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COMPLEX FORMATION OF INORGANIC PYROPHOSPHATE WITH MAGNESIUM

THE INFLUENCE OF IONIC STRENGTH, SUPPORTING MEDIUM AND TEMPERATURE

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SUMMARY

The influence of the composition of the medium, appropriate to PP_i-phosphohydrolytic activity determinations, on the distribution of the equilibrium species in mixtures of magnesium and PP_i has been investigated.

A spectrophotometric method, based on the competition of PP_i and 8-quinolinol for magnesium, was used. 8-quinolinol and its complex with magnesium were characterized at various temperatures and ionic strengths, and in different supporting media.

Magnesium binding by PP_i was measured in 50 mM Tris–HCl buffer at pH 8.6. The apparent stability constant K', decreased with increasing ionic strength, viz. from $2 \cdot 10^5$ M^{-1} at 10 mM to $0.4 \cdot 10^5$ M^{-1} at 250 mM. The relationship was linearised by the function $\log_{10}K'$ $(-\log_{10}f_{Mg^{2+}})$, where $f_{Mg^{2+}}$ is the activity coefficient of Mg^{2+}. Stability constants at zero ionic strength, estimated from extrapolation of the linear function, increased about 2-fold if the temperature was raised from 15 to 37 °C. At 37 °C and pH 8.6 the stability constant at zero ionic strength, obtained from the extrapolation of values determined in tetraethylammoniumbromide, was $4.7 \cdot 10^5$ M^{-1} (S.D: $0.3 \cdot 10^5$).

Concentrations of PP_i^{4-}, $MgPP_i^{2-}$ and Mg^{2+}, calculated on the basis of the determined stability constants, were significantly influenced by changes in ionic strength and temperature. Therefore, stability constants should be measured at the same conditions as those of the PP_i-phosphohydrolytic activity determination.

INTRODUCTION

Evidence has increasingly been furnished, that the roles of magnesium and inorganic pyrophosphate (PP_i) in enzymatic PP_i-phosphohydrolysis can be interpreted only in terms of the equilibrium species in mixtures of magnesium and PP_i, free PP_i, free Mg^{2+} and $MgPP_i$ complexes: the concentrations of these reactants are calculated from the total concentrations of magnesium and PP_i and from the stability constant of the $MgPP_i$ complexes [1–3]. Most often such stability constants have not been determined but values have been used, that were obtained by a potentiometric method at 25 °C in an unbuffered solution with an ionic strength of one adjusted by tetramethylammoniumchloride [4].

For complexes of magnesium with other ligands containing two or more phosphoryl groups it has been found, however [5, 6], that the values of the stability constant depend on ionic strength, on the composition of the medium, and on temperature. Stability constants of the $MgPP_i$ complex determined by different techniques and in various kinds of media were also not identical [7]. But apparently no study has been performed to elucidate in a systematic way the influences of the medium on the distribution of the different species in mixtures of magnesium and PP_i.

In connection with studies on the PP_i-phosphohydrolytic activity of human serum [8] values of the apparent stability constants have been determined at circumstances that are pertinent to the assay of the enzyme activity, i.e. at pH about 8.5–9.0. A spectrophotometric technique, based on the competition between PP_i and 8-quinolinol for magnesium has been used. The formation of complexes of magnesium and 8-quinolinol is accompanied by spectral changes [9], that are measured.

THEORY

8-Quinolinol (Ox) is an acid–base indicator that also forms chelates with divalent cations [10]; the spectrophotometric technique is based on the equation [10]:

$$\varepsilon_{st} - \varepsilon_{HOx} + (\varepsilon_{st} - \varepsilon_{H_2Ox^+})\,[H^+]/K'_1 + (\varepsilon_{st} - \varepsilon_{Ox^-})\,K'_2/[H^+] = 0 \qquad (1)$$

