MICROCALORIMETRIC STUDIES ON THE FORMATION OF MAGNESIUM COMPLEXES OF ADENINE NUCLEOTIDES

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Abstract.—Values for the thermodynamic quantities (ΔF, ΔH, ΔS) in reactions in which complexes of adenine nucleotides with magnesium ion ($ATPMg^{--}$, $ADPMg^{-}$, AMPMg) are formed have been obtained by a microcalorimetric technique by using an isothermic Calvet's apparatus.

Experimental values measured at ionic strength $\mu = 0.2$ indicate that complex formation reactions are driven by the entropic factor and that stability of complexes increases with length of the phosphate chain.

Most important reactions in the field of bioenergetics involve the participation of magnesium complexes of adenine nucleotides. Much work has been devoted to the determination of the thermodynamic quantities of these complexes. Yet there is a great disparity in the results available, *vide*, in a recent review by R. C. Phillips *et al.*[1]

We report the use of a microcalorimetric technique which permits direct determinations of the enthalpy of these complex formation reactions, as well as estimations of the apparent equilibrium constants.

Materials and Methods.—*Microcalorimeter:* The apparatus used was constructed by the "Institut de Microcalorimétrie et de thermogénèse" CNRS, Marseilles, France, according to the Calvet principle (cf. Calvet and Prat).[2] This microcalorimeter is an isothermic differential heat fluxmeter, the sensitivity of which is 2.9 μwatt per millimeter recorder deflection. The experimental device is shown in Figure 1. Figure 2 exhibits the artifact observed when 1 ml of buffer contained in the siphon is injected into the pyrex calorimetric cell containing 5 ml of the same buffer. The beginning of the recording shows the constant stirring heat flow.

Experimental procedure: Five ml of triethanolamine buffer containing appropriate concentrations of tetramethylammonium chloride and of magnesium ion are pipetted into the pyrex calorimetric cell. The empty siphon is held by a ground-glass stopper on this cell. The cell is put into the calorimeter for temperature stabilization for about 5 hr. After this, 1 ml of adenine nucleotide solution which has been equilibrated with buffer of the same pH and ionic strength is injected into the siphon. The experiment can be started by pumping the nucleotide solution from the siphon into the calorimetric cell when the recording has come back to the base line (about one hr after filling the siphon). Pumping time is ten min.

All experiments were made at 30°C, pH 8.5, and at ionic strength 0.2 maintained by the electrolyte support (tetramethylammonium chloride), the concentration of which varies with the concentration of nucleotides and of magnesium ion. The concentration of TEA buffer is constant and equal to 0.15 *M*.

Chemicals: The sodium salts of adenosine triphosphate (ATP) and of adenosine diphosphate (ADP) (chromatographically pure) were purchased from Schwarz BioResearch Inc., and the acid form of adenosine monophosphate "puriss grade" (AMP) from Fluka. Sodium ion was not eliminated because of its low affinity for adenine nucleotides[3] and its low concentration with respect to the electrolyte support.

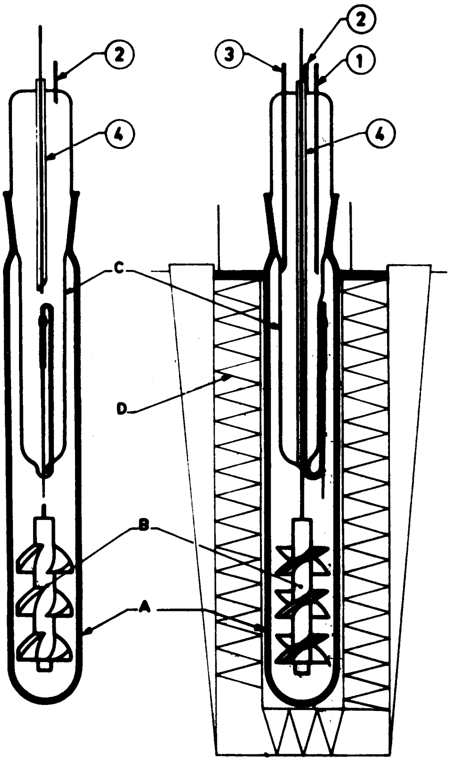

FIG. 1.—Experimental device used for the calorimetric determination, (A) Pyrex calorimetric cell. (B) Helix stirrer in Teflon. (C) Siphon: (1) tube for reactant introduction; (2) tube for the equilibration of the pressure between the siphon and the exterior; (3) tube for the equilibration of the pressure between the calorimetric cell and the exterior; and (4) access through the siphon for the stirrer axis. (D) Thermocouples surrounding the calorimetric cell.

