A Raman Spectroscopic Study of the Interaction of Ca^{2+} and Mg^{2+} with the Triphosphate Moiety of Adenosine Triphosphate in Aqueous Solution*

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ABSTRACT: The frequency of a Raman line of the phosphate group of ATP was measured as a function of pH in aqueous solutions (0.2–0.02 M) of ATP alone and of ATP complexes with Na^+, Mg^{2+}, and Ca^{2+}. The data were analyzed to determine the pK_a of $HATP^{3-}$, obtain the stability constants of ATP–M complexes (M = Na^+, Ca^{2+}, or Mg^{2+}), explain the dynamics (exchange rates) of the processes involved and develop a model to estimate ATP–M–ATP interactions. The log of the stability constants of ATP^{4-} with Ca^{2+} and Mg^{2+} are 3.9 ± 0.4 and 4.5 ± 0.3, respectively. It was concluded that the differences in the ATP–M–ATP interaction energies and in exchange rates for Ca^{2+} and Mg^{2+} complexes with ATP is much greater than the difference in their stability constants.

The effect of excess Na^+ on the M–ATP complexes is discussed. Parallels are drawn between these simple ATP–metal ion systems and some phenomena occurring in biological ATPase reactions.

When ATP is present in a biological system as an energy-transfer agent it is generally associated with a divalent cation, most often Mg^{2+} or Ca^{2+} (Bendall, 1969; Melancon and DeLuca, 1970; Perry, 1967). In some cases there is evidence that a complex of a divalent metal ion and a nucleotide triphosphate is the actual substrate for the enzyme (Larsson-Raznikiewicz and Malström, 1961; Melchior, 1965). In addition, the strong tendency for the formation of such complexes may be a factor in the control of the free concentration of divalent cations in the cell, and thus may play a role in interactions where binding of these ions to other molecules is involved (Weber, 1969; Ebashi, 1960; Portzehl *et al.*, 1964). Furthermore there are marked differences in the activating effect of the various metal ions on ATPase catalysis (Ebashi and Lipmann, 1962; Weber and Winicur, 1961; Weber *et al.*, 1964; Weber and Hertz, 1963) and on nonenzymatic ATP hydrolysis (Miller and Westbeimer, 1966a,b; Lowenstein, 1957). Thus the interactions between the various ionic forms of ATP and the divalent metal ions have to be considered in the search for an understanding at the molecular level of the biological interactions involving ATP. Metal ion complexes with ATP have been investigated extensively by various techniques. Most of the experiments that have yielded quantitative results for complex stability constants involved standard titration methods, *e.g.*, pH determinations (Smith and Alberty, 1956a,b; Kahn and Martell, 1962; Siegel *et al.*, 1967; Alberty, 1968) or optical density measurements in the presence of pH indicating dyes (O'Sullivan and Perrin, 1964). These methods, although sensitive to extremely low ATP concentrations, do not directly provide structural information about the complexes and, therefore, corresponding assumptions have to be introduced in order to interpret the data. Infrared (Brintzinger, 1963; Khalil and Brown, 1964) and nuclear magnetic resonance spectra (Cohn and Hughes, 1960, 1962; Happe and Ward, 1969) as well as detailed studies of optical rotation and difference spectra (Handschin and Brintzinger, 1962; Schneider *et al.*, 1964; Schneider and Brintzinger, 1964) can give structural information, but the sensitivity is usually lower than that of titrimetric techniques. Earlier nuclear magnetic resonance measurements have indeed indicated significant changes in the spectrum due to the interactions of ATP with physiologically important divalent cations, which yielded qualitative information on complex structure (Cohn and Hughes, 1960, 1962; Happe and Morales, 1966). The application of nuclear magnetic resonance results obtained on transition metal ions (Sternlicht *et al.*, 1965a,b, 1968) to Ca- and MgATP is questionable since large differences have been found in the effect of chemically similar ions on ATPase activity. In attempting to reproduce the earlier infrared results (Khalil and Brown, 1964) we obtained good agreement with those reported for solutions of ATP by itself; we were unable, however, to reproduce those given for the metal ion complexes. Due to the large frequency-dependent background, the interpretation of the infrared spectra even in D_2O solutions involves subjective judgments which limit their reliability for quantitative analysis. Laser Raman spectra of good quality can be obtained for aqueous solutions of ATP and other nucleotides (Lord and Thomas, 1967a,b; Yu, 1969; Rimai *et al.*, 1969a, 1970). We have studied the Raman spectra of ATP and complexes of the form ATP–M (where M = Ca^{2+} or Mg^{2+}) in search of information pertinent to the binding of ATP with the divalent cations. The measurements were extended to lower ATP concentrations than in previous Raman, infrared, or nuclear magnetic resonance work. The range covered in this work was 0.02 M ≤ [ATP] ≤ 0.3 M; the lower limit, at which most spectra were recorded, is within a factor of two from the average ATP concentration in tissues, such as the muscle, where there is high ATPase activity (Perry, 1967). The concentration dependence of the data yields important information on the interactions between ATP molecules. The discussion of the experimental results given below contains the arguments involved in the analysis of the data, a simple model for the fast-exchange mechanism, the calculation of ATP–M binding constants, and a model that enables one to estimate the ATP–M–ATP interaction energies.