K'_1 and K'_2 are the first and second acidic dissociation constants, respectively, of 8-quinolinol:

$$[H_2Ox^+] \overset{K'_1}{\rightleftharpoons} [H^+] + [HOx] \qquad (2)$$

$$[HOx] \overset{K'_2}{\rightleftharpoons} [H^+] + [Ox^-] \qquad (3)$$

ε_{st} is the absorbance, A_O, divided by the total concentration of 8-quinolinol, $[Ox]_t$

$$\varepsilon_{st} \triangleq A_O/[Ox]_t \qquad (4)$$

The molar absorption coefficients of the species of Eqn 1, indicated by suffixes, can be obtained from measurements of the absorbances as a function of pH [10]: $\varepsilon \triangleq \varepsilon_{Ox^-}$ (pH $\geqslant$ 12), $\varepsilon \triangleq \varepsilon_{H_2Ox^+}$ (pH $\leqslant$ 2). Measurements at pH values about 7.5, near to the isoelectric point, give an approximation of ε_{HOx}. K'_1 and K'_2 are now determined from the preliminary values of the ε values by measurements at pH 5 and pH 10, respectively (Eqn 1). More accurate values of the ε values, K'_1 and K'_2 appear by recalculation from preliminary values.

If magnesium is added an additional equilibrium must be considered [9, 11]:

$$[\mathrm{Mg}^{2+}] + [\mathrm{Ox}^-] \overset{k_1}{\rightleftharpoons} [\mathrm{MgOx}^+] \qquad (5)$$

$$[\mathrm{Mg}^{2+}] + [\mathrm{HOx}] \overset{k_1'}{\rightleftharpoons} [\mathrm{MgOx}^+] + [\mathrm{H}^+] \qquad (6)$$

The absorbance of the solution now is:

$$A = \varepsilon_{\mathrm{HOx}} \cdot [\mathrm{HOx}] + \varepsilon_{\mathrm{Ox}^-} \cdot [\mathrm{Ox}^-] + \varepsilon_{\mathrm{MgOx}^+} \cdot [\mathrm{MgOx}^+] + \varepsilon_{\mathrm{H_2Ox}^+} \cdot [\mathrm{H_2Ox}^+] \qquad (7)$$

If the solution is buffered at a pH value $\geqslant \mathrm{p}K'_1 + 2$, i.e. pH $\geqslant 7$, $\mathrm{H_2Ox}^+$ can be neglected, and the ratio of [HOx] to $[\mathrm{Ox}^-]$ is constant; thus the increase of absorbance, ΔA, depends on $[\mathrm{MgOx}^+]$ only:

$$[\mathrm{MgOx}^+] = (A - A_{\mathrm{O}})/(\varepsilon_{\mathrm{MgOx}^+} - \varepsilon_{\mathrm{st}}) = \Delta A/(\varepsilon_{\mathrm{MgOx}^+} - \varepsilon_{\mathrm{st}}) \qquad (8)$$

$\varepsilon_{\mathrm{MgOx}^+}$, the molar absorption coefficient of MgOx^+, is determined by the measurement of the absorbance of the solution in which the ratio of magnesium to 8-quinolinol is about 100 so that $[\mathrm{Ox}]_t \triangleq [\mathrm{MgOx}^+]$.

k'_1 of Eqn 5 equals:

$$k_1' = \frac{[\mathrm{MgOx}^+] \cdot [\mathrm{H}^+] \cdot (1 + K_2'/[\mathrm{H}^+])}{[\mathrm{Mg}^{2+}] \cdot ([\mathrm{Ox}]_t - [\mathrm{MgOx}^+])} \qquad (9)$$

The equation is based on the conservation equation of $[\mathrm{Ox}]_t = [\mathrm{HOx}] + [\mathrm{Ox}^-] + [\mathrm{MgOx}^+]$, and Eqns 3 and 5. $[\mathrm{MgOx}^+]$ is determined by Eqn 8, $[\mathrm{H}^+]$ is measured and $[\mathrm{Mg}^{2+}] = [\mathrm{Mg}]_t - [\mathrm{MgOx}^+]$.

By a combination of Eqns 3, 5 and 6

$$k_1 = k_1'/K_2' \qquad (10)$$

If $\mathrm{PP_i}$ is added, and the pH is kept at about 8.5–9.0 the conservation equations are:

$$[\mathrm{Mg}]_t = [\mathrm{Mg}^{2+}] + [\mathrm{MgOx}^+] + [\mathrm{MgPP_i}^*] \qquad (11)$$

$$[\mathrm{PP_i}]_t = [\mathrm{PP_i}^*] + [\mathrm{MgPP_i}^*] \qquad (12)$$

where $\mathrm{MgPP_i^*}$ expresses the various kinds of complexes between magnesium and $\mathrm{PP_i}$, i.e. $\mathrm{MgPP_i^{2-}}$, $\mathrm{MgHPP_i}^-$, $\mathrm{Mg_2PP_i}^0$, $\mathrm{Mg(PP_i)_2}^{6-}$, etc.

