Spectrophotometric Evidence for the Formation of a 2-Nickel–Adenosine Triphosphate Complex†

Thomas A. Glassman,‡ Jan Suchy, and Cecil Cooper*

ABSTRACT: The change in ultraviolet absorbance produced by the addition of Ni^{2+} to an ATP solution results from the formation of both a 1:1 Ni–ATP complex in which the phosphate bound metal interacts with the adenine ring and from a 2:1 Ni_2–ATP complex. The association constant for the Ni_2–ATP complex at 25°, pH 6.2, and ionic strength 0.1 M is 250 M^{-1}. Measurements made with a mixture of Ni^{2+} and adenosine showed no evidence of complex formation whereas there clearly was complex formation with a mixture of adenosine and Ni^{2+}–triphosphate. The association constant for the adenosine–Ni^{2+}–triphosphate complex at 25° and ionic strength 0.1 M is 75 ± 50 M^{-1}. The possible structures and significance of the 2:1 metal–ATP species in the regulation of enzymatic reactions are discussed.

All enzymatic reactions utilizing adenosine di- or triphosphates require divalent cations. At metal to nucleotide ratios around one, a complex of one metal to one ligand (ATP or ADP) is formed. Although this complex has been extensively studied, there remains much controversy over the structure of and the degree of metal ion–adenine ring interaction in the complex (Walaas, 1958; Watanabe *et al.*, 1963; Hotta *et al.*, 1961; Schneider *et al.*, 1964; Happe and Morales, 1966; Cohn and Hughes, 1962; Sternlicht *et al.*, 1965a,b; Glassman *et al.*, 1971; Kuntz *et al.*, 1972). At metal/ATP ratios greater than unity both nonenzymatic and enzymatic studies have suggested the existence of a complex containing two metal ions bound per ATP (Lowenstein, 1958; Liebecq and Jacquemotte-Louis, 1958; Handschin and Brintzinger, 1962; Kuby *et al.*, 1954; Noat *et al.*, 1970; Mohan and Rechnitz, 1972; Frey *et al.*, 1972).

In this paper, we will show that the difference in absorbance between a Ni^{2+}–ATP solution and an ATP solution at the same concentration, pH, and ionic strength results from the formation of the protonated and unprotonated 1:1 metal–ATP complexes in which the phosphate bound metal ion interacts with the adenine ring and at high metal to ligand ratios from the formation of 2:1 metal–ATP complex. We have also examined the effect that complexing Ni^{2+} to a triphosphate chain has on the ability of the metal to interact with the adenine ring of adenosine and ATP. The Ni^{2+} metal ion was used in this study because it produces a greater absorbance change with adenine nucleotides and nucleosides than most other divalent metal ions and because extensive magnetic resonance studies have been done on its interaction with ATP.

Experimental Section

The disodium salt of ATP, the barium salt of ADP, the disodium salt of AMP, and adenosine were purchased from Sigma Chemical Co. The method of Davoll and Lowy (1951) was used to prepare 9-(β-D-glucopyranosyl)adenine. This compound was phosphorylated by methods described in a previous communication (Hohnadel and Cooper, 1972). The resulting 6′-triphosphate derivative, gATP,[1] had the same ultraviolet spectrum as ATP, a phosphorus to adenine ratio of 3.0 ± 0.1, and gave a single spot on thin-layer chromatography in the following solvents: isobutyric acid–concentrated NH_3–water (66:1:33); 95% ethanol–1 M ammonium acetate, pH 7.5 (5:2); 1-propanol–concentrated NH_3–water (6:3:1).

The tetramethylammonium salts of the nucleotides were made by eluting the nucleotides from a Dowex 50 (H^+) column with quartz-distilled water and adjusting the pH of the solution with tetramethylammonium hydroxide (Eastman Chemical). Final concentrations were determined spectrophotometrically.

Nickel solutions were prepared from reagent grade $Ni(NO_3)_2 \cdot 4H_2O$ (Fisher Scientific Co.) and were standardized by atomic absorption against certified standards (Fisher Scientific Co.). Commercial sodium triphosphate, $Na_5P_3O_{10} \cdot 6H_2O$ (BIOS Laboratories), was purified by repeated crystallizations from water–ethanol solutions (Quimby, 1954). The final product was chromatographed on paper in isopropyl alcohol–20% trichloroacetic acid–concentrated NH_3 (75:25:0.25) and no mono- or pyrophosphate was detected after the chromatogram was developed with a modified Hanes-Ishewood spray (Bandurski and Axelrod, 1951).

Measurements were made by selecting an appropriate wavelength and reading the difference in absorbance between a Ni^{2+}–nucleotide or nucleoside solution and a solution of nucleotide or nucleoside at the same concentration, ionic strength and pH. Because the tetramethylammonium ion and $Ni(NO_3)_2$ have ultraviolet absorption, the absorbance of solutions of these ions was determined at the same concentrations used in the experiments and the appropriate corrections were made. For studies measuring the effect of pH on the change in absorbance, solutions were adjusted to pH 10 with tetramethylammonium hydroxide and titrated with nitric acid. For studies measuring the binding constants of the various complexes, the metal ion concentration was

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received January 23, 1973.* This work was supported by a research grant (GB-12747) from the National Science Foundation and a training grant (GM-00035) from the National Institutes of Health.

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[1] Abbreviations used: gATP, 9-(β-D-glucopyranosyl)adenine 6′-triphosphate.