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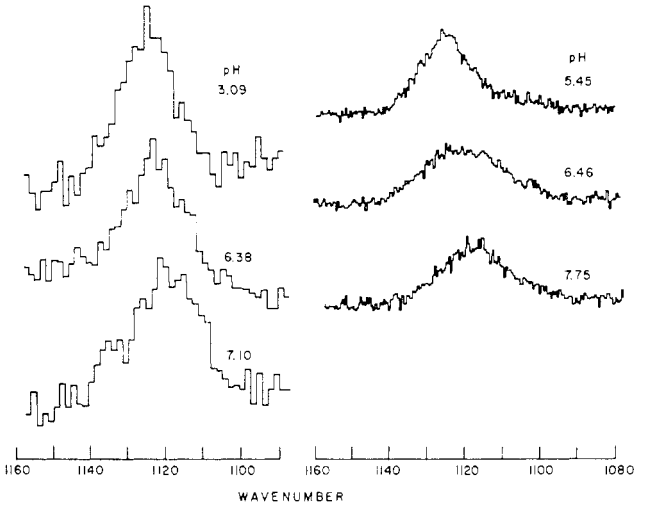

FIGURE 1: Recorder tracings of the Raman line characteristic of the triphosphate moiety of ATP: left, 0.02 M ATP in H_2O; right, 0.2 M ATP in D_2O.

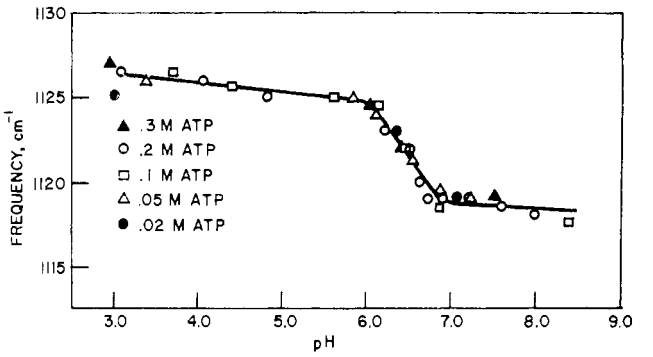

FIGURE 2: pH dependence of the center frequency of the Raman triphosphate line for various concentrations of $Na_2H_2ATP \cdot 3H_2O$ in aqueous solution. In this and similar figures the line connecting the data points is not theoretically determined.

Method and Materials

The Raman spectra were recorded by a standard experimental setup (Rimai *et al.*, 1969a). The excitation at 4880 Å generated by an argon ion laser was focused by a 30-cm focal length lens on a rectangular sample cell. The light scattered at 90° was focused on the entrance slit of a Jarrell Ash 75-cm Czerny-Turner double monochromator, and detected at the output slit by an FW-130-type photomultiplier tube and photon-counting electronics. The spectral slit widths, in the range of the Raman lines of interest, corresponded to a resolution of approximately 3 cm^{-1}. The laser powers used at the sample varied between 50 and 400 mW depending on ATP concentration; the source length was in the order of 6 mm. Most scans were taken at a speed of 10 cm^{-1}/min and with integration times between 0.5 and 5 sec. For the 0.02 M samples the phosphate indicator line frequencies were averaged from three consecutive runs.

Adenosine triphosphate, as $Na_2H_2ATP \cdot 3H_2O$ was purchased from Nutritional Biochemical Corp. The metal ions were introduced as reagent grade chloride salts. For the experiments with D_2O solutions, deuterium oxide of 99.77% isotopic purity was obtained from International Chemical and Nuclear Corp. The pH of the solutions was adjusted with the addition of Na_2O (K & K Laboratories) and was measured on a Beckman Expandomatic potentiometer. The samples were filtered into the scattering cell through a 100-mμ Millipore filter. The measurement of all the reported spectra was made at 25°, with the exception of some ATP spectra run at temperature intervals from 25 to 40°, in which no variation in the frequency of the pH-dependent phosphate line was observed. Corrections for the variation of pH with temperature were made from an experimentally determined calibration curve.

Experimental Results and Discussion

The region of the Raman spectrum of ATP that is most interesting for analytical studies is that between 600 and 1600 cm^{-1}. There the largest number of strong sharp lines is present, and effects due to molecular conformational change can be most easily detected (Rimai *et al.*, 1969a). In all the adenosine phosphates as well as in the complexes of ATP with Ca^{2+} and Mg^{2+} these lines can be classified into three categories (Lord and Thomas, 1967a; Rimai *et al.*, 1969a). (1) Lines that exhibit no marked pH dependence of their frequency (*e.g.*, the strong line at 727 cm^{-1}) and which probably correspond to vibrations of the base which do not involve the N-1 or the amino group at C-6. (2) Vibrations that change markedly only near a pH of 4.0, and are contributed by the normal coordinates of the base which are sensitive to the displacements of N-1 and/or the amine-substituted carbon, since $pK_a \sim 4$ corresponds to a proton dissociation from one of these two sites (Lord and Thomas, 1967a; Tsuboi *et al.*, 1962). (3) Lines that can be clearly associated with the phosphate moiety because they change in a pH region near the pK_a' of the secondary phosphate proton. ATP has one strong line of the third type at 1125 cm^{-1} when pH $\ll pK_a' \simeq 6.5$ and at 1117 cm^{-1} when pH $\gg pK_a'$. There is another weaker line that, by the same criterion, is identified with the phosphate moiety, at 1180 cm^{-1} below the pK_a' and at 1185 cm^{-1} above it. Its pH dependence seems to be similar to that of the strong line. There are several weaker lines that could possibly be associated with the phosphate; these have not been used in this investigation. We used the 1125-cm^{-1} phosphate line as a conformational indicator and we make the basic assumption that it follows the structural changes that affect the phosphate component of the molecules. This line is associated with the P=O bond stretching motion (Rimai *et al.*, 1969a) and, based on its strength and polarization, corresponds to a symmetric vibration. Such modes are sensitive to the state of ionization also in inorganic orthophosphate, pyrophosphate, and nucleotide mono- and diphosphates (Rimai *et al.*, 1969a). Figure 1 illustrates in detail the pH dependence of the phosphate indicator line in the region of dissociation of the terminal phosphate of ATP. In H_2O solutions the line shifts continuously with pH, without marked broadening. In D_2O solutions, a continuous frequency shift is still present but measurable line broadening is also evident. The ATP phosphate line in H_2O at all values of pH, and the line in D_2O far from the pK_a', can be fitted, within the noise level, to single Lorentzians. The broadened line near the pK_a' in D_2O, on the other hand, is well fitted by a superposition of two Lorentzians, closer in frequency than the lines at extreme pH.

