Inorganica Chimica Acta, 35 (1979) 15–21

A Thermodynamic Study of Magnesium(II) Interactions with Mono- and Dinucleotides*

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Received November 29, 1978

The equilibrium constants for the interaction between magnesium(II) and a number of mono- and dinucleotides (AMP, TMP, GMP, CMP, d-ApA, d-TpT, d-GpG, d-CpC, d-pTpT) have been determined accurately at 25.0 °C and an ionic strength of 0.2 M *by a computerized, multiparamagnetic curve fitting analysis of pH titration curves. The data are consistent with inner sphere binding of magnesium(II) with the phosphate group. The presence of an additional base in those dinucleotides without a terminal phosphate causes a decrease in the magnesium(II) association constant.*

Introduction

The growing availability of synthetically prepared oligonucleotides makes it feasible to undertake a study of their thermodynamic properties using techniques such as pH titrations, spectrophotometry, fluorescence, *etc.* [1]. This situation has not led, however, to a clear view of the binding of biologically important metals, such as magnesium, since much of the previous work is not in agreement in terms of binding models or magnitudes [2].

The importance of the magnesium(II) ion in biological systems cannot be overstressed [3]. Magnesium directly affects the stability of sequence specific oligonucleotides [4] as shown by the greater melting temperatures of the double helix in its presence. It is known that magnesium affects the specific recognition of many DNA–protein interactions [5], and controls the expression of the codon. If its concentration is changed or another ion is used, there are many triplet codes which are not degenerate and give rise to the expression of other amino acids. This is of particular importance in the understanding of molecular evolution and genetic control. Another example is given by the recognition of the magnesium–ATP complex by ATPase.

Short oligonucleotides of well defined base sequence are especially important since they provide a general method for insertion of a given sequence in a cloning vehicle [6]. An investigation of magnesium binding to short oligonucleotides is of fundamental importance for a quantitative analysis of its binding to longer sequences, and for the determination of the possible influence of the bases adenine, thymine, guanine, and cytosine. This type of information is not available in the literature.

Most of the studies to date have avoided magnesium partly because of the lack of spectral shifts resulting from the binding of magnesium to mononucleotides. The relatively small binding constant of magnesium has led to experimental difficulties. This may explain the large number of studies with preference of nickel(II) over magnesium(II), since it has a higher intrinsic binding constant with mononucleotides and shows a spectral shift in the UV upon complexation [7]. In the nickel(II) case, multiple metal ligand binding has been proposed.

In this study the magnesium(II) binding constant to, and the acid pK_as of the 5′-nucleotides AMP, TMP, CMP, GMP, the oligonucleotides d-ApA, d-TpT, d-pTpT, d-GpG, d-CpC, are calculated by a computer based analysis of the pH titration curve of each metal ligand complex. The use of a microelectrode and a pyrex vessel enabled titrations to be carried out with only 0.2 ml of sample for each experiment. Since each solution was on the average $\sim 10^{-4}$ *M*, approximately 20 nanomol of the valuable compounds were required for each titration.

Theory

When magnesium binds to oligonucleotides, there is an observable shift in the pH of the system due to the displacement of a hydrogen ion by the metal when it binds to the phosphate group (Fig. 1). In general, the nucleotides can be treated as H_2L acids (except TMP which has no amine proton and is represented as an HL acid) and appropriate equations can be derived to cover their dissociation and metal com-

*Taken in part from the M.S. Thesis of H. Trimm, Clarkson College (1978).

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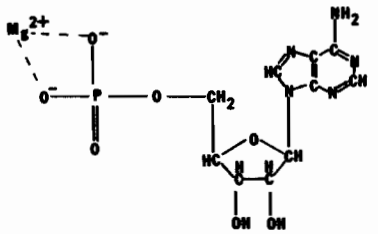

Fig. 1. Magnesium(II)–5'AMP complex.

plexation in solution. The following equilibria were considered under the conditions of the experiment for 5'-AMP (Fig. 2).