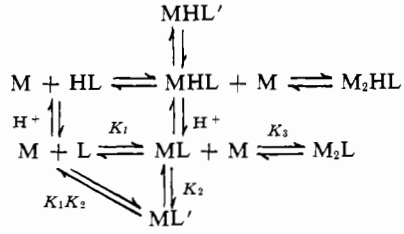
varied from 10 times the nucleotide or nucleoside concentration to either 100 times the nucleotide or nucleoside concentration or to an ionic strength of 0.1 M. For those studies involving nickel concentrations greater than 2×10^{-4} M, the ionic strength of the solution was maintained at 0.1 M with tetramethylammonium nitrate (Eastman Chemical Co.). For the experiments involving ATP at low concentration, no ionic strength adjustment was made. Binding constants for Ni^{2+}–triphosphate and adenosine were obtained by measuring the change in absorbance at 274 nm (pH 7.0 ± 0.2, 25°) of Ni^{2+}–triphosphate and 1.91×10^{-4} M adenosine solutions as the concentration of Ni^{2+}–triphosphate was varied from 1×10^{-3} to 5×10^{-3} M. Because the low solubility of Ni^{2+}–triphosphate at this pH prevented measurements at higher concentrations, the binding constant was also obtained by keeping the Ni^{2+}–triphosphate concentration constant at 1×10^{-3} M and varying the adenosine concentration from 0.05 to 0.25 M.. The latter measurements were made at 294 nm, pH 9.3 ± 0.3, and 25°. Measurements were made on a Gilford 2000 or Cary 14 spectrophotometer.

The fluorescence measurements were made with an Aminco-Bowman Spectrofluorometer. The samples were activated at 285 nm and the emission measured at 395 nm. The nucleotide concentration was 1.7×10^{-4} M and $Ni(NO_3)_2$ or $Mg(NO_2)_3$ was added to the appropriate metal/nucleotide ratios. All solutions contained 0.25 M tetramethylammonium chloride.

Theoretical

Most spectrophotometric techniques used for the determination of association constants are modifications of the Benesi–Hildebrand method (1949). The reciprocal absorbance changes are plotted against the reciprocal metal or ligand concentration and from the intercepts of these plots values for the association constant and extinction coefficient can be derived. In order to determine whether a complex has been formed, the change in absorption spectrum of a solution containing the reactants is compared to the spectra of the individual components. It is not necessary to have a new or shifted absorption band to establish the presence of a complex. What is needed is to show that a change in the absorption spectrum has occurred and that this change can be resolved into a single value for the association constant and a finite constant value for the extinction coefficient (Person, 1965). When this method is used for a system as complicated as the one described below, it is necessary to know what the intercept values actually represent. To do this we have derived the relationship between the change in absorbance to the metal and ligand concentration for the Ni^{2+}–ATP system.

The metal–ATP complexes can be related by the following reactions



where M = metal ion, L = ligand (ATP), ML = metal bound to phosphates of ligand only, ML′ = metal bound to phosphates and the adenine ring, and M_2L = two metals bound to the ligand. The HL, MHL, MHL′, and M_2HL species are all protonated at the phosphate chain.

The following equilibrium constants describe this scheme:

$$K_1 = (ML)/(M)(L),\ K_2 = (ML')/(ML),\ K_3 = (M_2L)/(M)(ML)$$

$$K_1K_2 = (ML')/(M)(L),\ K_1K_3 = (M_2L)/(M)^2(L)$$

$$K = K_1 + K_1K_2 = [(ML) + (ML')]/(M)(L)$$

$$K' = K_1K_3/K = K_3/(1 + K_2) = (M_2L)/[(M)(ML + ML')]$$

If M_T is the total metal ion concentration, L_T the total ligand concentration, M the free metal ion concentration, and L the free ligand concentration, then $M_T = M + ML + ML' + 2M_2L$ and $L_T = L + ML + ML' + M_2L$.

The protonated form of the 1:1 metal/nucleotide complex is not included in these equations because at pH 6, nucleotide concentrations around 1×10^{-3} M, and metal/nucleotide ratios of 10 it represents less than 5% of the total ligand concentration (Frey and Steuhr, 1972).

Assuming that the L and ML forms have the same extinction coefficient ϵ_L, that the extinction coefficient of ML′ is $\epsilon_{ML'}$ and that of M_2L is ϵ_{M_2L}, then the absorbance A of the sample cell is

$$A = \epsilon_L(L + ML) + \epsilon_{ML'}(ML') + \epsilon_{M_2L}(M_2L)$$

and the absorbance of the reference cell is

$$A = \epsilon_L(L_T)$$

Experimental conditions were selected so that $M_T \gg L_T$ and $M_T = M$. The above equilibrium constants can be solved for ML′ and M_2L in terms of M_T and L_T and when substituted into the above equations yield

$$\Delta A = \frac{\Delta\epsilon_{ML'-L}[K_1K_2(M_TL_T)] + \Delta\epsilon_{M_2L-L}[K_1K_3(M_T)^2(L_T)]}{1 + K(M_T) + K_1K_3(M_T)^2}$$

$$1/\Delta A = \frac{1 + K(M_T) + K_1K_3(M_T)^2}{\Delta\epsilon_{ML'-L}[K_1K_2(M_TL_T)] + \Delta\epsilon_{M_2L-L}[K_1K_3(M_T)^2(L_T)]}$$

where A = absorbance of sample cell − absorbance of reference cell. Literature values for K at 0.1 M ionic strength have varied from 35,000 M^{-1} (Brintzinger, 1961) to 100,000 M^{-1} (Kahn and Martell, 1967) so that at metal ion concentrations greater than 2×10^{-4} M

$$1 + K(M_T) = K(M_T)$$

$$1/\Delta A = \frac{1 + K_2 + K_3(M_T)}{\Delta\epsilon_{ML'-L}K_2(L_T) + \Delta\epsilon_{M_2L-L}K_3(M_TL_T)}$$