Tetramethylammonium chloride and TEA were purchased from Eastman Kodak and Merck, respectively. Magnesium sulfate (RP) was supplied by Prolabo.

Calculation: All calculations were made with a small computer (Programma 101 Olivetti).

Results.—Principle of calculation: At pH 8.5 adenine nucleotides are almost entirely dissociated, i.e., present as ATP^{4-}, ADP^{3-}, and AMP^{2-}, and we can consider that, as a first approximation, the quantity which is not dissociated is negligible. For simplicity we shall call A: adenine nucleotide, M: magnesium ion, and A.M: complex.

The reaction studied is:

$$A + M \rightleftarrows A.M$$

If a and b are the initial concentrations of adenine nucleotide and magnesium ion respectively, and x the concentration of complex at equilibrium, the apparent formation constant is:

$$K_{\text{app}} = \frac{x}{(a - x)(b - x)} \qquad (1)$$

When this reaction occurs at constant temperature and pressure in a calorimeter of utilizable volume (V) the heat evolved during the reaction is:

$$Q_1 = x \cdot \Delta H^\circ \cdot V \qquad (2)$$

where ΔH° is the enthalpy of the complex formation reaction. If a second experiment is done under the same conditions, but with a different initial concentration (c) of magnesium ion, the concentration of complex at equilibrium will be y, and

$$K_{\text{app}} = \frac{y}{(a - y)(c - y)} \qquad (3)$$

and

$$Q_2 = y \cdot \Delta H^\circ \cdot V \qquad (4)$$

From the relationships 1, 2, 3, and 4, we obtain:

$$\Delta H^\circ = \frac{(c - b)\,Q_1 Q_2 \pm \sqrt{\Delta}}{2(Q_1\,ac - Q_2\,ab)V} \qquad (5)$$

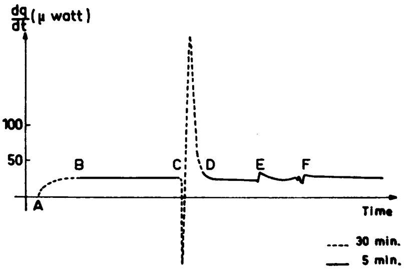

FIG. 2.—Artifacts produced by the stirring (AB), by the introduction of 1 ml of buffer into the siphon (CD) and by the injection of 1 ml of buffer into 5 ml of the same buffer from the siphon to the calorimetric cell (EF).

with $\Delta = Q_1^2 \cdot Q_2^2 (b^2 - 2bc + c^2) - 4(Q_1ac - Q_2ab)(Q_1 Q_2^2 - Q_2Q_1^2)$.

Experimental calorimetric determinations of Q_1 and Q_2 permit estimation of ΔH°, and the knowledge of ΔH° provides K_{app} from the relationships (1) and (2) or (3) and (4).

Experimental results.—(*a*) *ATPMg*$^{2-}$ *complex:* (1) ΔH° *measurement.* Assuming that, at the pH considered, ATP is entirely dissociated, the reaction of complex formation is: $ATP^{4-} + Mg^{2+} \rightleftharpoons ATPMg^{2-}$. In all experiments, we have chosen the adenosine triphosphate concentration (a) equal to 2.5×10^{-3} *M;* the magnesium ion concentration varies with the experiment; for Q_1 measurement we have $b = a$, and for Q_2, $c = 2a$. Figure 3 shows a thermogram of the $ATPMg^{2-}$ complex formation reaction. The area between the time axis and the curve represents the quantity of heat evolved during the reaction. The Q_1 and Q_2 values shown in Table 1 were obtained by integration of thermograms. The ΔH° obtained from equation (5) is equal to 4.16 kcal/mole. In reality, this value of ΔH° is only an apparent value, termed $\Delta H^\circ_{\text{app}}$ because, at pH 8.5, all ATP is not entirely dissociated and the true reaction scheme is:

$$\begin{array}{ccc} H^+ + ATP^{4-} + Mg^{2+} & \rightleftharpoons & ATPMg^{2-} + H^+ \\ \downarrow\uparrow & & \downarrow\uparrow \\ ATPH^{3-} + Mg^{2+} & \rightleftharpoons & ATPHMg^-. \end{array}$$