Figure 2 shows the phosphate Raman line frequency as a function of pH for four different Na_2ATP concentrations in H_2O. The data are independent of ATP concentration,

indicating that for ATP solutions without added salts the observed behavior is also expected at infinite dilution. A definite transition appears in the pH region where the secondary proton dissociates. The midpoint of the transition at pH 6.50 agrees well with the apparent acid dissociation constant pK_a' measured for this proton by many other techniques (Smith and Alberty, 1956a,b; Khan and Martell, 1962; Khalil and Brown, 1964). Most of the conclusions in this work have been drawn from data such as that in Figure 2. Based on the above results the transition midpoint was used to locate the apparent dissociation constants for the ATP metal ion complexes. The effects shown in Figures 1 and 2 can be qualitatively understood in terms of a fast-exchange model similar to that employed in the analysis of high-resolution nuclear magnetic resonance spectra (Kubo, 1961; Chan *et al.*, 1964). There are essentially two forms of ATP present, with the following average concentrations: N_1 for ions with protonated terminal phosphate, and N_2 for the deprotonated ions. If there are no transitions between the two forms their instantaneous concentrations are equal to N_1 and N_2, independent of time, and the spectrum of interest will consist of two lines at frequencies ω_1 and ω_2 with amplitudes proportional to N_1 and N_2. (The widths of the two lines are assumed equal.) This is the situation for pH $\ll$ pK_a' or pH $\gg$ pK_a', where $N_2 = 0$ or $N_1 = 0$, respectively, and for ADP and AMP (Rimai *et al.*, 1969a) where two distinct lines are observed which progressively exchange intensity as the pH is varied through the transition region. There always exist transitions between the two ionic forms, due to proton exchange between a site covalently bonded to the terminal phosphate (the instantaneous spectrum would correspond to that at pH $\ll$ pK_a'), and any other site where the covalent bond is broken (the instantaneous spectrum corresponds to that at $pK \gg pK_a$). These transitions are characterized by an exchange frequency, ω_e, and they impart a time dependence to the instantaneous concentrations of the two species. The effect of these fluctuations on the spectrum will depend on the magnitude of ω_e. If $\omega_e \ll |\omega_1 - \omega_2|$ one obtains an essentially static situation, with two discrete lines at ω_1 and ω_2. As ω_e increases toward $|\omega_1 - \omega_2|$ the two lines approach each other and eventually, in the limit of fast exchange, when $\omega_e > |\omega_1 - \omega_2|$ one is left with a single sharp line at a frequency ($N = N_1 + N_2$)

$$\omega = (\omega_1 + \omega_2)/2 + \frac{\Delta\omega}{2}(N_2 - N_1)/N \qquad (1)$$

The width of this component will be equal to that of the static lines. There will be another component, too broad, however, for detection under present conditions. From eq 1 we see that at the midpoint of the transition, $\omega = (\omega_1 + \omega_2)/2$ and $N_1 = N_2$. Comparing the results for ATP, where $|\omega_1 - \omega_2| = 5$ cm^{-1}, to those for ADP and AMP, where $|\omega_1 - \omega_2| = 18$ and 102 cm^{-1}, respectively, we set the following limits for ω_e of ATP in H_2O: $15 \times 10^{10} < \omega_e < 54 \times 10^{10}$ sec^{-1} From the data in Figure 1 for ATP in D_2O, we seem to have a case of intermediate exchange, with $\omega_e \simeq 15 \times 10^{10}$ sec^{-1}, in qualitative agreement with the expected slower jumping rate for the massive ion.

The exchange frequency involved here is much faster than that obtained from mobilities of protons in aqueous solutions, or from measurements of chemical dissociation rates by temperature-jump methods (Eigen and DeMeyer, 1963; Diebler *et al.*, 1960; Eigen and Hammes, 1960). However, this discrepancy can be explained if it is realized that the

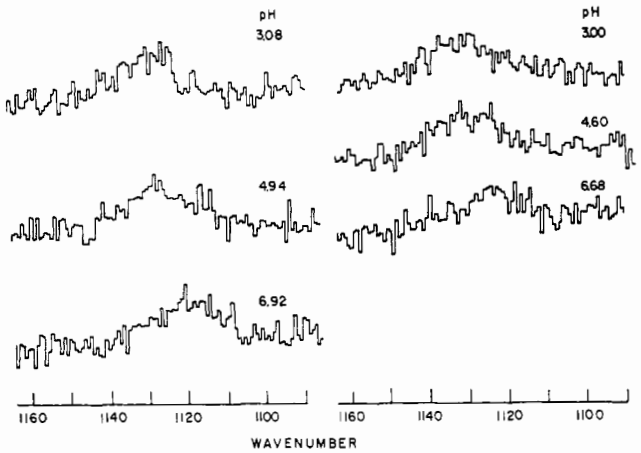

FIGURE 3: Recorder tracings of the Raman line associated with the triphosphate moiety of the Ca–ATP complex: left, 0.02 M ATP + 0.015 M $CaCl_2$; right, 0.02 M ATP + 0.02 M $CaCl_2$.