At the extremes when $1/(M_T)$ approaches zero and $1/\Delta A$ approaches zero

$$1/\Delta A = 1/\Delta\epsilon_{M_2L-L}(L_T)$$

$$1/(M_T) = -K' = \frac{-K_1K_3}{K} = -K_3/(1 + K_2)$$

At low concentrations of ATP and low M^{2+}/ATP ratios, the M_2L species will not be formed and

$$1/\Delta A = \frac{1 + K(M_T)}{\Delta\epsilon_{ML'-L}K_1K_2(M_TL_T)}$$

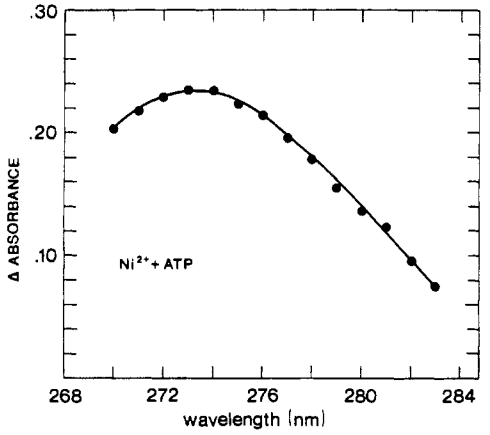


FIGURE 1: Change in absorbance, A, of a Ni^{2+}–ATP solution plotted against wavelength. The solution contained 0.20×10^{-3} M ATP and 20×10^{-3} M $Ni(NO_3)_2$ at a pH of 6.38 and ionic strength of 0.1 M.

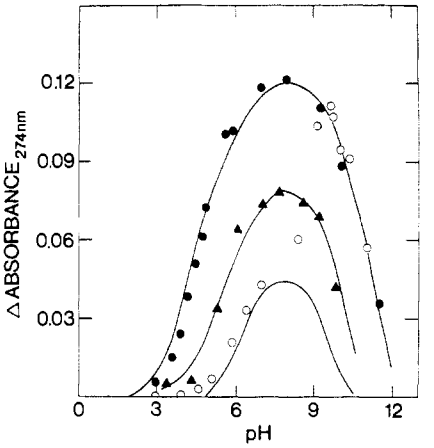


FIGURE 2: Change in absorbance of 1:1 Ni^{2+}–nucleotide solutions plotted against pH: (●) concentration of Ni^{2+} and ATP was 0.18×10^{-3} M; (▲) concentration of Ni^{2+} and ADP was 0.17×10^{-3} M; (○) concentration of Ni^{2+} and AMP was 0.19×10^{-3} M. Solid lines are absorbance changes calculated from published ionization and binding constants as explained in the text. The points are experimental values.

As $1/(M_T)$ approaches zero

$$1/\Delta A = \frac{1}{\alpha \Delta \epsilon_{ML'-L}(L_T)}$$

where $\alpha = K_2/(1 + K_2)$. As $1/\Delta A$ approaches zero, $1/(M_T) = -K$.

This extrapolation results in a binding constant for the entire 1:1 metal–ATP complex and does not depend on the equilibrium between the ML and ML′ species.

For gATP, the ML′ complex is probably not formed because of steric and conformational effects and the following is likely

$$M + L \overset{K_a}{\rightleftharpoons} ML + M \overset{K_b}{\rightleftharpoons} M_2L$$

Following the above reasoning as $1/\Delta A$ approaches zero, $1/(M_T) = -K_b$, and as $1/(M_T)$ approaches zero

$$1/\Delta A = \frac{1}{\Delta \epsilon_{M_2L-L}(L_T)}$$

The complex formation between Ni^{2+} and adenosine may be represented by

$$M + L \overset{K_c}{\rightleftharpoons} ML$$

and at the extremes $1/\Delta A = 1/\Delta \epsilon_{ML-L}(L_T)$ and $1/(M_T) = -K_c$.

Results

In Figure 1, the ultraviolet difference spectrum from 270 to 286 nm of an aqueous solution of 2×10^{-4} M ATP and 2×10^{-2} M $Ni(NO_3)_2$ is shown. When possible, absorbance measurements in the following experiments were made at 274 nm, a wavelength near the maximum of this curve. At higher concentrations of ATP, the absorbance at this wavelength was so large that accurate readings could not be obtained and therefore a wavelength as near as possible to the plateau region was selected.

The effect of pH on the change in absorbance of 1:1 Ni^{2+}–ATP, 1:1 Ni^{2+}–ADP, and 1:1 Ni^{2+}–AMP solutions is shown in Figure 2. For each curve, the points represent experimental data while the solid lines are calculated absorbance changes. The ionization and binding constants determined by Perrin and Sharma (1967), Kahn and Martell (1967), and Frey and Steuhr (1972) were used to calculate the concentrations of the various protonated and unprotonated metal–ligand species. It was initially assumed that the only absorbance change resulted from the unprotonated 1:1 metal–ligand complex or $\Delta A = X(ML + ML')$. The constant, X, was calculated from the experimental absorbance change at a pH where only the 1:1 unprotonated metal–ligand complex exists. In order to fit the experimental points, it was necessary to assume that the 1:1 metal–ADP and metal–ATP complexes protonated on the phosphate chain (MHL + MHL′) also contributed to the absorbance change or $\Delta A = X(ML + ML') + Y(MHL + MHL')$. An excellent fit resulted for Ni^{2+}–ATP when $X = 800$ and $Y = 2780$ and for Ni^{2+}–ADP when $X = 885$ and $Y = 3600$. In the theoretical section it was shown that for Ni^{2+}–ATP, the constant X corresponds to $\alpha \Delta \epsilon_{ML'-L}$. The value of 800 obtained in the above studies agrees very well with the value of 620 obtained from subsequent binding constant measurements (Table I). For Ni^{2+}–AMP with a value of $X = 5000$, the initial part of the experimental curve fits fairly well although the slight deviation indicates a previously unidentified protonated complex may exist. It is apparent that at the higher pH another species, as yet unidentified, is contributing to the absorbance change. This species is not the bis complex and since the maximum absorbance change of this unknown species occurs at pH 9.4, the pK_a for the hydrolysis of the hydrated Ni(II) ion, it might be a Ni^{2+}–AMP–OH^- species. The decrease in absorbance change at high pH in all cases was accounted for by the formation of $Ni(OH)_2$.