The dissociation of the free acid can be described as follows:

$$H_3L^+ \rightleftharpoons H^+ + H_2L \quad KA1 \tag{1}$$

$$H_2L \rightleftharpoons H^+ + HL^{-1} \quad KA2 \tag{2}$$

$$HL^{-1} \rightleftharpoons H^+ + L^{2-} \quad KA3 \tag{3}$$

The binding of ligand to a divalent metal in a 1:1 complex may be described by the equilibria:

$$M^{2+} + L^{2-} \rightleftharpoons ML \quad KM1 \tag{4}$$

$$M^{2+} + HL^- \rightleftharpoons MHL^+ \quad KM2 \tag{5}$$

The following mass balance equations have been used to reduce the system to one master variable, namely L^{2-}. CLIG is the analytical concentration of nucleotide and CMET is the analytical concentration of magnesium. All mathematical operations are represented by standard FORTRAN IV symbolism except for implied multiplication by parentheses. The concentrations of various species are denoted by square brackets. The equilibrium constants are valid under the experimental conditions used in this study, in which the ionic strength was kept constant at 0.2 *M* by the addition of sodium chloride.

$$CMET = [ML] + [MHL^+] + [M^{2+}] \tag{6}$$

$$CMET = M^{2+}([L^{2-}]KM1 + [H^+][L^{2-}]KM2/KA3 + 1.0) \tag{7}$$

$$[M^{2+}] = CMET/(1.0 + [L^{2-}](KM1 + [H^+]KM2/KA3)) \tag{8}$$

$$CLIG = [H_3L^+] + [H_2L] + [HL^-] + [L^{2-}] + [ML] + [MHL^+] \tag{9}$$

$$CLIG = [L^{2-}]\left(\frac{[H^+]^3}{KA1KA2KA3} + \frac{[H^+]^2}{KA2KA3} + \frac{[H^+]}{KA3} + 1.0\right) + [M^{2+}][L^{2-}]\left(KM1 + \frac{[H^+]KM2}{KA3}\right) \tag{10}$$

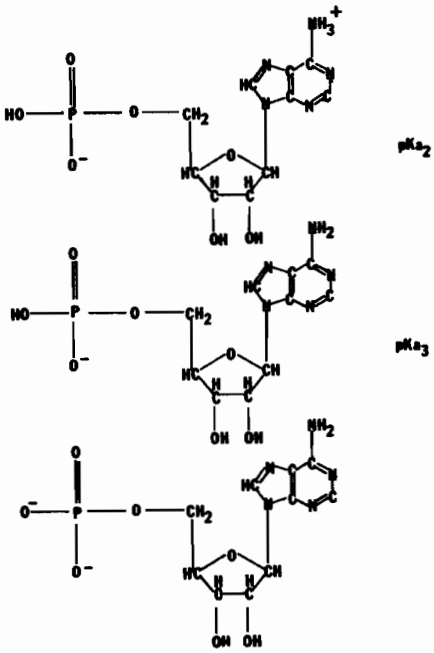

Fig. 2. Acid dissociation of 5'-AMP in solution.

By substituting eq. (8) into eq. (10) one obtains

$$CLIG = [L^{2-}]\left(\frac{[H^+]^3}{KA1KA2KA3} + \frac{[H^+]^2}{KA2KA3} + \frac{[H^+]}{KA3} + 1.0\right) + \frac{CMET}{1.0 + [L^{2-}]\left(KM1 + \frac{[H^+]KM2}{KA3}\right)}[L^{2-}]\left(KM1 + \frac{[H^+]KM2}{KA3}\right) \tag{11}$$

Multiplying through by $(1.0 + [L^{2-}](KM1 + [H^+]KM2/KA3))$ yields a quadratic in $[L^{2-}]$. After combining terms one obtains:

$$[L^{2-}]^2\left(KM1 + \frac{[H^+]KM2}{KA3}\right)\left(\frac{[H^+]^3}{KA1KA2KA3} + \frac{[H^+]^2}{KA2KA3} + \frac{[H^+]}{KA3} + 1.0\right) + [L^{2-}]\left(\frac{[H^+]^3}{KA1KA2KA3} + \frac{[H^+]^2}{KA2KA3} + \frac{[H^+]}{KA3} + 1.0 + \left(KM1 + \frac{KM2}{KA3}[H^+]\right)\right)$$

$$(CMET - CLIG)) - CLIG = 0 \qquad (12)$$

Trial solutions of the quadratic, $a[L^{2-}]^2 + b[L^{2-}] + c = 0$, show that only the positive root is valid for real systems.

$$[L^{2-}] = \frac{-b + SQR(b^2 - 4ac)}{2a}$$

In dealing with the numerical analysis of titration equations, it is important to guard against the error normally encountered when using small numbers. The above form of the quadratic would be unsatisfactory in some cases unless all variables and functions were declared double precision.