At ionic strength 0.2 and a temperature of 25°C. Phillips *et al.*[1, 4] give

$$pK_{ATPH^{3-}} = 7.05$$

$$pK_{ATPHMg^-} = 5.26.$$

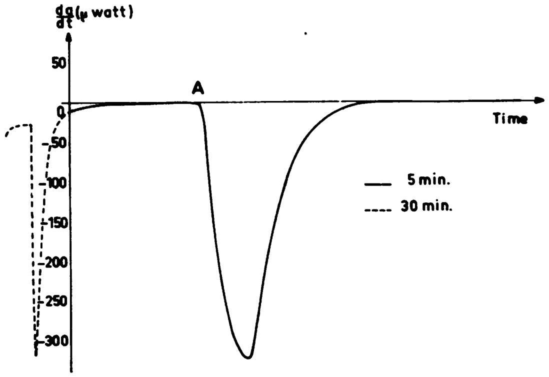

FIG. 3.—Thermogram of the formation of $ATPMg^{2-}$ complex formation. The dotted line represents the injection and the stabilization of 1 ml of ATP solution in the siphon (in this case the stabilisation is endother mic). The pumping of ATP solution begins from point A.

TABLE 1.

Exp. no.	Q_1 (cal)	Exp. no.	Q_1 (cal)	Exp. no.	Q_2 (cal)	Exp. no.	Q_2 (cal)
B59	0.0576	A42	0.0552	B53	0.0614	B82	0.0604
B60	0.0581	A43	0.0562	B54	0.0586	A37	0.0614
B61	0.0586	A44	0.0562	B55	0.0628	A38	0.0650
B62	0.0558	A65	0.0558	B56	0.0616	A39	0.0598
B63	0.0597	A66	0.0577	B57	0.0621	A40	0.0631
B64	0.0559	A67	0.0558	B78	0.0623	A61	0.0656
B83	0.0572	...	...	B79	0.0621	A62	0.0641
B84	0.0567	...	...	B80	0.0632	A63	0.0593
B85	0.0579	...	...	B81	0.0590	A64	0.0614
A41	0.0579	...	...	B82	0.0604	...	...

Q_1 mean = 0.0570 ± 0.0006 cal with a confidence value equal to 95%

Q_2 (mean) = 0.0620 ± 0.0009 cal with a confidence value equal to 95%

Q_1 and Q_2 experimental values of $ATPMg^{2-}$ complex formation reaction. These values are corrected for the heat of dilution of the reactants and for the heat of injection artifact.

Correction of ΔH° takes into account the neutralization heat of protons which are liberated during the reaction and the $ATPHMg^-$ complex formation.

Let us call: a, the total concentration of nucleotides before the reaction; α, the concentration of ATP^{4-} at equilibrium; α', the concentration of $ATPH^{3-}$ at equilibrium; x, the concentration of $ATPMg^{2-}$ at equilibrium; x', the concentration of $ATPHMg^-$ at equilibrium; $\Delta H^\circ{}_{A^{2-}}$, the enthalpy of the complex $ATPMg^{2-}$ formation reaction; $\Delta H^\circ{}_{A^-}$, the enthalpy of the complex $ATPMg^-$ formation reaction; ($ATPH^{3-} + Mg^{2+} \rightleftharpoons ATPHMg^- + 2.2$ kcal/mole according to the method of Phillips *et al.*); $\Delta H^\circ{}_p$, the enthalpy of ionization of $ATPH^{3-}$ (-1.68 kcal/mole: Phillips *et al.*); $\Delta H^\circ{}_n$, the enthalpy of ionization of the triethanolamine buffer 7.5 kcal/mole (our experimental determination).

The concentration of undissociated protons before reaction at any alkaline pH is equal to $ATPH^{3-}$ concentration, hence

$$\frac{[ATP^{4-}]}{[ATPH^{3-}]} = \frac{10^{-pK_{ATPH^{-3}}}}{10^{-pH}}. \tag{6}$$

At pH 8.5 $[ATPH^{3-}] = [ATP^{4-}]/28$ and $[ATPH^{3-}] = a/29$. When equilibrium is reached, equation (6) becomes:

$$\frac{\alpha}{\alpha'} = 10^{-pK_{ATPH^{-3}}}/10^{-pH} \tag{7}$$

and

$$x/x' = 10^{-pK_{ATPHMg^-}}/10^{-pH}. \tag{8}$$

At pH 8.5, the indissociated proton concentration is

$$[ATPH^{3-}] + [ATPHMg^-] = \alpha' + x' = \frac{\alpha}{28} + \frac{x}{1740}.$$