In the theoretical section, it was assumed that when one metal is bound to ATP, only the ring-bound species will contribute to the change in absorbance. Molecular models show that for steric and conformational reasons, there is little probability that the phosphate bound metal ion in gATP can interact with the adenine ring. Since the binding constants for ATP and gATP primarily reflect the association of the metal ion and the phosphate chain, they should

TABLE I: Calculated Association Constants and Extinction Coefficients for Various Complexes with Ni^{2+}.

Compound Concn (M)	Wavelength (nm)	Association Constant		$\Delta\epsilon_{M_2L-L}$, $M^{-1} \times 10^{-3}$	$\alpha\Delta\epsilon_{ML'-L}$, $M^{-1} \times 10^{-3}$	$\Delta\epsilon_{M_2L-L}$ from Figure 1, $M^{-1} \times 10^{-3}$
		2:1 Complex M^{-1}	1:1 Complex M^{-1}			
ATP (2×10^{-3})	283	$K' = (M_2L)/(M)(ML + ML')$		0.37		0.38
		$K' = 250$				
ATP (1×10^{-3})	281	$K' = 250$		0.50		0.58
ATP (5×10^{-4})	277	$K' = 250$		0.85		0.98
ATP (5×10^{-5})	274	$K' = 150$			0.62	
ATP (2×10^{-5})	274	$K' = 150$	$K = (ML + ML')/(M)(L) = K_1 + K_1K_2$	1.25	0.62	1.17
			$K = 37,000$			
gATP (1×10^{-3})	281	$K_b = (M_2L)/(M)(ML) = 200$		0.22		
Adenosine–PPP_i (2×10^{-3})	274		$K_c = (ML)/(M)(L) = 75$	3.85		

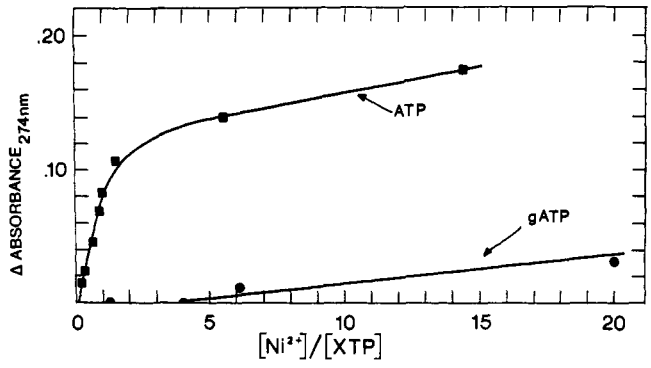


FIGURE 3: Change in absorbance of Ni^{2+}–ATP and Ni^{2+}–gATP solutions plotted against the Ni^{2+}–nucleotide ratio. The nucleotide concentration was 0.19×10^{-3} M and the pH 5.5 in both cases.

be approximately the same for the 1:1 metal–nucleotide complex. The effect of adding Ni^{2+} to 0.188×10^{-3} M gATP and ATP solutions at pH 5.5 is dramatically different (Figure 3). At Ni^{2+} to gATP ratios up to 4, no change in absorbance occurs while the curve for Ni^{2+}–ATP is biphasic with an initial sharp increase in absorbance as metal ion is added. Since there is no reason to doubt that metal ion is bound to the phosphates of gATP, forming the 1:1 metal–nucleotide species will not change the absorbance of the solution unless the phosphate-bound metal ion can interact with the adenine ring. This also implies that the complex in which the metal binds only to the phosphates, ML, has the same extinction coefficient as the free ligand species, L. At higher Ni^{2+} to gATP ratios, a change in optical density occurs. Since these values were all corrected for metal absorption, this suggests that an M_2L species is formed.

It has been postulated above that when Ni^{2+} is added to a solution of ATP, the ML′ and M_2L species are responsible for the changes in absorbance of the difference spectra. If it is assumed that the association constants for the 1:1 and 2:1 metal–nucleotide complexes are different by at least a few orders of magnitude then there should be a set of conditions where just the ML′ or M_2L complexes affect the change in absorbance. Under these conditions, plots of $1/\Delta A$ *vs.* $1/(Ni^{2+})$ should be linear and determined by their respective association constants and extinction coefficients. At concentrations between these extremes, plots of $1/\Delta A$ *vs.* $1/(Ni^{2+})$ should be the summation of these two linear plots. In Figure 4, $1/\Delta A$ *vs.* $1/(Ni^{2+})$ has been plotted for ATP concentrations of 5×10^{-4}, 1×10^{-3}, and 2×10^{-3} M. In each experiment the metal ion concentration was varied from ten times the nucleotide concentration to a maximum Ni^{2+} concentration giving an ionic strength of 0.1 M. Because of differences in the absorbance of the ATP blank, the measurements were done at 277, 281, and 283 nm, respectively. These plots yield straight lines with an extrapolated value for the association constant of the M_2L species of 250 M^{-1}. In Table I the extinction coefficients obtained at the different wavelengths (Figure 4) are compared to $\Delta\epsilon_{M_2L-L}$ values calculated from the difference spectrum of an aqueous solution of 2×10^{-4} M ATP and 2×10^{-2} M $Ni(NO_3)_2$ (Figure 1).