The final equation needed to fully describe the system is the statement of electroneutrality:

$$2[M^{2+}] + [MHL^{+}] + [H_3L^{+}] + [Na^{+}] + [H^{+}] = [OH^{-}] + [Cl^{-}] + [HL^{-}] + 2[L^{2-}] \qquad (13)$$

The sodium term in equation (13) arises from the fact that the nucleotide is added as the sodium salt and the solution may be titrated by sodium hydroxide. The chloride term is due to the addition of the metal as magnesium chloride and the solution may be titrated by hydrochloric acid.

The form of the equation used to analyze the data is obtained by combining eq. (12) and eq. (13) so that the only remaining variable is the concentration of hydrogen ion which is determined by the pH titration. For simplicity, the solution of the quadratic will be expressed as $[L^{2-}]$, but note that by eq. (12) this is just a function of constants and the hydrogen ion concentration.

$$F([H^{+}]) = 2.0*\left(\frac{CMET}{1 + [L^{2-}]\left(KM1 + \frac{KM2}{KA3}[H^{+}]\right)}\right) + \frac{CMET\,[L^{2-}]KM2}{1 + [L^{2-}]\left(KM1 + \frac{KM2}{KA3}[H^{+}]\right)}\left(\frac{[H^{+}]}{KA3}\right) + \frac{[H^{+}]^3[L^{2-}]}{KA1KA2KA3} + CBASE + X(CLIG) + [H^{+}] - \left(K_w/[H^{+}] + 2.0*(CMET) + CACID + 2.0*[L^{2-}] + \frac{[H^{+}]}{KA3}[L^{2-}]\right) = 0 \qquad (14)$$

Equation (14) enables, in principle, the calculation of $[H^{+}]$ if the numerical values of the remaining parameters are known. In practice, the values of some of the variables (*e.g.* KM1, KM2, *etc.*) may not be known, and a program can be written to calculate one $[H^{+}]$ value corresponding to each addition in a titration, using an initial estimate of the unknown parameters. The multiparametric curve fitting program uses a nonlinear least squares analysis in conjunction with the method of steepest descent to simultaneously vary the unknown parameters until the best fit is achieved [8]. Since an exact solution for a Nth order equation is not readily available, it is much easier to solve equation (14) by numerical means with the aid of a computer. The difficulty in finding the derivatives of this function makes the use of the Newton–Raphson technique much more dif-