The quantity (z) of neutralized protons all of which arise from $ATPH^{3-}$ is

$$z = \left(\frac{a}{29} - \frac{\alpha}{28} - \frac{x}{1740}\right)V \doteq \left(\frac{x}{29} - \frac{0.016x}{29}\right)V.$$

and the neutralization heat (Q_n) evolved during the reaction is

$$Q_n = z(\Delta H^\circ_p - \Delta H^\circ_n)$$

$$Q_n = V\left(\frac{x}{29} - \frac{0.016x}{29}\right)(\Delta H^\circ_p - \Delta H^\circ_n).$$

With this correction equation (2) becomes

$$Q = V[x\Delta H^\circ_{A^{2-}} + x'\Delta H^\circ_{A^-} + z(\Delta H^\circ_p - \Delta H^\circ_n)]$$

and

$$Q = xV\left[\Delta H_{A^{2-}} + \frac{\Delta H^\circ_{A^-}}{1740} + \left(\frac{1}{29} - \frac{0.016}{29}\right)(\Delta H^\circ_p - \Delta H^\circ_n)\right]\cdot \tag{9}$$

Since $\Delta H^\circ_{A^{2-}}$, $\Delta H^\circ_{A^-}$, ΔH°_n, and ΔH°_p are constant, the measured heat is always proportional to the concentration of the ATPMg^{2-} complex.

We can now estimate the enthalpy of ATPMg^{2-} formation, i.e., the true ΔH°, using equation (9) (in this case only the positive root is meaningful)

$$\Delta H^\circ_{\text{app}} = \Delta H^\circ_{A^{2-}} + \frac{\Delta H^\circ_{A^-}}{1740} + \left(\frac{1}{29} - \frac{0.016}{29}\right)(\Delta H^\circ_p - \Delta H^\circ_n) \tag{10}$$

As $\Delta H^\circ_{A^-}$ is of the same order of magnitude as $\Delta H^\circ_{A^{2-}}$, the term $\Delta H^\circ_{A^-}/1740$ is negligible with respect to $\Delta H^\circ_{A^{2-}}$, and the $\Delta H^\circ_{A^{2-}}$ value, corrected for neutralization, is 4.47 kcal/mole. Yet we see in equation (10) that term 0.016/29 $(\Delta H^\circ_p - \Delta H^\circ_n)$ is also negligible and equation (10) can be simplified to

$$\Delta H^\circ_{\text{app}} = \Delta H^\circ_{A^{2-}} + \frac{1}{29}(\Delta H^\circ_p - \Delta H^\circ_n) \tag{11}$$

The $\Delta H^\circ_{A^{2-}}$ value in this equation is also equal to 4.47 kcal/mole, the difference between values of $\Delta H^\circ_{A^{2-}}$ obtained from equation (10) or (11) being only of the order of 0.005 kcal/mole.

(2) *Equilibrium constant measurement.* For measurement of the apparent equilibrium constant at $\mu = 0.2$ we have to use $\Delta H^\circ_{\text{app}}$ and the relationships 1 and 2, or 3 and 4. The result obtained by this means is K_{app} measured ($K_{\text{app meas.}}$) = 47.100 M^{-1}. This value, like that of ΔH°, must be corrected for ATPH^{3-} and ATPHMg$^-$ complex formation. For ionic strength 0.2, the true K_{app} is:

$$K_{\text{app}} = \frac{[\text{ATPMg}^{2-}]}{[\text{ATP}^{4-}]\cdot[\text{Mg}^{2+}]} \tag{12}$$

If b_t is the total Mg concentration, at equilibrium we have:

$$[\text{Mg}^{2+}] = b_t - x - x' \tag{13}$$

and

$$[\text{ATP}^{4-}] = a - \alpha' - x - x' \tag{14}$$

Replacing α' and x' by their values as a function of α and x (equations (7) and (8)), we have from (12), (13), and (14)

$$K_{\text{app}} = \frac{x(1 + 10^{(\text{pK}_{\text{ATPH}^{3-}} - \text{pH})})}{[b_t - x\,(1 + 10^{(\text{pK}_{\text{ATPHMg}^-} - \text{pH})})][a - x\,(1 + 10^{(\text{pK}_{\text{ATPHMg}^-} - \text{pH})})]} \tag{15}$$

From equation (15) $K_{\text{app}} = 49.300\ M^{-1}$

(*b*) *ADPMg⁻ complex:* In this case $b = a$, $c = 2a$, and $a = 4.10^{-3}\ M$. Table 2 shows values of Q_1 and Q_2 for ADPMg⁻ complex formation reactions. From these values we have $\Delta H^\circ_{\text{app}} = 2.99$ kcal/mole.