The apparent, composite stability constant [7] of magnesium with $\mathrm{PP_i}$ is

$$K' = \frac{[\mathrm{MgPP_i}^*]}{[\mathrm{Mg}^{2+}] \cdot [\mathrm{PP_i}^*]} \qquad (13)$$

By a combination of Eqns 11, 12 and 13

$$K' = \frac{[\mathrm{Mg}]_t - [\mathrm{Mg}^{2+}] - [\mathrm{MgOx}^+]}{[\mathrm{Mg}^{2+}]\cdot([\mathrm{PP_i}]_t - [\mathrm{Mg}]_t + [\mathrm{Mg}^{2+}] + [\mathrm{MgOx}^+])} \tag{14}$$

Determinations of K', therefore, comprise a) measurement of the increase in absorbance, ΔA, resulting from the addition of magnesium to a solution containing 8-quinolinol and PP_i; from Eqns 4 and 8 calculation of $[MgOx^+]$; b) Calculation of $[Mg^{2+}]$ from Eqn 9; k'_1 has been previously determined in an experiment where 8-quinolinol only is titrated by magnesium (cf. text to Eqn 9). $[H^+]$ is measured; c) K' is now calculated from Eqn 14.

MATERIALS AND METHODS

Reagents

8-Quinolinol was from BDH, Analar grade. Working solutions were standardised by measurements of the absorbance, 360 nm, at pH 12. A stock solution of $MgCl_2$ about 25 mM was made from 1 g of dry MgO dissolved in 50 ml 1 M HCl and made up to exactly 1000 ml with water. The concentration of magnesium was calculated from the actual amount of MgO weighted out. The determination of the concentration of magnesium by atomic absorption spectrometry and by complexometric titration was also made. The same stock solution of $MgCl_2$ was used throughout. $Na_4P_2O_7 \cdot 10\ H_2O$ was an Analar grade reagent from BDH. Tris-(hydroxymethyl)-aminomethane was a Trizma base from Sigma. The ionic strength was adjusted by tetraethylammoniumbromide from Fluka (purum grade), by KCl, KNO_3 and NaCl from Merck (suprapur and A.R. grade). Water was double distilled.

Equipment

A Zeiss spectrophotometer, type PMQ II supplied with a hydrogen lamp and a thermostated cell-holder was used. The height of the light beam was 3.5 mm and the slit width was 20 nm. Quartz cells of 4.5 ml capacity and with a 10-mm light path were used. The temperature of the cell housing was kept within ± 0.1 °C. The pH of solutions were measured with a type 26 pH-meter (Radiometer) supplied with a combined glass reference electrode, type GK2322 C. The calibration of the pH-meter was performed with two or more certified phosphate buffers of differing pH values [12].

Titrations by Mg

The quartz cells were filled with 3 ml of solutions containing 0.33 mM 8-quinolinol, 0.05 M Tris–HCl buffer (pH 8.6) and one of the background salts, KCl, KNO_3, NaCl or tetraethylammoniumbromide, to adjust the ionic strength, I. The absorbance was read (A_O) when temperature equilibrium had been reached. Now 25-μl aliquots of $MgCl_2$ titration solutions were added by a calibrated constriction pipette, and changes in absorbances were read (ΔA). Four additions were made to each of the cells; the concentrations of $MgCl_2$ titration solutions were 3 mM to 12 mM, depending on the concentrations of PP_i. The read off values of ΔA were corrected for volume changes. When titrations were finished the pH and $[Cl^-]$ of the solutions were measured for use in calculations and for the control of ionic strength, respectively.

TABLE I

MOLAR ABSORPTION COEFFICIENTS ($M^{-1}\cdot cm^{-1}$) AT 360 nm OF 8-QUINOLINOLS

Values were determined at ionic strengths from 15 to 400 mM, and at temperatures of 15, 24 and 37 °C. *n* is the number of determinations. Forms of 8-quinolinol are represented by the suffix of ε. Cf. Eqns 1 and 8 in theory section.