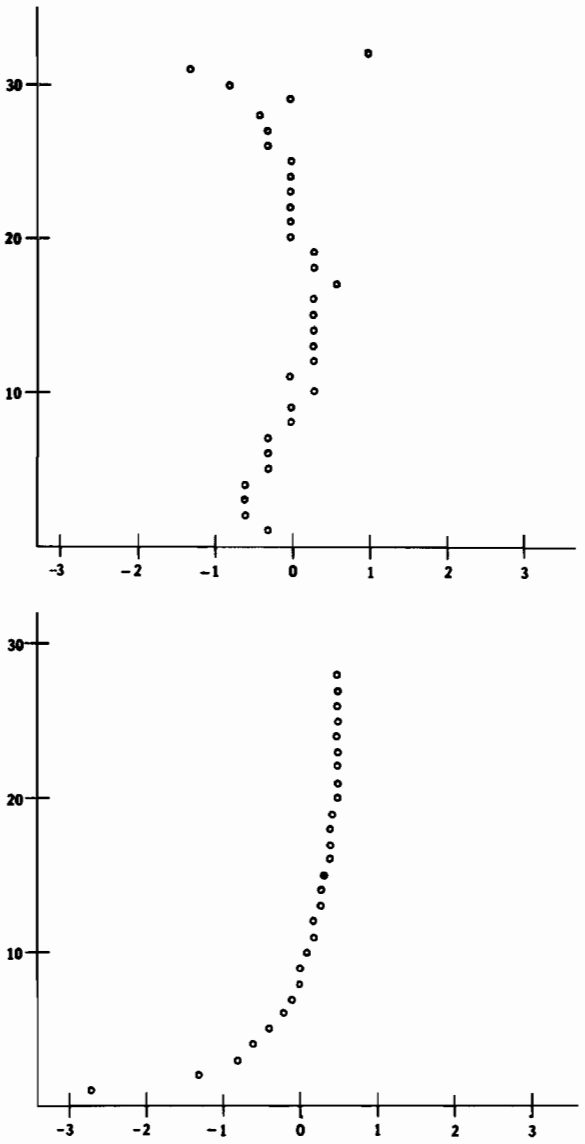

Fig. 3. a) Random deviation pattern of magnesium–AMP titration curve fit. b) Systematic deviation due to an incorrect value for the percentage of sodium ions per ligand (parameter X in eq. (14)). Both plots are the titration point number *versus* the difference between the calculated and observed value of the pH, divided by the standard deviation of the fit.

TABLE I. Summary of Equilibrium Constants for Mono- and Dinucleotides (25.0 °C, ionic strength: 0.2 *M*.)

Nucleotide	pKA1	pKA2	KM1	KM2
5'-AMP	3.57	6.37	67.4	1.1
5'-TMP		6.15	57.3	4.3
5'-CMP	4.47	6.69	65.0	.51
5'-GMP	2.27	6.37	64.6	.50
d-ApA	3.57		65.3	1.3
d-TpT			52.1	5.0
d-GpG	2.30		40.5	6.0
d-CpC	4.45		25.0	3.7
d-pTpT			85.0	15.0

ficult than a simple brute force method, in which the correct value of $[H^+]$ is calculated by iteration so that $F[H^+] = 0$ in equation (14). The variable X in eq. (14) is the number of mol of sodium per mol of nucleotide. Since this can vary from lot to lot, it is made a free parameter. Any parameter, which is not a linear combination of another parameter, may be determined simultaneously from a single titration curve by using eq. (14) in conjunction with a multiparametric curve fitting analysis. The use of a large number of parameters to describe the experimental curve cannot be made indiscriminately. It should be established that the adjustable parameters are appropriately correlated to the experimentally measurable quantity. Independent criteria, such as the calculation of acid pKs in the presence and absence of magnesium(II), as well as comparison with literature values wherever feasible, were applied to check the internal consistency of this method. A further control is provided by the deviation pattern that is obtained (Figs. 3a and 3b). Some note must be made that these equations assume the magnesium(II) ion binds to the nucleotide via the phosphate group. All experimental evidence so far confirms this method. First, the metal binding constant increases dramatically from the monoprotic to the unprotonated form of AMP as shown in the work of Khan and Martell [9] and this work (Table I). Second, the same authors show that the magnesium is especially stable in the case of ATP. Third, it has been found in this work (see Table I) that the absence of a terminal phosphate group in a dinucleotide results in a great decrease in the metal binding constant. The equilibrium constant for $Mg^{2+} + HPO_4^{2-}$ has a value of 75 at an ionic strength of 0.2 *M* and 25 °C [12].

Experimental

All water used in this study was first prefiltered, run through an ion exchange column, and then doubly glass distilled in a Corning AG-11 still. The water was always used fresh to prevent carbonate contamination. The mononucleotides were all Sigma grade and in the form of the sodium salt. The dinucleotides were from Collaborative Research and in the form of the ammonium salt. Both were present at the lyophilized powder. The concentrations of the solutions are shown in Table III. The magnesium chloride, sodium chloride, sodium hydroxide, and hydrochloric acid were prepared from Baker reagent grade chemicals. The pH calibration buffers were potassium hydrogen phthalate, Fisher acidimetric standard grade, and sodium tetraborate decahydrate, Baker reagent grade.

Solution concentrations were determined spectrophotometrically on a ZEISS PMQ3 by measurement of absorbance at 260 nm. Titrations were performed in a pyrex microtitration vessel equipped with a nitrogen gas inlet, a microelectrode, and a dispensing syringe (Fig. 4). A volume of 0.2 ml was all that was needed. The mixture was purged with nitrogen and an

TABLE II. Comparison of Observed and Calculated Equilibrium Constants for Magnesium(II).