configurational change that shifts the vibrational frequency from ω_1 to ω_2 does not require complete dissociation of the proton from the hydration volume of the ATP ion, but only a small displacement from the covalently bonded site to a nearest H-bonded site still inside this volume (Eigen and DeMeyer, 1958; Albery, 1967). Evidence for very fast proton transfer within such regions is available: from line broadening of Raman lines of anions in water solution $\omega_e \simeq 1\text{–}30 \times 10^{11}$ sec^{-1} (Kreevoy and Mead, 1962a,b); from the width of Raman transitions contributed by proton transfers in crystals, whose ordered structure, although longer range, bears similarity to the organized atomic arrangement in the hydration volume, $\omega_e \simeq 10^{12}$ sec^{-1} at room temperature in NH_4Cl (Rimai *et al.*, 1969b); from polarographic measurements $\omega_e \simeq 10^{10}\text{–}10^{13}$ sec^{-1} (Strehow, 1963). In addition, the large widths of the stretching and bending OH vibrations in water ($\Delta\omega \simeq 30\text{–}100$ cm^{-1}), and the extremely broad infrared spectrum found for the hydronium ion (Eigen and DeMeyer, 1958) provide evidence for the presence of such rapid transfer processes. Finally, since we depend on a combination of jumping rates for protonation and deprotonation (Eigen and DeMeyer, 1963), and on the concentrations of the species involved,

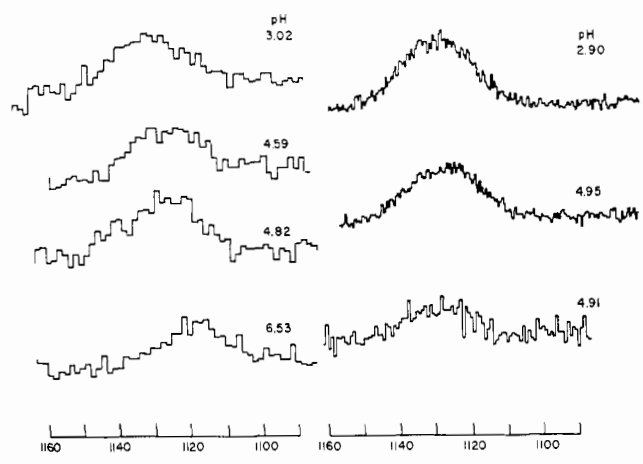

FIGURE 4: Recorder tracings of the Raman line associated with the triphosphate moiety of the MgATP complex: left, 0.02 M ATP + 0.02 M $MgCl_2$; upper and mid right, 0.2 M ATP + 0.2 M $MgCl_2$ + 0.2 M NaCl; lower right, 0.02 M ATP + 0.015 M $MgCl_2$.

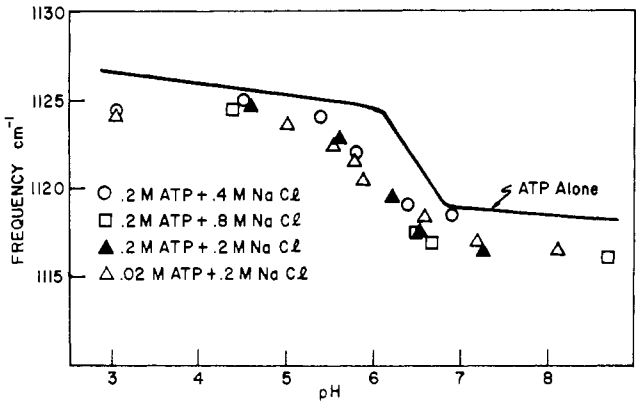

FIGURE 5: Center frequency of the Raman triphosphate line of ATP solutions for various additions of NaCl, as a function of pH.

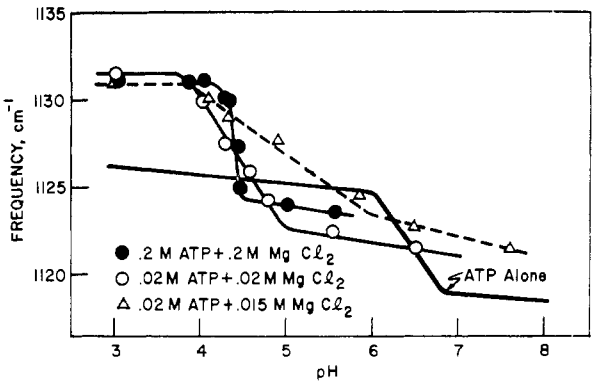

FIGURE 7: Center frequency of the Raman triphosphate line *vs.* pH for several concentrations of the MgATP complex.

the individual jumping rates can only be determined from detailed concentration-dependent measurements, which are not warranted since the present experiments can only set limits for the exchange frequency.

The fact that the pK_a', as obtained by other techniques, is also given by the midpoint of the Raman transition cannot be rigorously derived from the above model. However, the accurate agreement with previously reported values for the ATP solutions of Figure 2 supports well this argument. Figures 3 and 4 show sample data, and Figures 5, 6, 7, and 8 show Raman titration curves for various ATP–metal ion combinations in H_2O. The apparent dissociation constants pK_a' obtained from these data are summarized in Table I.

Since all measurements were made with the disodium salt of ATP, we had to correct for the Na^+ binding, in order to obtain the true pK_a for the secondary proton (Smith and Alberty, 1956a; Khalil and Brown, 1964). Though a comparative analysis of the data for two samples with equal total ATP concentration $[ATP]_0$, but different total sodium concentrations $[Na]_{0,1}$ and $[Na]_{0,2}$, one can obtain the binding constant K_{Na} of sodium to the phosphate moiety of ATP, and the true dissociation constant

$$K_a = ([ATP^{4-}][H_p^+])/([H_pATP^{3-}]) \quad (2)$$

The assumptions are similar to those used for the analysis of titration curves, and for the interpretation of the infrared intensity measurements (Smith and Alberty, 1956a; Khalil and Brown, 1964). (1) All primary protons on the phosphate moiety of ATP are assumed dissociated, which is reasonable for pH > 3; (2) Na^+ binds only to ATP^{4-}; (3) Only a monomeric complex is formed. Our ATP concentration dependence measurements indicate the latter certainly to be valid for samples with no excess of sodium salt (Figure 2) and for all the samples at 0.02 M Na_2ATP. Applying the charge and mass balance equations, the definition of the pK_a' in the presence of Na^+ ions (Khalil and Brown, 1964), the following condition at the pK_a'

$$[HATP^{3-}] = [ATP^{4-}] + [NaATP^{3-}] = [ATP]_0/2 \quad (3)$$

and the expression of K_a' in terms of K_a and the sodium binding constant

$$K_a' = K_a\{1 + K_{Na}[Na^+]\} \quad (4)$$

to a pair of solutions, one obtains explicitly

$$K_a = K_{a,1}' - \{([Na]_{0,1}/([Na]_{0,1} - [Na]_{0,2})) \times (K_{a,1}' - K_{a,2}') \times (1 - [Na]_{0,2}/([ATP]/2))\} \quad (5)$$