	ε_{Ox^-} ($M^{-1}\cdot cm^{-1}$)	$\varepsilon_{H_2Ox^+}$ ($M^{-1}\cdot cm^{-1}$)	ε_{HOx} ($M^{-1}\cdot cm^{-1}$)	ε_{MgOx^+} ($M^{-1}\cdot cm^{-1}$)
Mean	2710	1710	89	2290
S.D.	42	78	8	68
Range	2670–2830	1540–1850	75–103	2120–2370
n	23	19	16	19

Calculations

Values of the constants were calculated on a desk computer after programming. Parameters of linear regression equations and their errors were obtained by the use of appropriate programs from the IBM 1130 scientific subroutine package (statistics).

RESULTS

I. Characterization of 8-quinolinol and its complex with magnesium

The molar absorption coefficients of 8-quinolinols are given in Table I. They were independent of temperature and of ionic strength.

Values of K'_2 (Eqn 3) and k'_1 (Eqns 6 and 9) were not influenced by the type of background salt, but depended on ionic strength, Table II. Plots of $-\log_{10}K'_2$ and

TABLE II

INFLUENCE OF TEMPERATURE AND IONIC STRENGTH ON THE VALUES OF K'_2 AND k'_1

K'_2 and k'_1 values were determined at six ionic strengths from 15 to 300 mM; the background salt was KCl. The relationships between the constants and the ionic strengths were expressed by equatoins for linear regression: $-\log_{10}$ (constant) $= \alpha\cdot I^{\frac{1}{2}} + \log_{10}$ (constant at zero ionic strength). α is the regression coefficient; *r* is the correlation coefficient. Figures in brackets are the standard error of the mean of the coefficients. $K'_{2.0}$ and $k'_{1.0}$ are the values of constants at zero ionic strength, obtained by extrapolation of the functions. *n* is the number of experiments.

Function	Temperature (°C)		
	15	24	37
$-\log_{10}K'_2$ ($I^{\frac{1}{2}}$)			
n	3	2	4
α	−0.25 (0.02)	−0.29 (0.02)	−0.36 (0.03)
r	−0.987	−0.996	−0.992
$K'_{2.0}$, M	$1.45\cdot10^{-10}$ ($0.04\cdot10^{-10}$)	$1.70\cdot10^{-10}$ ($0.08\cdot10^{-10}$)	$2.29\cdot10^{-10}$ ($0.05\cdot10^{-10}$)
$-\log_{10}k'_1$ ($I^{\frac{1}{2}}$)			
n	2	2	6
α	0.55 (0.03)	0.43 (0.06)	0.46 (0.03)
r	0.997	0.983	0.996
$k'_{1.0}$	$5.1\cdot10^{-6}$ ($0.1\cdot10^{-6}$)	$5.8\cdot10^{-6}$ ($0.2\cdot10^{-6}$)	$7.2\cdot10^{-6}$ ($0.1\cdot10^{-6}$)

of $-\log_{10}k'_1$ against $I^{1/2}$ were linear. By extrapolation of the linear functions, values of the constants at zero ionic strength, $K'_{2.0}$ and $k'_{1.0}$, were found at three temperatures, Table II. If the values of $k'_{1.0}$ were corrected, according to Eqn 10, for competition between magnesium and hydrogen for 8-quinolinol values of $k_{1.0}$ (Eqn 5) could be determined; at 37 °C, $3.16 \cdot 10^4$ (S.D. $0.11 \cdot 10^4$) M^{-1}; at 24 °C, $3.47 \cdot 10^4$ (S.D. $0.22 \cdot 10^4$) M^{-1}; at 15 °C, $3.55 \cdot 10^4$ (S.D. $0.12 \cdot 10^4$) M^{-1}. From these values the thermodynamics of magnesium binding by 8-quinolinol were estimated, $\Delta H^o = -1$ kcal·mole^{-1} and ΔS^o 20 cal·mole^{-1}·°K^{-1}.