Equilibrium Constant, K	$Z_1Z_2 = -2$	$Z_1Z_2 = -4$	$Z_1Z_2 = -6$
Outer sphere, calculated by eq. 18	1.64	8.44	43.5
Average value measured for mononucleotides		63.5	
Average value measured for dinucleotides	45.7		
Average value measured for ADP [2]			10^3

TABLE III. Molar Extinction Coefficient Values and Concentrations of Nucleotides Used in the Present Study.

Nucleotide	$\epsilon \times 10^{-4}$ [11]	Concentration used (*M*)
5'-AMP	1.54	3.37×10^{-3}
5'-TMP	0.84	2.25×10^{-3}
5'-CMP	0.74	1.04×10^{-3}
5'-GMP	1.14	1.89×10^{-3}
d-ApA	3.08	7.92×10^{-4}
d-TpT	1.68	1.26×10^{-3}
d-GpG	2.28	2.58×10^{-4}
d-CpC	1.48	8.88×10^{-4}
d-pTpT	1.68	1.42×10^{-3}

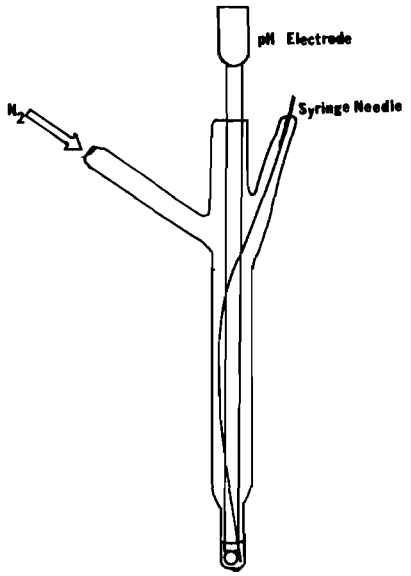

Fig. 4. Microtitration vessel.

inert atmosphere was kept over the solution during the course of the reaction. The solution was mixed at a constant rate with a Teflon micro-stir bar. The addition of titrant was made with a Hamilton 1725-LT, gastight, .025 ml syringe held in a Hamilton PB600-I repeating dispenser. Each addition of titrant was 0.00500 ml. The pH of the system was measured using a Thomas 4094-S.10, 0 to 12 pH combination microelectrode connected to a Sargent-Welch model PAX digital pH meter. All measurements were done at 25.0 °C. The temperature was controlled by circulating water from a HAAKE model FS thermostat through the jacket of the titration vessel.

Results

pH Titrations

Each solution of oligonucleotide and magnesium was titrated by acid or base to give a large pH range for the curve fit analysis. The direct titration of the oligonucleotide by metal produced only a small shift to lower pH and proved to be inadequate for extracting the parameters desired. The computer analysis of the titration data turned out to be quite necessary for the interpretation of the curves since no inflection points were visible in the more dilute cases (Fig. 5). Using the multiparametric curve fit analysis, the metal binding constant, acid pK_as, and even ligand concentrations, could be determined by a single run. The results of the computer analysis are summarized in Table I. It is important to note that this method provides an independent means of determining, with good accuracy (~2%) the concentration of oligonucleotide in solution. The molar extinction coefficients and nucleotide concentrations used in this study are shown in Table III.

The results of Table I show a consistent binding constant for magnesium, in the case of mononucleotides, which reinforces the model of magnesium binding to the phosphate group. The pK_a values for the mono- and dinucleotides are all internally consistent and show good reproducibility. The theoretical treatment of the data can be seen to fit the experimental points to well within the experimental uncertainty (Fig. 6).