$K_{a,1}'$, $K_{a,2}'$ are the apparent dissociation constants measured by the Raman data, on the two solutions. With $[ATP]_0$ = 0.02 M, $[Na]_{0,1}$ = 0.04 M, $[Na]_{0,2}$ = 0.24 M, and using data of Figure 5 and Table I, we obtain

$$K_a = 1.165 \times 10^{-7}\ M^{-1}\ (pK_a = 6.93)$$

in reasonably good agreement with previously reported values (Smith and Alberty, 1956a; Alberty, 1968). We also obtain, using eq 4: K_{Na} = 25.8 M^{-1}. This value is somewhat higher than K_a = 14.3 ± 4.0 M^{-1}, reported by Smith and Alberty and may reflect in addition to possible residual concentration effects still present at 0.02 M ATP, the fact that our measurements observe only the phosphate end of the molecule whereas potentiometric titration measures the effects of pH on the whole molecule.

In attempting to analyze, by the same method, the data for $[ATP]_0$ = 0.2 M (Figure 5 and Table I) we obtained the absurd result of $K_a < 0$ suggesting that even with monovalent ions, when in excess concentration, there are strong effects involving interactions between the ATP molecules.

The divalent ions M = Ca^{2+} or Mg^{2+} are expected to bind

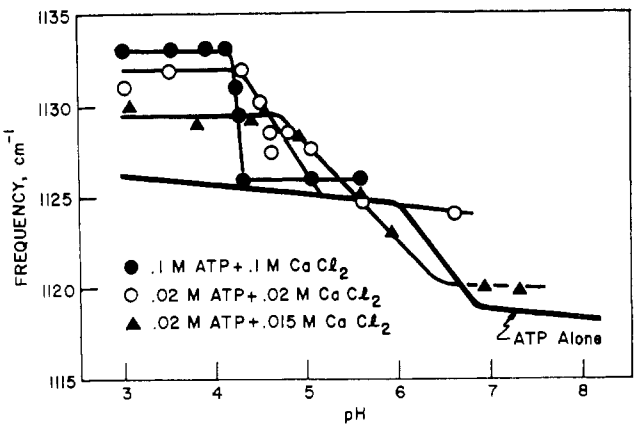

FIGURE 6: Center frequency of the Raman triphosphate line *vs.* pH for several concentrations of the CaATP complex.

TABLE I: Apparent Acid Dissociation Constants Obtained by Raman Titration of the Phosphate Moiety of Solutions of ATP and Its Complexes with Ca^{2+} and Mg^{2+}.

Sample	pK_a'
0.3 M ATP	6.50
0.2 M ATP	6.50
0.1 M ATP	6.50
0.05 M ATP	6.50
0.02 M ATP	6.50
0.2 M ATP + 0.2 M $MgCl_2$	4.42
0.1 M ATP + 0.1 M $CaCl_2$	4.21
0.02 M ATP + 0.02 M $MgCl_2$	4.47
0.02 M ATP + 0.02 M $CaCl_2$	4.71
0.02 M ATP + 0.015 M $MgCl_2$	4.92
0.02 M ATP + 0.015 M $CaCl_2$	5.56
0.2 M ATP + 0.2 M NaCl	6.00
0.2 M ATP + 0.4 M NaCl	5.91
0.2 M ATP + 0.8 M NaCl	5.95
0.02 M ATP + 0.2 M NaCl	6.02
0.2 M ATP + 0.2 M $MgCl_2$ + 0.4 M NaCl	4.54
0.02 M ATP + 0.02 M $MgCl_2$ + 0.04 M NaCl	4.65
0.1 M ATP + 0.1 M $CaCl_2$ + 0.2 M NaCl	>5.0
0.02 M ATP + 0.02 M $NaCl_2$ + 0.04 M NaCl	4.82

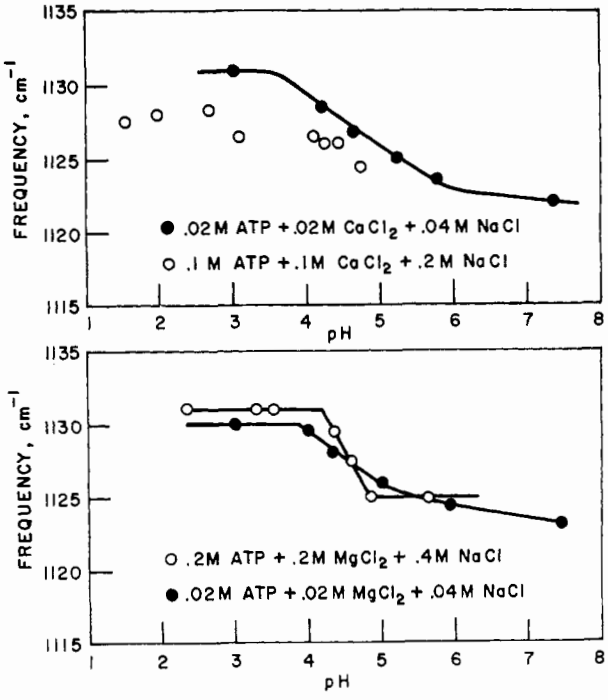

FIGURE 8: Top, pH dependence of the center frequency of the Raman triphosphate line of CaATP solutions with excess NaCl. Bottom, same as above, for MgATP solutions.