II. Determination of the apparent stability constant of the MgPP$_i$ *complex*

Fig. 1 illustrates the procedures, that were performed when a single value of K' was determined (Eqn 14). The value of k'_1 (Eqns 6 and 9), that was calculated from curve a, was $4.6 \cdot 10^{-6}$ (S.D. $0.14.10^{-6}$). The mean values of K' calculated from curves b and c were identical, $0.42 \cdot 10^5$ (S.D. $0.04 \cdot 10^5$) M^{-1} and $0.46 \cdot 10^5$ (S.D. $0.04 \cdot 10^5$) M^{-1}, respectively. The values of K' only showed random variations with increasing concentrations of total magnesium, i.e. with increasing the ratio of MgPP$_i$* to PP$_i$* (cf. Eqn 13).

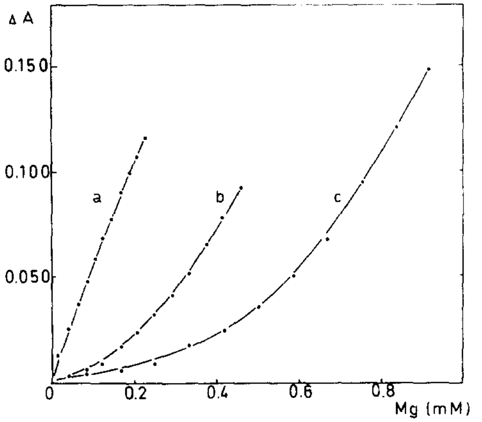

Fig. 1. Magnesium titration of solutions containing 8-quinolinol and PP$_i$. MgCl$_2$ titration solutions used in Curves a, b and c were 3, 6 and 12 mM, respectively. Concentrations of PP$_i$ in Curves a, b and c were zero, 0.34 and 0.68 mM, respectively. Assay solutions contained 0.33 mM 8-quinolinol, 50 mM Tris–HCl (pH 8.5, 37 °C), and the ionic strength was adjusted to 0.175 M by KCl. ΔA is the change in absorbance and Mg is the final, total concentration of magnesium.

The values of K' depended on ionic strength (Fig. 2). From the insertion in Fig. 2 it appears, that this relationship could be linearised by the function $\log_{10}K'$ ($-\log_{10} f_{Mg^{2+}}$). $f_{Mg^{2+}}$ is the activity coefficient of Mg^{2+}; it was calculated from the Debye–Hückel equation [13].

$$-\log_{10} f_{Mg^{2+}} = A \cdot z^2_{Mg} \cdot I^{\frac{1}{2}}/(1 + B \cdot \mathring{a} \cdot I^{\frac{1}{2}})$$

where A and B are temperature dependent constants, $\mathring{a}$ is the ion size parameter of the hydrated Mg^{2+} in Angstroms, z_{Mg} is the charge of the Mg^{2+}, and I is the ionic strength (M). Values of K' at zero ionic strengths, K'_0, were estimated from extrapolation of the linear function $\log_{10}K'$ ($-\log_{10} f_{Mg^{2+}}$), and are given in Tables III and IV.

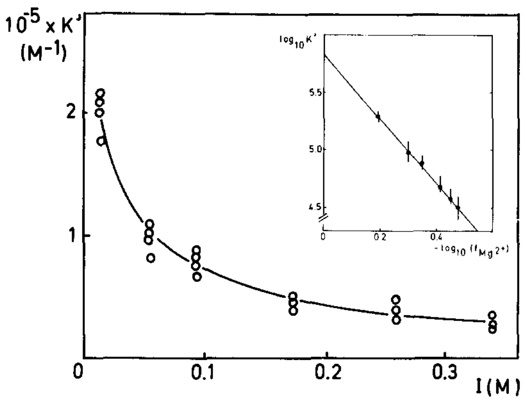

Fig. 2. The dependence of K', the apparent stability constant of the MgPP$_i$ complex, on ionic strength adjusted by KCl. Temperature was 37 °C and pH 8.5–8.6 in 50 mM Tris–HCl. The insertion shows the transformation of data to a linear plot of $\log_{10}K'$ against $(-\log_{10}f_{Mg^{2+}})$, cf. the text. The correlation coefficient was −0.998, the equation of linear regression was $\log_{10}K' = -2.78\,(-\log_{10}f_{Mg^{2+}}) + 5.84$, and K'_O, the constant at zero ionic strength, was $6.7 \cdot 10^5$ M^{-1}.