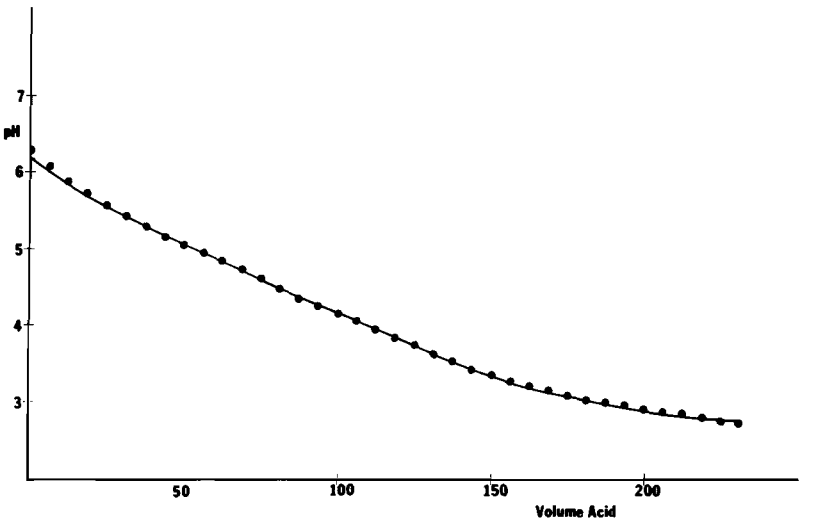

Fig. 5. pH *versus* microliters of 0.0100 *M* HCL where [CMP] = 1.04×10^{-3} *M*, [$MgCl_2$] = 0.200 *M*, and the total volume is 0.500 ml. The theoretical curve given by eq. (14) is superimposed on the experimental points.

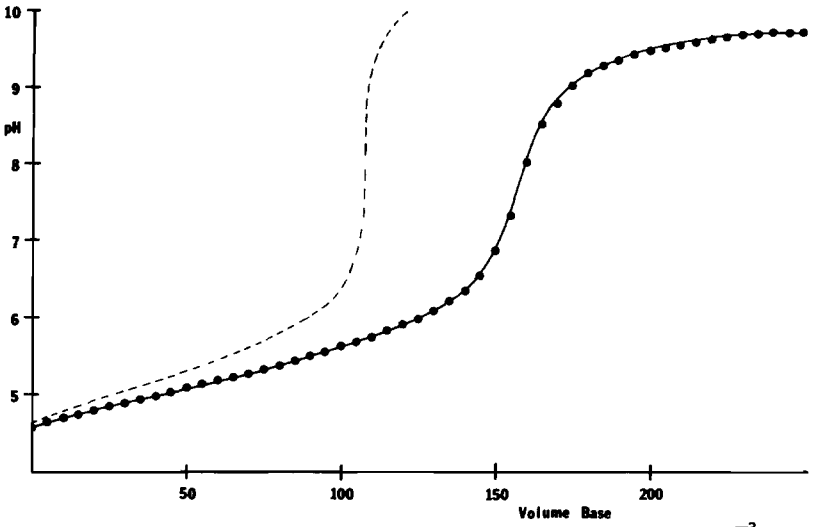

Fig. 6. pH *versus* microliters of 0.0100 *M* NaOH where [AMP] = 3.37×10^{-3} *M*, [$MgCl_2$] = 0.200 *M*, and the total volume is 0.500 ml. The theoretical curve given by eq. (14) is superimposed on the experimental points. The dashed line indicates the calculated curve if a M_2L binding constant of 1.0 is assumed. The larger the value of K_{M_2L} the more deviation from the experimental points.

Discussion

It is interesting that the metal binding constants for all the nucleotides are quite similar, which might be expected for the metal–phosphate binding model (Fig. 1). These constants are slightly lower than those reported by Khan and Martell [9]. One assumption made in their work is that the AMP obtained from Sigma is the monosodium salt. The process Sigma uses for isolating their nucleotides involves precipitating the acid in high salt concentrations and then lyophilizing the crystals. Their typical AMP lots are assayed at 1.5 mol of sodium per mol AMP, which accounts for the inclusion of the adjustable parameter X in eq. (14).

It may be of even more interest that the values of the metal binding constant can change, two-fold, in the case of dinucleotides, depending on the base adenine, thymine, guanine, or cytosine. This shows that although the metal phosphate binding is the predominant interaction, there is a modification of this affinity depending on the nature of the base. This effect can lead to more pronounced differences in longer oligonucleotide sequences. The intrinsic binding constants determined here can be useful in the analysis of the interaction between magnesium(II) and DNA sequences of longer chain length. The series 5′-TMP, d-TpT, d-pTpT is an example of these two factors. 5′-TMP can bind with magnesium better than d-TpT because of the extra oxyanion present. This is shown by the higher binding constant in Table I. d-pTpT has a higher binding constant than 5′-TMP, probably due to the extra phosphate group.