effectively to ATP, both below and above the pK_a of the terminal phosphate (Alberty, 1968; Phillips, 1966; Rimai *et al.*, 1970). Thus two binding constants have to be determined, K_{M_1} and K_{M_2}, respectively. They can be obtained by solving a set of simultaneous equations, derived again from mass and charge balance conditions, and the relations describing the various chemical equilibria. The equations are summarized in the Appendix. The relations are nonlinear, and unlike the simpler case of Na^+ binding no explicit algebraic solution could be obtained. We have applied a systematic trial and error procedure to data on pairs of 0.02 M ATP solutions, with respective additions of 0.015 and 0.02 M $CaCl_2$ and $MgCl_2$ (data in Table I). The results were: for MgATP, log K_{M_1} = 1.7 ± 0.2; log K_{M_2} = 4.5 ± 0.3; for CaATP, log K_{M_1} = 1.0 ± 0.4; log K_{M_2} = 3.9 ± 0.4. We see in the first place that for pH > pK_a the Mg^{2+} binding is slightly stronger than that of Ca^{2+}. This is so despite the proximity of the pK_a's at 0.02 M equimolar MATP, and contrary to what one would expect from the values of pK_a's at 0.1 or 0.2 M ATP. In this connection it should be noted that, concentration effects aside, the depression of the pK_a' upon complexing is a function of the relative values of complex stability below and above the proton dissociation point and thus in order to obtain the constants themselves it is not sufficient to measure the pK_a' depression for only the equimolar solution (Khalil and Brown, 1964). The present results are in reasonable agreement with the following values, obtained recently under similar assumptions by the analysis of titration data (Khan and Martell, 1962): MgATP, log K_{M_2} = 4.22, log K_{M_1} = 2.24; CaATP, log K_{M_2} = 3.97, log K_{M_1} = 2.13; and by spectrophotometry of 8-hydroxiquinoline (O'Sullivan and Perrin, 1964): MgATP, log K_{M_2} = 4.31–4.90, log K_{M_1} = 2.84; CaATP, log K_{M_2} = 4.47; but much higher than an earlier titrimetric value (Smith and Alberty, 1956b): MgATP, log K_{M_2} = 3.47; and those obtained from infrared spectra in D_2O (Khalil and Brown, 1964): MgATP, log K_{M_2} = 3.6; CaATP, log K_{M_2} = 3.0. Since the present results stem from a rather precise spectroscopic determination of frequency shifts, they provide strong support for the group of higher values in the preceding list.

It should be clear from the data of Figures 2, 6, and 7 that whereas the Raman titration curve is independent of ATP concentration in the disodium salt solutions, this is not so for the equimolar Ca^{2+} and Mg^{2+} solutions. The pK_a' values become concentration dependent (Table I) and, more importantly, there is a marked sharpening of the transition upon increase of concentration. This result strongly suggests the presence of a cooperative effect involving interactions between ATP ions induced by the divalent cations, and may be interpreted in the following manner. As the pH is increased through the transition region, the fraction of the ATP^{4-} ions that has already lost the proton facilitates the dissociation of the remaining ones. The interactions are such that the molar free energy of the dissociated form E_2 is lowered relative to E_1, that of the protonated form, as the concentration of the latter decreases. In the presence of interactions induced by metal ions at concentration M, the free molar energies, E_i (i − 1, 2 for the two ionic species of ATP), can be related to the energies in the absence of interaction $E_{i,0}$, and the concentrations of the two species N_i (i − 1, 2) by

$$E_1 = E_{10} + M(C_{11}N_1 + C_{12}N_2)$$
$$E_2 = E_{20} + M(C_{12}N_1 + C_{22}N_2) \quad (6)$$

where the C_{ij} are interaction constants in a first-order approximation. Using the Boltzmann relation $N_1/N_2 = \exp\{-\beta[E_1 - E_2]\}$ we readily obtain for each pH an implicit equation for the concentration difference $D = N_1 - N_2$

$$\frac{N + D}{N - D} = \left(\frac{N + D_0}{N - D_0}\right) \exp\{-\beta M\{[1/2(C_{11} + C_{22}) - C_{12}] \times D + 1/2(C_{11} - C_{22})N\}\} \quad (7)$$

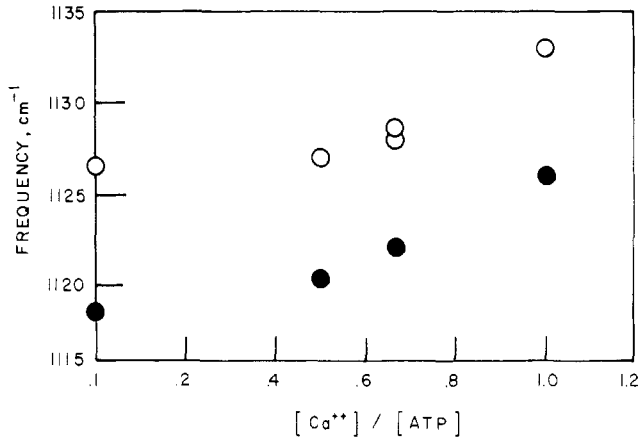

FIGURE 9: Center frequency of the Raman triphosphate indicator line of the CaATP complex, at pH $\ll$ pK_a' (open circles) and at pH $\gg$ pK_a' (solid circles), as a function of $[Ca^{2+}]_0/[ATP]_0$.