Plots of $1/\Delta A$ *vs.* $1/(Ni^{2+})$ at 5×10^{-5} and 2×10^{-5} M ATP are shown in Figure 5. For these measurements 5- and 10-cm light-path cuvets were used. It was not possible to obtain accurate measurements at lower ATP concentrations. It appears that as the concentration of ATP is lowered from 5×10^{-5} to 2×10^{-5} M, plots of $1/\Delta A$ *vs.* $1/(Ni^{2+})$ approach linearity and it is probable that if measurements

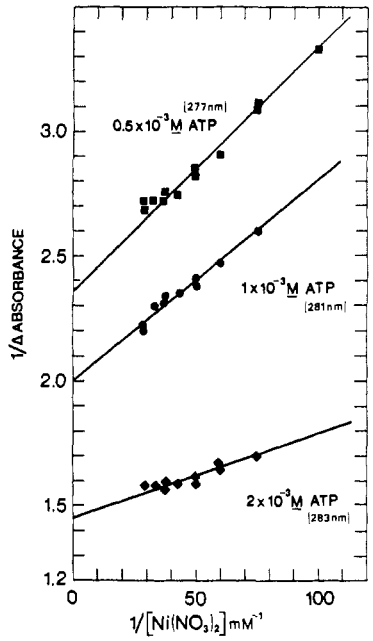


FIGURE 4: $1/\Delta A$ of Ni^{2+}–ATP solutions plotted against $1/(Ni^{2+})$. The pH was 6.2 ± 0.2 and the ionic strength was adjusted to 0.1 M with tetramethylammonium nitrate.

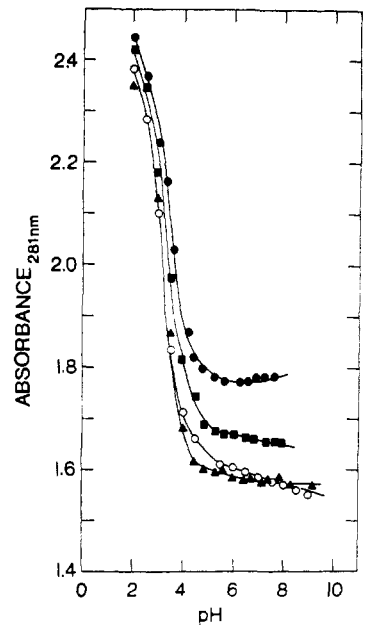


FIGURE 6: Absorbance of Ni^{2+}–adenosine and adenosine solutions plotted against pH: (■) 0.91×10^{-3} M $Ni(NO_3)_2$ and adenosine; (▲) 0.10×10^{-3} M adenosine; (○) 0.91×10^{-3} M sodium triphosphate and adenosine; (●) 0.91×10^{-3} M sodium triphosphate, $Ni(NO_3)_2$ and adenosine.

could have been made at lower ATP concentrations, a straight line representing the formation of the ML and ML′ species would have been obtained. The curved line at 2×10^{-5} M ATP can be resolved into two straight lines. Values for the extinction coefficient and binding constant of the M_2L species are assumed and the series of straight lines representing these values is plotted. Points along these lines are then subtracted from corresponding points on the experimental curve and $1/\Delta A$ values from these differences are plotted against the reciprocal Ni^{2+} ion concentration. For ATP at 2×10^{-5} M, this treatment resolved the curve into two straight lines, one with an association constant of 150 M^{-1}, the other with an association constant of approximately 37,000 M^{-1}; the extinction coefficients are given in Table I. The association constant of 150 M^{-1} is for the formation of the M_2L complex. The ionic strength for this determination was less than 0.1 M and one might have expected a value higher than the 250 M^{-1} which was obtained at an ionic strength of 0.1 M. Because the former measurements were made at much lower concentrations than needed for the optimal study of the M_2L complex and because of the error associated with resolving the experimental curve into its components, we interpret this experiment as showing that there are two processes contributing to the change in absorbance of the Ni^{2+}–ATP system. The value for the 2:1 metal–ATP association constant is roughly in the correct range of the value obtained under better conditions while the value for the association constant of the 1:1 metal–ATP complex is consistent with literature values for this complex (Brintzinger, 1961; Kahn and Martell, 1967; Frey and Steuhr, 1972).

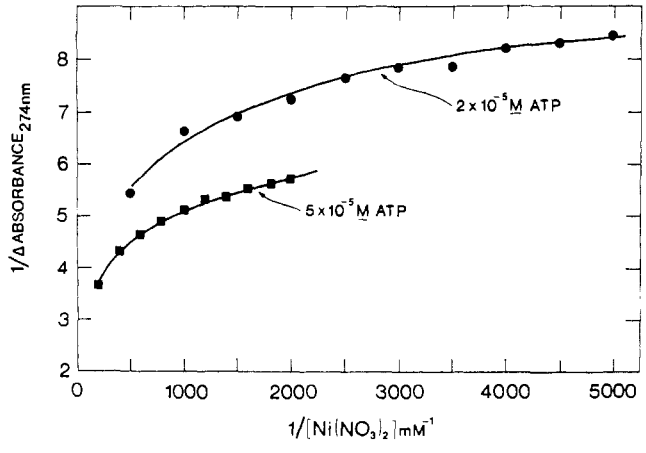


FIGURE 5: $1/\Delta A$ of Ni^{2+}–ATP solutions plotted against $1/(Ni^{2+})$.