The parameters of the linear regression equations, i.e. the function $\log_{10}K'$ $(-\log_{10}f_{Mg^{+2}})$, that described the relation between K' and ionic strength were influenced by the kind of background salt, that was used for adjusting ionic strength, Table III. The estimated values of K'_0, were, therefore, different.

The equilibrium between magnesium and PP$_i$ was investigated at three temperatures, Table IV. Temperature-induced changes of pH in the solutions, that contained 50 mM Tris–HCl, were about −0.03 pH units/°C. These changes of pH were compensated for by adjusting the pH in all three solutions to 8.6 at 37 °C. The thermodynamics of the MgPP$_i$ complex formation were calculated from the values of K'_0, that were estimated at three temperatures, Table IV. The enthalpy change was,

TABLE III

INFLUENCE OF THE COMPOSITION OF THE SUPPORTING MEDIUM ON K', THE STABILITY CONSTANT OF THE MAGNESIUM COMPLEX WITH PP$_i$

The K' values were determined at six ionic strengths from 15 to 250 mM, adjusted with each of the four types of background salt. The temperature was 37 °C, pH 8.5–8.6 in 50 mM Tris–HCl buffer. The relation between K' and ionic strength was expressed by the equation for a linear regression, $\log_{10}K' = \alpha \cdot (-\log_{10}f_{Mg^{2+}}) + \log_{10}K'_0$. α is the regression coefficient. $f_{Mg^{2+}}$ is the activity coefficient of Mg^{2+} calculated from the ionic strength according to the Debye–Hückel equation (particulars in the text). r is the coefficient of correlation. Figures in brackets are the standard error of the mean of the coefficients. K'_0 is the value of K' at zero ionic strength. n is number of experiments.

Function $\log_{10}K'$ $(-\log_{10}f_{Mg^{2+}})$	Type of background salt			
	KCl	KNO$_3$	NaCl	Tetraethylammoniumbromide
n	4	2	2	4
α	−2.78 (0.09)	−2.57 (0.17)	−3.51 (0.06)	−1.94 (0.11)
r	−0.998	−0.995	−0.999	−0.995
K'_0, M^{-1}	$6.76 \cdot 10^5$ $(0.34 \cdot 10^5)$	$6.92 \cdot 10^5$ $(0.32 \cdot 10^5)$	$8.51 \cdot 10^5$ $(0.22 \cdot 10^5)$	$4.68 \cdot 10^5$ $(0.11 \cdot 10^5)$

TABLE IV

THE DEPENDENCE OF K' ON TEMPERATURE

The K' values were determined at six different ionic strengths from 15 to 200 mM: the pH was 8.6 at 37 °C in 50 mM Tris–HCl buffer; ionic strengths were adjusted with KCl. Notations and abbreviations are the same as used in Table III.

Function	Temperature (°C) 15	 24	 37
$\log_{10}K'$ ($-\log_{10}f_{Mg^{2+}}$)			
n	2	2	3
a	−1.96 (0.10)	−2.58 (0.53)	−2.75 (0.18)
r	−0.998	−0.960	−0.996
K'_0, M^{-1}	$2.88 \cdot 10^5$ ($0.07 \cdot 10^5$)	$4.37 \cdot 10^5$ ($0.86 \cdot 10^5$)	$6.61 \cdot 10^5$ ($0.46 \cdot 10^5$)

$\Delta H^{o'} = 6.5$ kcal·mole^{-1}; the changes in free energy, $\Delta G^{o'}$, were at 37 °C −8.6 kcal·mole^{-1}, at 24 °C −7.7 kcal·mole^{-1}, and at 15 °C −7.2 kcal·mole^{-1}. The change in entropy, $\Delta S^{o'}$, was, at the three temperatures, 48 cal·mole^{-1}·°K^{-1}. Thus magnesium binding by PP_i seems to be an exergonic and endothermic process accompanied by a large increase in entropy.