Potentiometric microtitrations are a fast, convenient method to obtain pK_a values and binding constants of oligonucleotides. They may also be used to determine the concentration of ligand in solution. This is especially important in the case of higher oligonucleotides where molar extinction coefficients are not known. The microtitration can easily be applied to oligonucleotides of a dilute concentration ($\sim 10^{-4}$ *M*) using a volume of only 0.2 ml. This means that in only one experiment the values of the metal binding constant and the pK_as can be determined, all with just $\sim 2 \times 10^{-8}$ mol of ligand of interest. This is of pivotal importance in studies on biologically important oligonucleotides which are available in minute quantities only.

The sensitivity and accuracy of this method are due to two factors. First, the pH electrode can measure the concentration of hydrogen ion very precisely (relative pH = ±0.001) over a concentration range of more than eight decades. Second, the computer analysis allows for the determination of certain parameters within the error limits imposed by the experimental data. In the past, the actual concentration of oligonucleotide in solution was not known exactly and usually was the greatest cause for uncertainty in the calculations. In the present case, this can be determined independently as a free parameter in a curve fit analysis.

The multiparametric curve fit uses the mathematical model described by eq. (14) to generate a theoretical curve. The parameters are then adjusted by the method of steepest descent to give the best correlation between the theoretical and experimental data. A plot of the deviation of each point (given by the difference between the calculated and observed value divided by the standard deviation of the fit) *versus* the point number should give a random

pattern whose maximum deviation is the uncertainty of the data (Fig. 3a). If any pattern develops, there is an error in the mathematical model. In the case of AMP Fig. 3a is the actual plot of the deviation pattern. In Fig. 3b the sodium term X in eq. (14) is assigned an incorrect value and in Fig. 6 the 2:1 metal complex M_2L^{2+} is assumed in the model. Clearly, the latter two cases introduce a systematic error resulting from an incorrect mathematical treatment and will cause a pattern in the deviation plot.

The problems associated with this technique are common to all oligonucleotide work and are accentuated by the sensitivity of this method. Any additional acid–base species present in the oligonucleotides had to be accounted for in eq. (14) since they would affect the pH. The acid dissociation of ammonia had to be taken into account since typically some of the ologonucleotides were present as the ammonium salt. This was included in the system of equations by adding $[NH_4^+]$ to eq. (14) and solving the mass balance for ammonia using its acid dissociation

$$NH_4^+ \rightleftharpoons NH_3 + H^+ \quad KA4 = 5.5 \times 10^{-10} \quad (15)$$

$$\text{CAMMONIA} = NH_4^+ + NH_3 = [NH_4^+] + \frac{[NH_4^+]\,KA4}{[H^+]} \quad (16)$$

$$[NH_4^+] = \text{CAMMONIA} / \left(1 + \frac{KA4}{[H^+]}\right)$$

The analytical concentration of ammonia could be determined as a free parameter. This drawback becomes serious only when the species are unknown, or in concentrations similar to that of the oligonucleotide.

The small concentrations involved made it necessary to use a very stable electrode-pH meter combination with an inert atmosphere. Contamination with air, or any other impurities, had to be avoided as this would produce a drift in the pH of the system. It should also be mentioned that this technique can be scaled down to the titration of less than 40 μl of total volume by the use of a micro combination pH probe (Microelectrodes, Inc., MI-410), and a suitable titration vessel.

It is interesting to note that the binding constants in Table I are much higher than those calculated by eq. (18) for an outer sphere complex [10].

$$K_{os} = \frac{4\pi Na^3}{3000} \exp(-U_a/kT) \quad (18)$$

where

$$U_a = \frac{(Z1)(Z2)e^2}{aD} - \frac{(Z1)(Z2)e^2\kappa}{D(1+\kappa a)}$$

and

$\kappa^2 = 8\pi Ne^2\mu/1000\,kTD$
N = Avogadro's number
a = distance of closest approach (~5 Å)
e = electronic charge
k = Boltzmann Constant
D = dielectric of the medium
T = absolute temperature
μ = ionic strength.

By substituting the proper values into eq. (18) one can solve for the outer sphere binding constant for the magnesium(II)–AMP complex. The results of these calculations are shown in Table II for various metal ligand charge complexes. In all cases, K_{os} is much less than the observed metal binding constant indicating that the binding is inner sphere.

Acknowledgment

This work was supported by NIH Grant No. 1 R01 GM24523-01. We are grateful to Prof. Louis Meites for helpful discussions on the multiparametric curve fitting calculations. Thanks are due to Dr. S. A. Narang for supplying us with some of the dinucleotides.

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