4N \cdot Br$ as supporting electrolyte. The obvious agreement between the values of K_{app} and ΔH obtained with two completely different technique suggests that for I 0.2 and temperature 30 °C the most probable value of K_{app} is 45 000–50 000 M^{-1}.

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