Using $\text{pK}_{\text{ADPHMg}} = 5.31$ (ref. 1), $\text{pK}_{\text{ADPH}^{2-}} = 6.78$ (ref. 4), and ΔH° ionization of $\text{ADPH}^{2-} = -1.37$ kcal/mole (ref. 1) equations analogous to the preceding equations give the thermodynamic quantities of the ADPMg⁻ complex formation reaction. True $\Delta H^\circ = 3.15$ kcal/mole, $K_{\text{app meas}} = 4.720\ M^{-1}$, and $K_{\text{app}} = 4.950\ M^{-1}$.

(*c*) *AMPMg complex:* In this case $a = b$, $c = 8a$, and $a = 4.10^{-3}\ M$.

The complex formation reaction is simpler than the two preceding reactions because only one species of complex has to be considered

$$\begin{array}{c} \text{H}^+ + \text{AMP}^{2-} + \text{Mg}^{2+} \rightleftharpoons \text{AMPMg} + \text{H}^+ \\ \upharpoonleft\downharpoonright \\ \text{AMPH}^- \end{array}$$

and equation (15) in this case becomes

$$K_{\text{app}} = \frac{x(1 + 10^{(\text{pK}_{\text{AMPH}^-} - \text{pH})})}{(b_t - x)(a - x)} \tag{16}$$

Table 3 shows values and means of Q_1 and Q_2 for the AMPMg complex formation reaction. With $\text{pK}_{\text{AMPH}^-} = 6.47$ (ref. 4) and ΔH of AMPH⁻ ionization equal to -0.85 kcal/mole[5] and using equations (11) and (16), we find that the thermodynamic quantities of the AMPMg complex formation reaction are: $\Delta H^\circ_{\text{app}} = 1.70$ kcal/mole; $\Delta H^\circ = 1.78$ kcal/mole; $K_{\text{app meas}} = 63\ M^{-1}$; and $K_{\text{app}} = 64\ M^{-1}$.

Discussion.—The main purpose in microcalorimetric experiments is to measure the true ΔH° of reactions. In our case we see that ΔH° increases with the length of phosphate chain (Fig. 4). These results do not agree with those of Burton[6] (5 kcal/mole ATPMg²⁻ and 6 kcal/mole ADPMg⁻) and of Taqui Khan and Martel[9] (3.4 kcal/mole for ATPMg) who obtained ΔH° by the Vant'Hoff

TABLE 2.

Exp. no.	Q_1 (cal)	Exp. no.	Q_1 (cal)	Exp. no.	Q_2 (cal)	Exp. no.	Q_2 (cal)
B64	0.0558	A45	0.0579	B71	0.0686	A54	0.0679
B65	0.0583	A46	0.0579	B72	0.0684	A55	0.0675
B66	0.0569	A47	0.0579	B73	0.0688	A56	0.0708
B67	0.0595	A48	0.0573	B74	0.0693	A58	0.0706
B68	0.0558	A49	0.0583	B75	0.0686	A59	0.0683
B69	0.0560	A50	0.0562	B76	0.0674	...	...
B70	0.0569	A51	0.0562	A53	0.0666	...	...

Q_1 (mean) = 0.0572 ± 0.0006 cal with a confidence value equal to 95%

Q_2 (mean) = 0.0685 ± 0.0007 cal with a confidence value equal to 95%

Q_1 and Q_2 experimental values of ADPMg⁻ complex formation reaction. Values corrected as for Table I.

TABLE 3.

Exp. no.	Q_1 (cal)	Exp. no.	Q_2 (cal)
B90	0.0070	B94	0.0264
B91	0.0072	B95	0.0266
B92	0.0067	B96	0.0263
B93	0.0065	B97	0.0270
A70	0.0067	B98	0.0264
Q_1 (mean) = 0.0070 ± 0.0003 cal with a confidence value equal to 95%		Q_2 (mean) = 0.00265 ± 0.0003 cal with a confidence value equal to 95%	

Q_1 and Q_2 experimental values of AMPMg complex formation reaction. Values corrected as for Tables 1 and 2.