The association constant of Ni^{2+}–gATP was obtained by plotting $1/\Delta A$ *vs.* $1/(Ni^{2+})$ at 1×10^{-3} M gATP and pH 6.0. These plots are straight lines; the binding constant for this complex is about 200 M^{-1} and the change in extinction coefficient at 281 nm is 0.22×10^{-3} M^{-1}.

Because the M_2L complex might be formed between a non-phosphate-bound metal and the adenine ring of ATP, the interaction of Ni^{2+} with adenosine was compared to the interaction of Ni^{2+}–triphosphate with adenosine. In Figure 6, the effect of pH on the absorbance of a 1:1 Ni^{2+}–adenosine solution at 0.91×10^{-3} M is compared to the effect of pH on an adenosine solution at the same concentration and ionic strength. Figure 6 also shows that when inorganic triphosphate was added to each solution, a greater change in the absorbance is seen. This implies that either the interaction between Ni^{2+}–triphosphate and adenosine is greater than the interaction between Ni^{2+} and adenosine or that the extinction coefficient of Ni^{2+}–triphosphate–adenosine is greater. Binding constants for the Ni^{2+}–triphosphate–adenosine complex were measured by plotting $1/\Delta A$ at 274 nm against $1/(Ni^{2+}$–triphosphate) at 1.96×10^{-4} M adenosine, pH 6.5 and ionic strength 0.1 M. A value of 25 M^{-1} was obtained. In order to measure the binding constant of the complex at higher concentrations, plots of $1/A$ against 1/(adenosine) at 1×10^{-3} M Ni^{2+}–triphosphate, pH 9.3, ionic strength 0.1 M were made by varying the adenosine concentration from 0.05 to 0.25 M. These measurements yielded a binding con-

stant for Ni^{2+}–triphosphate to adenosine of 75 ± 50 M^{-1}. When the same conditions were used for Ni^{2+}–adenosine no change in absorbance occurred.

Discussion

Ni_2–ATP Complex. The preceding spectrophotometric analysis of the effect of Ni^{2+} on the ultraviolet absorption spectrum of ATP has provided evidence for the existence of a Ni_2–ATP complex. At ATP concentrations of 2×10^{-3}, 1×10^{-3}, and 5×10^{-4} M, plots of $1/\Delta A$ *vs.* $1/(Ni^{2+})$ yielded straight lines which extrapolated to a binding constant of 250 M^{-1}. The extinction coefficients obtained in these experiments agreed to within 15% when adjusted to the same wavelength. This fulfills the criteria of Person (1965) for spectrophotometrically establishing the presence of a complex. Mohan and Rechnitz (1972) using a Ca^{2+} selective membrane electrode obtained an association constant for Ca_2ATP at zero ionic strength of 1100 M^{-1}. Their association constant becomes 160 M^{-1} at an ionic strength of 0.1 M when calculated by the extended Debye–Huckel equation (Frey *et al.*, 1972). This latter value is very similar to the 250 M^{-1} we obtained for Ni_2–ATP at an ionic strength of 0.1 M.

Several possibilities exist for the structure of the Ni_2–ATP complex: (1) the second nickel ion displaces the phosphate bound nickel ion at N-7; (2) the second nickel ion binds to N-1 or N-3 while the phosphate-bound nickel ion remains complexed at N-7; (3) the second nickel ion also binds to the phosphate chain and either one or both nickel ions interact with the adenine ring; (4) the second nickel ion also binds to the phosphate chain and interacts with the adenine ring of another ATP molecule, *i.e.*, an $(M_2L)_n$ complex is formed.

The Ni_2–ATP complex probably does not involve binding of free Ni^{2+} to the adenine ring since we detected no interaction between Ni^{2+} and adenosine. There was only an interaction between Ni^{2+} and adenosine when triphosphate was present. However it is possible that the association constants for Ni^{2+}–adenosine and Ni^{2+}–triphosphate–adenosine are similar but that the extinction coefficient of the Ni^{2+}–triphosphate–adenosine complex is much larger and allows us to measure the association constant. This seems unlikely because we have preliminary nuclear magnetic resonance experiments showing that Ni^{2+} has only a small and equal effect on the chemical shift of the H-2 and H-8 protons of adenosine while Ni^{2+}–triphosphate causes a much larger shift of the H-8 proton.[2] This indicates that when Ni^{2+} is complexed to triphosphate the ability of the nickel to bind to the adenine ring is considerably enhanced and the binding becomes more specific for the N-7 position. If free Ni^{2+} does not bind to the adenine ring in ATP then the formation of the M_2L species involves either increasing the tendency of the second Ni^{2+} to bind to the adenine ring by having it complexed to the nucleotide phosphates or in some way increasing the tendency of the adenine ring to bind to the second Ni^{2+}. The latter possibility may occur if the adenine ring is already complexed to one metal ion as in the case of Ni–ATP. This seems unlikely since the association constants for Ni_2–gATP and Ni_2–ATP are almost identical and the Ni–gATP complex probably does not involve a ring interaction. In addition, Frey and Steuhr (1972) obtained identical association constants for Mg_2–ATP and Mg_2–CTP and it is very unlikely that the metal ion in a 1:1 metal–CTP complex interacts with the ring to a significant degree (Glassman *et al.*, 1971).