III. Concentrations of the equilibrium species in mixtures of magnesium and PP_i

At pH 8.6 (37 °C), which equals pH 9 (22 °C), complexes of magnesium with HPP_i^{3-} can be neglected [5, 7]. The concentrations of the equilibrium species, that are

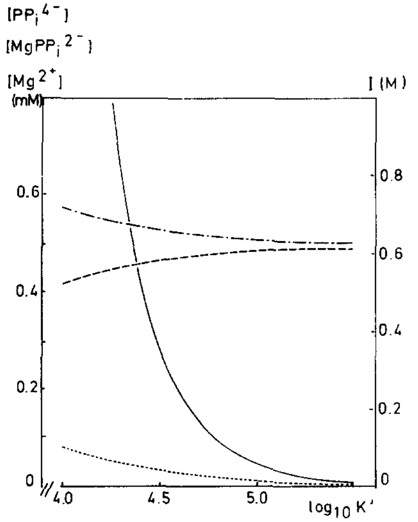

Fig. 3. The distribution of equilibrium species in mixtures of 0.5 mM magnesium and 1.0 mM PP_i as a function of I, the ionic strength, adjusted by KCl. On the right y-axis values of I are found, and the connected values of K' are found at the x-axis. From the values of K' were calculated the concentrations of the equilibrium species indicated at the left y-axis; — · —, PP_i^{4-}; — — —, $MgPP_i^{2-}$; ······, Mg^{2+}; ———, I.

free Mg^{2+}, free PP_i^{4-} and $MgPP_i^{2-}$ complex mainly, in mixtures of magnesium and PP_i were calculated from the total concentrations of PP_i and magnesium and from the previously determined values of K', the stability constant of the $MgPP_i$ complex. It appears from Fig. 3, that the dependence of K' values on ionic strength (cf. Fig. 2), significantly influenced the distribution of the equilibrium species. It was found too, that the changes in K' resulting from temperature changes (cf. Table IV), influenced the distribution of the equilibrium species.

DISCUSSION

With any method a careful consideration is necessary whether the effects of metal–ligand interaction at various conditions will be quantitatively reflected in the measured property. The first part of the study, in which 8-quinolinols were characterized, showed that the method employed fulfils the above demand; although account must be taken of the dependence of K'_2 and k'_1 on ionic strength and temperature. The values of the constants were consistent with previous reported values determined at 20 °C [9] and at 37 °C [11]. In addition the determinations in this study at three temperatures allowed an estimation of the thermodynamic parameters [14].

The values of the apparent stability constant of $MgPP_i$ complexes, which were determined in the second part of the study were independent of the magnesium to PP_i concentration ratio; thus no indications were found for the formation of 1:2 and 2:1 complex species. Values of K' should be considered as composite stability constants due to formation of $MgPP_i^{2-}$ as well as $MgHPP_i^{-}$; at pH about 9 only small amounts of the latter species will be formed.

Linearisation of the relation between the value of the apparent stability constant and the ionic strength by the function $\log_{10}K'$ $(-\log_{10}f_{Mg^{2+}})$ was valid within the whole range of ionic strengths investigated, from 10 mM to 400 mM. It is not possible from the function to draw any conclusions with regard to the mechanism of the complex formation. Firstly, because the measured property, the change in absorbance, equals concentration and not activity, and secondly because the Debye–Hückel equation, that was used in the calculation of the Mg^{2+} activity coefficient, is strictly valid only at ionic strengths less than about 10 mM [15].

The kind of background salt used influenced the parameters of the function $\log_{10}K'$ $(-\log_{10}f_{Mg^{2+}})$. The explanation probably is that Na^+ and K^+, in addition to their unspecific effects via their contribution to the ionic strength, bind to PP_i in competition with magnesium [16]. Tetraethylammoniumbromide does not bind, and the value of K'_O, obtained by extrapolation from values with this type of background salt, should be considered as a reliable estimate of the true value, $4.7 \cdot 10^5$ M^{-1}.

The thermodynamics of magnesium binding by PP_i showed, that the process was accompanied by a large increase in entropy; this is consistent with the chelate-formation mechanism [17] which results in dehydration of magnesium. The qualitative and quantitative effects on the equilibrium between magnesium and the ligand with changes in temperature and ionic strength were of the same kind as found with ATP [11], ADP [18], and 2,3-diphosphoglycerate [19] as the ligands. It seems allowable, therefore, to consider such changes characteristic of magnesium binding by compounds having two or more phosphoryl groups.

The last part of the study demonstrated, that variation in ionic strength influenced the distribution and the concentration of the equilibrium species in mixtures of magnesium and PP_i. Changes in temperature and ionic medium, which also result in the variation of the values of the stability constants, will influence the distribution qualitatively in the same way.

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