where $D_0 = N[\exp(-\beta E_{10}) - \exp(-\beta E_{20})]/[\exp(-\beta E_{10}) + \exp(-\beta E_{20})]$ is the population difference at the same pH if the interaction were switched off, and $\beta = 1/RT$. From eq 1 we know that D is proportional to the frequency deviation $x = \omega - (\omega_1 + \omega_2)/2, D = (2N/\Delta\omega)x$. Thus eq 7 yields an implicit transcendental equation for x, provided we know $D_0 = (2N/\Delta\omega)x_0$. If the interactions were switched off the frequency deviations would be independent of concentration, and equal to their low concentration limit. Thus, to analyze the data for the equimolar high concentration Mg and Ca samples we use, for x_0, the frequency deviations in the 0.02 M equimolar solutions. Without trying to solve the equation, which would be unwarranted due to the inherent inaccuracies in the model, we obtained estimates of C_{ij} by comparing the frequency deviations and slopes at the pH = pK_a' of the high concentration samples (where $x = 0$) with their low concentration counterparts. From the pK_a' of Table I we estimate for MgATP: $^1/_2(C_{11} - C_{22}) \simeq 3.4 \times 10^3$ cal/(mole)^2. From the slopes of data in Figure 7 we obtain $^1/_2(C_{11} + C_{22}) - C_{12} = -2.36 \times 10^2$ cal/(mole)^2. If we next assume that the major sources of the interaction is electrostatic, the three coupling constants should increase with the total negative charge of the two interacting ATP molecules

$$|C_{11}| < |C_{12}| < |C_{22}| \qquad (8)$$

and thus by neglecting C_{11} we can get the following estimates: $C_{22} = -7 \times 10^3$ cal/(mole)^2, $C_{12} = -1.5 \times 10^3$ cal/(mole)^2 both corresponding to attractive interactions.

The situation for CaATP is rather different. Here, to a good approximation, from the data of Figure 6, at pK_a' of the 0.2 M solution, $x_0 = \Delta\omega/2$ and $D_0 = N$, and thus from eq 7 it follows that $\beta MN(C_{11} - C_{22}) \rightarrow \infty$ which essentially means that the interaction energy represented by $MN(C_{11} - C_{22})$ is much larger than the room temperature molar equipartition energy $\beta = RT$. The fact that $D_0 = N$ combined with the slope data also yields $^1/_2(C_{11} + C_{22}) - C_{12} \rightarrow \infty$ and we are forced to conclude that these interaction constants are much larger for CaATP than for MgATP. If relation 8 is again assumed, the interactions are also attractive in this case.

These conclusions may very well have a fundamental biological implication. It has been shown by a number of experiments that myosin as an ATPase enzyme has two binding sites for ATP which are close together so interactions between them are probably significant (Hotta, 1961; Kiely and Martonosi, 1968; Murphy and Morales, 1970). From the foregoing conclusions one may then hypothesize that the activating effect of Ca^{2+} ions on myosin ATPase, and perhaps even on the muscle contraction phenomenon, would be related to its large enhancement of the interaction between the two ATP molecules bound to proximate sites on the enzyme.

These interactions may not result in a higher order dependence on ATP concentration in the rate equation for the enzyme, if at the ATP concentrations studied the nonhydrolytic site is saturated. As we have seen, such an interaction effect is much smaller for Mg than for Ca, whereas the differences in the binding strengths of these ions to individual ATP molecules is relatively insignificant. Perhaps even in other cases (Lowenstein, 1967) the differences between Mg^{2+} and Ca^{2+} as ATPase activators could be associated with their differences in inducing ATP–ATP interactions. With respect to biological systems, the results on samples with large excess NaCl may also be of importance (Table I and Figure 8). For 0.2 M MgATP excess salt causes a small increase in pK_a' and a smoothing of the transition, whereas for CaATP the upshift of pK_a' is so large that the transition disappears from the solubility region. Such effects are weaker for 0.02 M complex concentrations. It is unlikely that the Cl counter ions would be involved since the results vary markedly as the cation is changed from Na^+ to Ca^{2+} to Mg^{2+} even though the Cl concentration remains constant (Figures 5 and 8). These observations indicate that the excess salt tends to weaken the divalent cation binding as well as the induced ATP–ATP interactions, the effect being much stronger for Ca than Mg. The modifying effect of alkali ions on some CaATPases may be related to these phenomena (Bendall, 1969; Perry, 1967). It is also indicated that anomalous salt concentration effects only occur at a NaCl level much higher than those at which the stability constants were determined. This fact in conjunction with the agreement between the pK_a' for the Na_2ATP solutions obtained here and those obtained at even lower (more biological) ionic strengths (Smith and Alberty, 1956b; Khan and Martell, 1962; O'Sullivan and Perrin, 1964) lends support to the contention that the present results do have applicability under realistic biological conditions.

The frequency shifts of the indicator line measured upon complexing with Mg^{2+} and Ca^{2+}, are also of some significance. The fact that such shifts are present in both the dissociated and protonated forms is indicative of relatively strong complexing in both forms. Even in protonated ATP the binding constants are sufficiently large, so that in an equimolar solution essentially all the ATP is complexed, and we expect a single line if there is only one type of complex. Assuming, however, that there exists only one complex species and that exchange is slow we expect the progressive appearance of a second line corresponding to uncomplexed ATP when the relative concentration of the metal ion is decreased. This is clearly not the case for CaATP, as data of Figure 9 indicate, and thus one obtains some evidence for a high exchange frequency involved in the formation of CaATP complexes. Such a result is not in contradiction with that obtained by the temperature-jump technique for the deprotonated form of the complex (Diebler *et al.*, 1960).

A numerical estimate of this exchange frequency cannot be made from the present data; the signal to noise of the CaATP data (Figure 3) is not sufficiently good to ascertain possible line shape and width changes. Thus, the frequency

of 10^{11} sec^{-1} derived by direct application of the fast-exchange model can only serve as an upper limit. For MgATP, the data of Figure 4 shows for pH > pK_a' an asymmetry in the line, indicating the presence of more than one vibrational frequency. Thus, there may be more than one type of MgATP complex and the Mg^{2+}-exchange frequency is not sufficiently high to average out the structure in the spectrum. This result seems to agree with that from relaxation data for MgATP in the same pH region, which indicates a relatively slow exchange process (Diebler *et al.*, 1960).