It is tempting to evaluate the extinction coefficient data in Table I in terms of two metal ions being bound to the adenine ring in the Ni_2–ATP complex. At 281 nm, $\Delta\epsilon_{M_2L}$ is 0.5×10^3 for ATP and 0.22×10^3 for gATP. Since Ni_2–gATP does not involve a back bound complex with the adenine ring while ATP does, the higher extinction coefficient of ATP suggests that two metal ions may be bound to the ring in the Ni_2–ATP complex. At 274 nm, $\Delta\epsilon_{M_2L}$ for Ni_2–ATP is 1.25×10^3 and $\alpha\Delta\epsilon_{ML'}$ for Ni–ATP is 0.62×10^3 where $\alpha = K_2/(1 + K_2)$. If K_2 is much larger than 1, then the extinction coefficient for the 2:1 complex is twice that of the 1:1 complex and could mean that two metal ions are bound to the ring in Ni_2–ATP. This approach must be qualified. It is not known whether the second metal ion in Ni_2–ATP forms an inner or outer sphere complex with the adenine ring. Even if this were known, there is no theoretical basis for saying which type of complex would have a greater extinction coefficient. If the second metal ion formed an inner sphere complex with the adenine ring and if an inner sphere interaction has a greater extinction coefficient then the above results would also be consistent with one metal ion being bound to the adenine ring in Ni_2–ATP.

The above arguments suggest by exclusion that in Ni_2–ATP both metal ions may be bound to the phosphate chain. It is possible that having two metal ions bound to the phosphates disrupts the anti-gauche conformation of the phosphate chain (Sundaralingam, 1969) and allows either both metal ions or, in the case of gATP and CTP, one metal ion to interact with the adenine ring. It is also possible that the second metal ion binds to the phosphate chain and interacts with an adenine ring of another molecule. The low concentrations at which our and Mohan and Rechnitz's association constants were obtained make this latter structure less likely.

Ni–ATP Complex. Much of the controversy about the 1:1 metal–ATP complex has been whether the phosphate-bound metal ion interacts with the adenine ring and the extent of this interaction. We have assumed that when Ni^{2+} is bound to gATP, the metal-bound phosphate can not interact with the adenine ring for steric and conformational reasons and that the binding of Ni^{2+} to the phosphates of gATP and ATP is roughly equivalent. From studying atomic models and from previously published stability constant measurements (Walaas, 1958), these assumptions seem justified. Our spectrophotometric and nuclear magnetic resonance data show that in the Ni–ATP complex, the phosphate bound metal interacts with the adenine ring. As was shown in the theoretical section, measurements of the change in optical density at low ATP concentrations do not reflect the equilibrium between the ML and ML′ species but are influenced only by the overall stability constant for the formation of the 1:1 metal–ATP complex. It is not possible to measure the equilibrium constant of the $ML \rightleftharpoons ML'$ reaction or the fraction of ring interaction in the 1:1 metal–ATP complex in this system unless one has an independent method for determining the extinction coefficient of the ML′ species or the maximum change in absorbance when just the ML′ species exists. The above equilibrium is not dependent on metal ion or ligand concentration, *i.e.*, except for the effects from changing the activity of the solvent, *the ratio of ML′ to ML will be the same no matter how much metal or ligand is in the system.*

In order to estimate the maximum absorbance change of the ML′ species, Schneider *et al.* (1964) measured the difference spectra of 2×10^{-4} M adenosine with 1, 2, and 3 M

[2] G. P. P. Kuntz, T. A. Glassman, C. Cooper, and T. J. Swift, unpublished results.

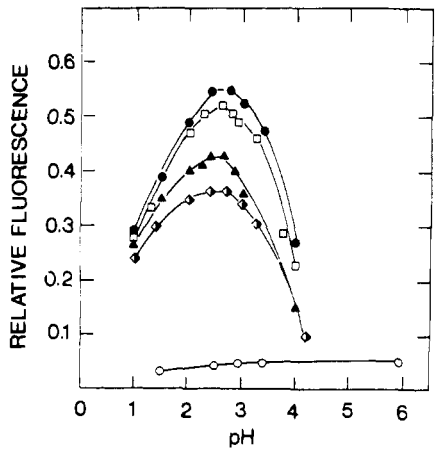


FIGURE 7: Intensity of fluorescence of ATP and metal–ATP solutions plotted against pH: (●) ATP; (□) Mg^{2+}/ATP = 2.2; (▲) Mg^{2+}/ATP = 22; (□) Ni^{2+}/ATP = 0.91; (◇) Ni^{2+}/ATP = 9.1; all solutions were 0.170×10^{-3} M ATP; (○) 0.5 M TMA-Cl.

$Ni(NO_3)_2$ solutions. They extrapolated to the absorbance change at infinite metal ion concentration and assumed that this represented the maximum absorbance change from having adenosine 100% complexed with metal ion. By comparing this value with the change in absorbance of various 1:1 metal–ATP solutions, they calculated the per cent ring interaction in the 1:1 metal–ATP complexes. There are several reasons for questioning the validity of their results. They had an extremely high ionic strength in their adenosine solutions which was not corrected as they increased the metal ion concentration and they compared changes from extrapolated values at infinite metal ion concentrations to absorbance changes of a 1:1 Ni^{2+}–ATP solution at 2×10^{-4} M. Although the pH of the adenosine–Ni^{2+} solutions was not reported, they presumably did the experiments at a low pH to prevent formation of $Ni(OH)_2$. We were only able to repeat their observations when the pH of the Ni^{2+}–adenosine solutions was below 2. At this pH the adenine ring is protonated and, if a complex is formed, it is probably different from the complex formed by the interaction of the phosphate bound metal and the adenine ring of ATP at higher pH. We were unable to demonstrate any binding of Ni^{2+} to adenosine at the higher pH's used in the work described in this paper. It is also possible that at the high metal concentrations used by Schneider *et al.* (1964) more than one metal ion is bound to the adenosine ring and that extrapolating to the change in absorbance at infinite metal concentration is not a suitable standard for determining the maximum complexation of metal ion to the adenine ring in the 1:1 metal ion–ATP complex. Another important consideration is our finding that there is a marked effect of triphosphate on the ability of Ni^{2+} to react with the adenine ring of adenosine. It is questionable that a solution of Ni^{2+} plus adenosine may be used as a control for the degree of interaction of Ni^{2+} with ATP. The work of Sternlicht *et al.* (1965a,b) and our earlier nuclear magnetic resonance studies (Glassman *et al.*, 1971; Kuntz *et al.*, 1972) indicated that the 1:1 metal–ATP complexes of Co^{2+}, Ni^{2+} and possibly Mn^{2+} exist almost entirely in the ring bound form. Schneider *et al.* (1964) using the ultraviolet spectroscopic method described above concluded that the ring-bound forms of these metal–ATP complexes were present as only 3% (Mn^{2+}), 12% (Co^{2+}), and 20% (Ni^{2+}) of the total complex. Although the results in this paper do not quantify the amount of the ring bound complex, they do raise serious objections to the validity of the conclusions of Schneider *et al.*