TABLE 4.

Reaction	K_{app} M^{-1}	ΔF Kcal/mole	$\Delta H°$ Kcal/mole	ΔS (e.u.)
$ATP^{4-} + Mg^{2+} \rightleftharpoons ATPMg^{2-}$	49 300	−6.51	4.47	36.2
$ADP^{3-} + Mg^{2+} \rightleftharpoons ADPMg^{-}$	4 950	−5.12	3.15	27.3
$AMP^{2-} + Mg^{2+} \rightleftharpoons AMPMg$	64	−2.50	1.78	14.1

Thermodynamic data for the magnesium complex formation reactions of ATP^{4-}, ADP^{3-}, and AMP^{2-} at 30 °C and for an ionic strength of 0.2.

relationship but at an ionic strength of 0.1. However, the fact that $\Delta H°$ increases with the length of phosphate chain is in good agreement with the work of Phillips *et al.*[1] The latter investigators, determining $\Delta H°$ of $ADPMg^-$ and $ATPMg^{2-}$ complex formation by the Vant'Hoff relationship, concluded that it was necessary to convert the apparent equilibrium constant (K_{app}) into the true equilibrium constant (K_0), i.e., the constant at infinite dilution. Indeed, the Vant'Hoff relationship gives 3.6 kcal/mole and 3.3 kcal/mole, respectively, for the ΔH of ADP and ATP magnesium complex formation reactions at ionic strength 0.1, if Log K_{app} is plotted versus $1/T$, and 4.3 kcal/mole and 5.1 kcal/ mole, respectively, for the same reactions if Log K_0 is plotted versus $1/T$. However, these last values are about 1 kcal/mole higher than ours.

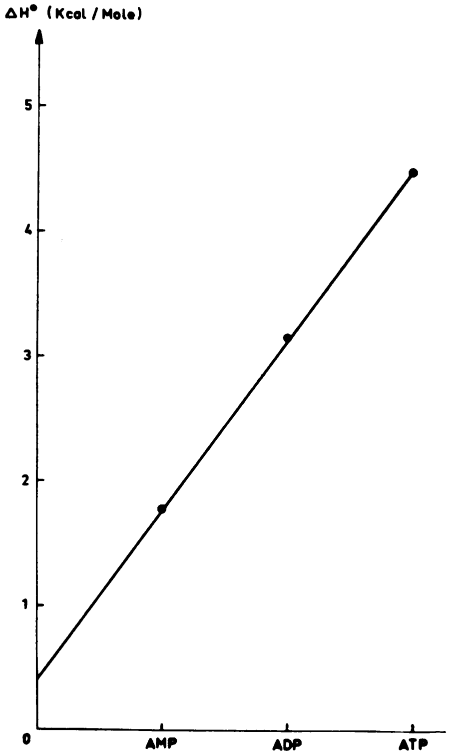

FIG. 4.—Experimental data demonstrating that the $\Delta H°$ values (enthalpy of the complex formation reactions) are proportional to the length of the phosphate chain.

Secondly, equation (9) demonstrates that complex concentration is linearly proportional to the heat evolved during the reaction. This fact permits the determination of K_{app} for experimental ionic strength by enthalpic titration. The values obtained for $ATPMg^{2-}$ and

$ADPMg^-$ formation by our method are in good agreement with the most probable values in the literature[1, 6–9] for an ionic strength of about 0.2. Our K_{app} of AMPMg is close to that of Taqui Khan *et al.*[10] and of Smith and Alberty.[11] With K_{app} and ΔH°, the entropy and the energy variation of reactions studied can be estimated by:

$$\Delta F = -RT \text{ Log } K_{app} \text{ and } \Delta S = \frac{\Delta H - \Delta F}{T}$$

Table 4 shows the respective values of ΔF and ΔS, demonstrating that complex formation is driven by the entropic factor; it also shows a good correlation between ΔS and the length of phosphate chain. This fact can probably be correlated with the rearrangement of H_2O molecules during the complex formation reaction. Finally it should be pointed out that our K_{app} values can be corrected to K_0 according to the results of Phillips *et al.*[4] These corrected K_{app} values will be published elsewhere.

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