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Appendix

It has to be considered that for both Ca^{2+} and Mg^{2+} complexes the pK_a' of the secondary phosphate proton, H_P, is lowered into the region where the adenine proton, H_A, is also undergoing dissociation. The interaction of the two equilibria has to be accounted for even in the present cases where the two moieties do not apparently interact at a molecular level (Rimai and Heyde, 1970).

Let $B = [H^+]/K_B$ where K_B is the dissociation constant of the adenine moiety. The equations then are, in addition to the mass balance condition for the added metal chloride:

$$[H_PMATP^-] + \frac{[ATP^{4-}][H^+]}{K_a} = [ATP^{4-}](1 + [Na^+]K_{N_a}) + [MATP^{2-}]$$

and

$$\frac{[ATP]_0}{1 + B} = [ATP^{4-}]\left(1 + \frac{[H^+]}{K} + [Na^+]K_{Na}\right) + [H_PMATP^-] + [MATP^{2-}]$$

$$K_{M_1} = \frac{[H_AH_PMATP] + [H_PMATP^-]}{[M^{2+}]([H_AH_PATP^{2-}] + [H_PATP^{3-}])}$$

$$K_{M_2} = \frac{[H_AMATP^-] + [MATP^{2-}]}{[M^{2+}]([H_AATP^{3-}] + [ATP^{4-}])}$$

Thus we have five equations with the following six unknowns: $[A_PMATP^-]$, $[MATP^{2-}]$, $[ATP^{4-}]$, $[M^{2+}]$, K_{M_1}, K_{M_2}. With two systems of equal $[ATP]_0$, (say 0.02 M) and different $[MCl_2]_0$, (0.02 and 0.015 M in the present work), we have a total of ten equations for ten unknowns, since K_{M_1} and K_{M_2} are invariant.

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Bioconversion of Tyrosine into the Propylhygric Acid Moiety of Lincomycin*

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ABSTRACT: *Streptomyces lincolnensis* has been shown to accumulate propyl- and ethylproline in media that are sulfur limited. These two compounds are proposed as intermediates to the propylhygric and ethylhygric acid moieties of lincomycin (Ia) and 4′-depropyl-4′-ethyllincomycin (Ib), respectively.

L-Tyrosine or L-dihydroxyphenylalanine addition to cultures stimulates propylproline and ethylproline production. This observation led to the hypothesis that the amino acid portions of Ia and Ib are derived from intermediates in the pathway from tyrosine to melanin. Preliminary experiments demonstrated that L-[1-^{14}C]tyrosine and L-[^{15}N]-tyrosine are efficiently incorporated into the propylhygric acid moiety of Ia and the ethylhygric acid moiety of Ib. From the comparison of the incorporations of L-[U-^{14}C]-tyrosine and L-[1-^{14}C]tyrosine into Ia, we conclude that seven carbon atoms of tyrosine are incorporated into the propylhygric acid moiety of Ia. Therefore tyrosine must undergo a cyclization (to form a ring containing nitrogen) and partial degradation (to lose two carbon atoms) to yield ultimately the propylhygric acid moiety of Ia. These results are completely consistent with our hypothesis involving the melanin pathway and furthermore provide evidence for a novel conversion of tyrosine into new amino acids.

Previous knowledge of the structure of lincomycin has been published (Schroeder *et al.*, 1967; Magerlein *et al.*, 1967), and the biosynthesis of lincomycin has been reviewed (Eble, 1967). The experiments of Eble and coworkers with radioactive isotopes indicated that several logical precursors of the propylhygric acid (IIa) moiety of lincomycin (Ia), *e.g.*, L-[U-^{14}C]proline, hydroxy-L-[2-^{14}C]proline, L-[U-^{14}C]-glutamic acid, and 5-amino[4-^{14}C]levulinic acid were not incorporated to any great extent (see Chart I). On the other hand, their studies with radioactive pyruvate indicated that the carboxyl carbon and carbons-2 and -3 of the proline ring were probably derived from this intermediate. Further studies (Argoudelis *et al.*, 1969) showed that both the CCH_3 and NCH_3 group of the IIa moiety were derived from methionine. However, the methylene carbons of the propyl side chain were not derived from methionine. Their methylation studies with the ethylhygric acid (IIb) moiety of 4′-depropyl-4′-ethyllincomycin (Ib) likewise showed that only the CCH_3 and NCH_3 groups were derived from methionine. These results indicated that Ib was not a precursor of Ia, but that both the IIa and IIb moieties were derived in the same manner except from precursors differing by one carbon atom. In the present study we have investigated the role of L-tyrosine as a precursor to the amino acid moieties of Ia and Ib.

Experimental Section

Culture. Streptomyces lincolnensis

Fermentation Procedures. Seed cultures were grown in a medium consisting of dextrose monohydrate (20 g/l.), NZ amine B (Sheffield Chemical) (5 g/l.), and Yeasttolac (A. E. Staley Manufacturing Co.) (10 g/l.). Incubation was at 28° for 48 hr on a rotary shaker (250 rpm). Complex fermentation medium consisting essentially of starch, Pharmamedia (Traders Oil Mill Co.), and inorganic salts was employed for some preparations.

Chemically defined medium (CDM) (glucose, 30 g/l.; trisodium citrate, 3 g/l.; $ZnSO_4 \cdot H_2O$, 1 mg/l.; $FeSO_4 \cdot 7H_2O$, 1 mg/l.; $MgSO_4 \cdot 7H_2O$, 1 g/l.; NaCl, 0.5 g/l.; NH_4NO_3,

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