The fluorescence studies of Watanabe *et al.* (1963) investigated the interaction of phosphate-bound Mg^{2+} ion with the adenine ring of ATP. Their conclusions were based on the assumption that if a phosphate-bound metal ion interacts with the adenine ring, an increase in fluorescence should occur. We repeated their studies with both Mg^{2+} and Ni^{2+}. The Ni^{2+} served as a control since it is a metal ion that has been shown by nuclear magnetic resonance studies to have a strong interaction with the adenine ring (Glassman *et al.*, 1971). As seen in Figure 7, both Mg^{2+} and Ni^{2+} fail to increase the fluorescence of ATP. Although it is still possible that Mg^{2+} does not interact with the adenine ring in the Mg–ATP complex, this technique is not capable of establishing this point.

The presence of a M_2L complex with a stability constant around 250 M^{-1} might permit control of enzymatic reactions by alterations in the ratio of metal ion to nucleotide in the system. Enzymatic studies (Kuby *et al.*, 1954; Noat *et al.*, 1970) have indicated that the M_2L species inhibits certain reactions. Small changes in metal ion or nucleotide concentrations seem unlikely to affect enzymatic rates if only the 1:1 metal–nucleotide complex was formed. These complexes have stability constants in the range of 30,000–100,000 M^{-1} and large changes in concentrations would be needed to influence the system. However, if the M_2L complex is formed and is inhibitory, then relatively small changes in metal ion or nucleotide concentration might influence enzyme reactions.

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The Affinity and Temporal Variation of Isoelectric Fractions of Rabbit Anti-Lactose Antibody†

Asoke C. Ghose and Fred Karush*,‡

ABSTRACT: A killed vaccine of *Streptococcus faecalis*, strain N, has been used in rabbits to produce anti-lactose antibody of restricted heterogeneity. The specifically purified anti-lactose antibody preparations have been further fractionated by preparative isoelectric focusing to yield antibody populations exhibiting functional homogeneity. The binding of a monovalent lactose-containing hapten was measured by equilibrium dialysis to provide affinity values for the isoelectric fractions and evidence of homogeneous reactivity. All of the rabbits studied showed a multiplicity of isoelectric fractions with anti-lactose specificity early in the response. The three animals selected for detailed study differed in the range of affinity observed with the isolated fractions reflecting, presumably, a difference in their genetic capability to produce anti-lactose antibody. The maturation of the response in these animals involved the preferential emergence of the isoelectric fractions of maximum affinity. However, only in the case in which the individual expressed a wide range of affinity, 100-fold in the association constant, was the development of a monoclonal response approached. When restimulation was carried out a year after the initial exposure to the vaccine and following the decline of serum antibody, the same isoelectric fractions found in the early response were observed. Their quantitative distribution, however, was modified in favor of a larger fraction of antibody of the higher affinities. The absence of new isoelectric fractions indicated that no significant somatic diversification had occurred during the year of adult life with respect to reactivity with the lactose determinant.

The study of the homogeneous antibody products of single clones of antibody-producing cells has emerged in recent years as a powerful tool for the analysis of the cellular parameters of antibody synthesis. These studies have utilized monofocal splenic fragments cultured *in vitro* (Klinman, 1969) and the selection and propagation *in vivo* of a single antibody-forming clone (Askonas *et al.*, 1970). At the same time the use of bacterial vaccines has become an effective technique for the induction of high levels of rabbit antibody with restricted heterogeneity specific for carbohydrate antigens. With such antibody preparations it has proved possible to isolate homogeneous 7S antibody directed against streptococcal cell wall determinants (Krause, 1970) and against capsular polysaccharides of the pneumococcus (Pincus *et al.*, 1970).

The recent discovery of a strain of *Streptococcus faecalis* (strain N), whose cell wall contains lactose as the immunodominant group (Pazur *et al.*, 1971, 1973), has expanded the utility of bacterial vaccines to the production of anti-lactose antibody of restricted heterogeneity. Although functionally homogeneous antibody has been observed in only one rabbit immunized with *S. faecalis* (see below), the application of preparative isoelectric focusing (Freedman and Painter, 1971) has allowed the separation of purified anti-lactose antibody into homogeneous fractions. We have undertaken to characterize these fractions with respect to their affinity for monovalent hapten and to follow the temporal variations of these monoclonal products over a period of about 1 year. The main purpose of the study was to assess the significance of the affinity of the secreted product of a clone for the natural history of the clone. The emergence of dominant clones, their finite life span and the persistence of clones reveal a high degree of complexity in their selection involving probably both

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